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THÈSE

EN VUE DE L'OBTENTION DU DIPLOME DE DOCTORAT

Domaine : Biologie Filière : Sciences biologiques
Spécialité : Biochimie Appliquée

Présentée par

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Thème

Étude de l'extraction par solvant assistée aux microondes et ultrasons des substances bioactives de jujube (*Zizyphus lotus* L.) : Optimisation, characterization par LC-DAD-ESI-MS/MS et évaluation *in vitro* de quelques activités biologiques.

Soutenue le : 19/05/2022 **Devant le Jury composé de :**

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Mr Smail MEZIANE	MCA	Univ. de Lorraine	Invité

Année Universitaire :2022 /2021

Scientific Publications

1- **Response Surface Methodology Optimization of Microwave-Assisted Polysaccharide Extraction from Algerian Jujube (*Zizyphus lotus* L.) Pulp and Peel.** *Journal of Pharmaceutical Innovation*, https://link.springer.com/article/10.1007/s12247-020-09475-9. **Farida Berkani**, Farid Dahmoune, Sabiha Achat, Sofiane Dairi, Nabil Kadri, Sabrina Zeghichi-Hamri, Amina Abbou, Imane Benzitoune, Khadidja Adel, Hocine Remini, Amine Belbahi & Khodir Madani.

2- **Ultrasound Assisted Extraction of Phenolic Compounds from a Jujube By-Product with Valuable Bioactivities.** *Journal of process*, 10.3390/pr8111441. **Farida Berkani,** Maria Luisa Serralheiro, Farid Dahmoune, Asma Ressaissi, Nabil Kadri and Hocine Remini.

3- **New bioactive constituents characterized by LC–MS/MS in optimized microwave extract of jujube seeds (Zizyphus lotus L.).** *Journal of Food Measurement and Characterization*, 10.1007/s11694-021-00903-z. **Farida Berkani,** Farid Dahmoune, Maria Luísa Serralheiro, Asma Ressaissi, Sofiane Dairi, Nabil Kadri, Hocine Remini, Amina Abbou, Khodir Madani.

4- ***Ziziphus lotus* (L.) Lam. plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality: An overview.** *North African Journal of Food Nutrition Research.* https://www.researchgate.net/publication/353440527_. **Farida Berkani,** Maria Luísa Serralheiro, Farid Dahmoune, Malik Mahdjoub, Nabil Kadri, Sofiane Dairi, Sabiha Achat, Hocine Remini, Amina Abbou, Khadidja Adel, Khodir Madani.

5- **LC-ESI-MS/MS analysis, Biological effects of phenolic compounds extracted by microwave method from Algerian Zizyphus lotus fruits**. *Journal of Food Measurement and Characterization*. 10.1007/s11694-022-01437-8. **Farida BERKANI**, Farid DAHMOUNE, Nabil KADRI, Maria Luísa SERRALHEIRO, Asma RESSAISSI, Amina ABBOU, Mouna KACI, Smail MEZIANE, Sabiha ACHAT, Nourelimane BENZITOUNE, Meriem ADOUANE, Khodir MADANI , Lotfi MOUNI.

6- **Phytochemical profile by LC-MS/MS and biological activities of polyphenols extracted from *Zizyphus lotus* L. endocarps using optimization of microwave**

extraction. **Farida BERKANI**, Farid DAHMOUNE, Maria Luísa SERRALHEIRO, Nabil KADRI, Sabiha ACHAT, Asma RESSAISSI, Sofiane DAIRI, Hocine REMINI, Khadidja ADEL, Khodir MADANI (Submitted).

7- **Comparison between antioxidative, cardiovascular-protective and antiproliferative activities of *Zizyphus lotus* L. pulps, seeds and endocarps extracted by ultrasound and microwaves under response surface methodology.** **Farida BERKANI**, Maria Luísa SERRALHEIRO, Farid DAHMOUNE, Nabil KADRI, Sabiha ACHAT, Asma RESSAISSI, Sofiane DAIRI, Hocine REMINI, Khodir MADANI (Submitted).

Communications

1- Drying kinetics by oven and microwave of Opuntia Ficus indica peels for recovery of antioxidants : Comparison of ultrasound, microwave and conventional assisted extraction. Congrès international sur la rencontre sur l'agriculture et la biologie. *Constantine 5-7 Mai 2018 (Algeria):* communication affichée. **Berkani Farida,** Chahi Faouzi, Achat Sabiha, Benazzouze Leila, Hadjout Linda, Madani Khodir, Dahmoune Farid.

2- Effect of ultrasound bath assisted extraction on the antioxidant activity of *Ziziphus lotus* : Optimization study. Séminaire national de biochimie et doctoriale de biologie et santé. *Université de Blida1, 19-20 Jun 2018 (Algeria):* communication affichée. **Berkani Farida,** Moussa Hamza, Siad Rabie, Dahmoune Farid, Abbou Amina, Adel Khadidja, Mameri Amal, Mouni Lotfi.

3- Enrichissement de l'huile d'olive extra vierge par les composants bioactifs du mélange de pelure et de pulpe de *Ziziphus lotus* . Salon national des produits de la recherche scientifique. *Alger, 2-4 July 2018* (*Algeria*)*:* communication affichée. **Berkani Farida,** Dahmoune Farid, Mouni Lotfi.

4- Certificate of attendance to the Algerian American Fondation (AAF 2018) summer university. *Alger, 8-14 Juillet 2018* (*Algeria*) *:* participation aux autres activités de l'école et présentation de thèse en 180 secondes. **Berkani Farida.**

5- Optimization of ultrasound assisted extraction of antioxidants from *Ziziphus lotus* pulp and peel using combination of Plackett and Burman Design and Full Factorial Design.

Algerian American Fondation (AAF 2018) summer university. *Alger, 8-14 Juillet 2018* (*Algeria*)*:* communication affichée**. Berkani Farida.**

6- Valorization of Algerian fruit for recovery of antioxidants compounds , evaluation and optimization of microwave, ultrasound application to solvent extraction using response surface methodology. Third Africa International Allelopathy Congress. *Blida, 24-26 Novembre 2018 (Algeria):* communication affichée. **Berkani Farida,** Dahmoune Farid, Mouni Lotfi, Achat Sabiha, Madani Khodir.

7- Jujube « *Ziziphus lotus* » a fruit with a nutrious and antioxidant potential . Deuxième journée sur l'agro-alimentaire. *Blida, 27,28 Novembre 2018 (Algeria):* communication affichée. **Berkani Farida,** Dahmoune Farid, Chelouche Raziqa, Derar Ryma, Mouni Lotfi, Madani Khodir.

8- Formation aux doctorants. Colloque national d'accompagnement des doctorants. *Bouira, 09 et 10 Décembre 2018 (Algeria).* **Berkani Farida.**

9- Optimization of microwave assisted extraction of flavonoids from *Ziziphus lotus* fruit pulp and peel using response surface methodology. Séminaire international les produits de terroir, un outil de développement de l'agriculture de montagne. Chemini, Béjaia ,*15,16 Décembre 2018 (Algeria):* communication affichée. **Berkani Farida**, Adel Khadidja, Abbou Amina, Dahmoune Farid, Mouni Lotfi, Madani Khodir.

10- Direct enrichment of olive oil in jujube fruit by ultrasound assisted maceration : Optimization study. Journées doctoriales sur « l'innovation et le transfert technologique » Béjaia Les *15 et 16 Juillet 2019 (Algeria) :* communication affichée. **Berkani Farida,** Achat Sabiha, Ait Ouali Nadia, Djerrada Meriem, Aiche Arezki, Dahmoune Farid, Abbou Amina, Adel Khadidja, Takka Melissa, Madani Khodir.

11- Effect of microwave pretreatment on bioactive compounds extraction from jujube by-products : Optimization sudy. Seminaire National sur la gestion integrée des Déchets Ménagers et Industriels. (SNGIDMI) à Ait Toudert, *Tizi Ouzou, 25 et 26 septembre 2019* (Algeria): communication affichée. **Berkani Farida**, Dahmoune Farid, Dairi Sofiane, Adel Khadidja, Abbou Amina, Achat Sabiha, Benzitoune Imane, Takka Melissa, Mouhoubi Khokha, Lahdiri Nassim, Madani Khodir.

12- *In vitro* Antioxidants, Anticancers, and Antiacetylcholinesterase activities of pulp, seed and endocarp of jujubes. Premières journées doctorales de la Faculté des Sciences de la Nature et de la Vie et des Sciences de la Terre. Univ. Bouira, 04-05 mars 2020 *(Algeria*): communication orale. **Berkani Farida.**

13- Online Mini Symposium at Food Structure & Functionality, Tuesday October 20, 2020. **Berkani Farida.**

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Dedication

A dream that is being fulfilled thanks to God the Almighty, this work consisting of the doctoral thesis is finally finished. This dissertation is dedicated :

To ***my Dear Parents Yahia BERKANI & Rachida FERKANE***

In my dear father's memory. Although I didn't want to see you go, I am at peace knowing that you are in a far better place. You will be forever missed. May your soul rest well in heaven, this work is the fruit of his advice which will remain forever engraved in my heart and mind. To my beloved mother, may God protect her. I thank her infinitely for her love, support and sacrifices. I would always be grateful to her. This work is the expression of my deep respect and witness of my gratitude

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To my ***Darling grand mother Yemma Zahra***
May God protect her and care her for all of us

To all my ***Colleagues & Friends***

« Le futur appartient à ceux qui croient en la beauté de leurs rêves »
Eleanor Roosevelt

List of Abbreviations

MAE	Microwave assisted extraction
UAE	Ultrasound assisted extraction
CSE	Conventional solvent extraction
TPC	Total phenolic content
TFC	Total flavonoid content
TTC	Total condensed tannins content
GAE	gallic acid equivalent.
QE	Quercitin equivalent
CE	Catechin equivalent
TE	Trolox equivalent
EDD	Ethylene-diamine-tetra-acetic acid equivalent
Zl	*Zizyphus lotus*
ZLPS	*Zizyphus lotus polysaccharide*
ZLP	Zizyphus lotus pulp
ZLS	Zizyphus lotus seed
ZLE	Zizyphus lotus endocarp
RSM	Response surface methodology
R^2	Determination coefficient
RMSE	Root mean square error
CCD	Central composite design
BBD	Box Bohken Design
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ABTS	2, 2'-9-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
FRAP	Ferric- reducing antioxidant power
FIC	Ferrous ions chelating
AChE	Acetylcholinesterase
AD	Alzheimer disease
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
LDL	Low Density Lipoproteins
HDL	High Density Lipoproteins
HepG2	Human hepatocyte carcinoma cell line
MCF-7	Breast cancer cell line
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
HPLC	High performance liquid chromatography
RP-HPLC-DAD	Reverse phase-High performance liquid chromatography -diode array detector
HPLC /MS	High-performance liquid chromatography-liquid chromatography mass spectrometry
[M - H]-	Negative mode
[M + H]+	Positive mode
R_t	Retention time

Table of Contents

List of Tables

Part I (Literature Review)		
Chapter II. Zizyphus lotus plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality : An overview		***Page***
1	Classification and vernacular names of *Zizyphus lotus* plant.	***47***
2	Chemical composition of *Z. lotus* in different part of jujube.	***49***
3	Most classes of polyphenols isolated from *Zizyphus* species.	***50***
4	Different green extraction techniques of antioxidants from *Zizyphus* plant	***56***
Part II (Material and Methods)		
5	The independent variables and its levels of factors influencing microwave and ultrasound assisted extraction for jujube phenolic extracts.	***74***
6	The independent variables and its levels of factors influencing microwave assisted extraction for jujube polysaccharide extract.	***74***
Part III (Results and discussion)		
Chapter I. Valorization of Zizyphus lotus pulp and peel for the recovery of phenolics and polysaccharides: optimization of microwave extraction process and evaluation of some biological activities		
9	Results of single-factor experiments for MAE from *Zizyphus lotus* pulp and peel.	***92***
10	Central composite design with the observed responses for yield of phenolic compounds (TPC, TFC, TTC) from *Z. lotus* pulp and peel using MAE.	***95***
11	Estimated regression coefficients for the quadratic polynomial model and the analysis of variance (ANOVA) for the experimental results of TPC, TFC and TTC from *Z. lotus* pulp and peels.	***96***
12	Comparison of extraction yield of bioactive compounds from *Z. lotus* pulp and peel by microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), and conventional solvent extraction (CSE). Results are expressed as means standard deviation.	***103***
13	Identification proposal of the phenolic compounds present in the *Z. lotus* pulp and peel by LC–MS/MS in ESI negative and positive mode. Peaks with a minus/plus superscript were analyzed in negative/positive mode. Compounds are indicated by retention time (R_t) order and numbered according to the appearance in each chromatogram, negative $[M-H]^-$ or positive mode $[M+H]^+$.	***106***
14	Response surface central composite design and results for	***117***

	extraction yield of *ZLPS.*	
15	Analysis of variance (ANOVA) for response surface quadratic model for extraction of *ZLPS.*	***118***
16	Predicted and experimental values of the responses at optimum conditions.	***120***
17	Quantitative analysis of sugars and protein in *ZLPS.*	***121***
18	Effect of *ZLPS* on heat induced protein denaturation (a), hypotonicity induced haemolysis (b) and heat induced haemolysis (c).	***125***
Chapter II. Optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from Zizyphus lotus seed using Response Surface Methodology and its biological effects		
19	Optimized MAE procedure of phenolic constituents from jujube seeds.	***130***
20	Regression coefficients estimation and their *p*-values for the reduced fitted quadratic model for microwave assisted ethanol extraction processing of phenolic compounds from jujube.	***131***
21	Phenolic yields of jujube seeds samples and some biological activities under optimum extraction conditions.	***135***
22	Phytochemical profile of *Z. lotus* seeds extracts under optimal conditions. Peaks with a minus/plus superscript were analyzed in negative/positive mode.	***141***
23	Box–Behnken design with the observed responses and predicted values of TPC from seeds using UAE.	***148***
24	Analysis of variance (ANOVA) for the experimental results obtained by using UAE.	***149***
25	Biological activities of *Z. lotus* seeds extract using UAE. Results are expressed as means ± standard deviation.	***153***
Chapter III. Valorization of Zizyphus lotus endocarp for the recovery of Total phenolic Compounds using optimized microwave and ultrasound process : Comparison to Zizyphus lotus pulp and seed extracts on their biological properties		
26	Box–Behnken design with the observed responses and predicted values of TPC from endocarp using UAE.	***160***
27	Analysis of variance (ANOVA) for the experimental results obtained by using UAE.	***161***
28	Biological activities of *Zle* endocarp extract using UAE. Results are expressed as means ± standard deviation.	***165***
29	Central composite design with the observed responses and predicted values of TPC from seeds using MAE.	***167***
30	Analysis of variance (ANOVA) for the experimental results obtained by using MAE.	***168***
31	Phenolic yields of jujube endocarp samples and some biological activities under optimum extraction conditions by MAE.	***171***

32	Identification proposal of bioactive compounds present in the *Z. lotus* endocarp MAE by LC–MS/MS in ESI negative and positive mode. Peaks with a minus/plus superscript were analyzed in negative/positive mode. Compounds are indicated by retention time (Rt) order and numbered according to the appearance in each chromatogram, negative [M − H]− or positive mode [M + H]+. NF : not fragmented.	***177***
Chapter IV. Comparison between optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from Zizyphus lotus L using Response Surface Methodology and its biological effects		
33	TPC, TFC and TTC recovery extracted by UAE and MAE from different parts of *Z. lotus.*	***186***
34	Effect of DPPH and FRAP assays for different parts of *Z. lotus* extracted by UAE and MAE.	***187***
35	Effect of HepG2 and MCF-7 cells tests for different parts of *Z. lotus* extracted by UAE and MAE.	***188***
36	Heatmap representing the intensity of all compounds present in jujube samples by LC–MS/MS in ESI negative mode. Intensity: 175000; 100.	***192***
37	Heatmap representing the intensity of all compounds present in jujube samples by LC–MS/MS in ESI positive mode. Intensity: 175000; 100.	***193***

List of Figures

Part I (Literature Review)		
Chapter I. polyphenol and polysaccharide –Generalities		***Page***
1	Phenolic compound classes.	***6***
2	Equilibrium between antioxidant compounds and pro-oxidants (ROS)	***11***
3	Mechanisms for the antioxidant activity	***12***
4	Structural organization of the plant wall	***25***
5	Primary structure of HG	***27***
6	Structure of rhamnogalacturonane I and II	***29***
7	Possible hydrogen bonds between a catechin dimer and a homogalacturonan	***33***
Chapter II. Zizyphus lotus plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality : An overview		
8	Green Food Processing: evolution or revolution	***41***
9	(A) Development and collapse of cavitation bubbles, and (B) schematic depicting classically thought bubble collapse at the solid surface	***43***
10	A brief description of phenomenon in the cell generated by microwave irradiation	***46***
11	*Zizyphus lotus* plant	***47***
Part II (Material and Methods)		
12	Map representing the areas of harvesting of the jujubes.	***67***
13	Parts of *Z. lotus* fruits.	***68***
14	Parts of *Z. lotus* plant used during experimental work (Photos from BBBS-Lab).	***68***
15	Single-factor experimental procedure of phenolics for both MAE and UAE of different jujube parts.	***70***
16	Preliminary study of polysaccharides extraction using MAE process and other extraction process of ZLP extracts.	***71***
17	Layout of the Central Composite Design (CDD) for 2 variables at 5 levels.	***72***
18	(a) the cube for BBD and three interlocking 22 factorial design (b)	***73***
19	MAE apparatus used during extraction of both polysaccharides and phenolic compounds (Photo from BBBS-Lab).	***75***
20	Ultrasonic equipment used during extraction of phenolic compounds (Photo fromGVRNAQ-Lab).	***76***
21	Reaction of reduction of DPPH radical	***78***
22	Reaction between ABTS radical and antioxidant compound	***79***
23	Principle of MTT assay	***83***
Part III (Results and discussion)		
Chapter I. Valorization of Zizyphus lotus pulp and peel for the recovery of phenolics and polysaccharides: optimization of microwave extraction process and evaluation of some		

	biological activities	
24	Graphical abstract of polysaccharides and phenolics extracted from jujube pulp and peel using microwave process.	***90***
25	Response surface analysis for the total phenolic compounds from *Z. lotus* pulp and peel with microwave assisted extraction with respect to ethanol concentration and solvent-to-solid ratio (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and microwave power (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).	***98***
26	Response surface analysis for the total flavonoids from *Z. lotus* pulp and peel with microwave assisted extraction with respect to ethanol concentration and solvent-to-solid ratio (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and microwave power (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).	***100***
27	Response surface analysis for the total condensed tanins from *Z. lotus* pulp and peel with microwave assisted extraction with respect to ethanol concentration and solvent-to-solid ratio (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and microwave power (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).	***102***
28	Total ionic chromatogram and ionic chromatograms extracted from ions identified in *Z. lotus* pulp and peel : (a) LC-MS/MS (-) and (b) LC-MS/MS (+).	***105***
29	Heatmap of the chemical profile and of *Z. lotus* pulp and peel extracts. Mean values refer to colors from minimum displayed in light orange to maximum represented with dark orange.	***110***
30	Effect of microwave power (a), irradiation time (b), and water to the raw material ratio (c) on the extraction yield of ZLPS (%)	***115***
31	The response surface plot (a, b, and c) indicating the effects of factors (X1, microwave power; X2, irradiation time; X3, liquid/solid ratio)	***120***
32	FTIR spectrum of the ZLPS extract	***122***
33	Comparison of DPPH*. radical scavenging activity (a), ABTS.+ scavenging activity (b), and FRAP scavenging activity of ZLPS and positive control (ascorbic acid) in the various concentrations	***123***
	Chapter II. Optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from Zizyphus lotus seed using Response Surface Methodology and its biological effects	
34	Graphical abstract of microwave and ultrasound assisted extraction of polyphenols from jujube seeds	***127***
35	Response surface plots for the total phenolic recovery from jujube seeds extracted by microwave extraction method with respect to ethanol concentration and irradiation time (A); ethanol concentration and microwave power (B); microwave power and irradiation time (C).	***133***

36	Total ionic chromatogram and ionic chromatograms extracted from ions identified in *Z. lotus* seeds samples : (A) LC-MS/MS (-) and (B) LC-MS/MS (+).	***136***
37	Heatmap of the chemical profile and of *Z. lotus* seeds samples. Mean values refer to colors from minimum displayed in beige to maximum represented with dark brown.	***139***
38	Response surface analysis for the total phenolic yield from *Z.lotus* seeds with UAE with respect to sonication time and ethanol concentration (a); solvent-to-solid ratio and ethanol concentration (b); sonication temperature and ethanol concentration (c); solvent-to-solid ratio and sonication temperature (d); solvent-to-solid ratio and sonication time (e); sonication temperature and sonication time (f).	***151***
Chapter III. Valorization of Zizyphus lotus endocarp for the recovery of Total phenolic Compounds using optimized microwave and ultrasound process : Comparison to Zizyphus lotus pulp and seed extracts on their biological properties		
39	Graphical abstract of microwave and ultrasound assisted extraction of polyphenols from jujube endocarps.	***158***
40	Response surface analysis for the total phenolic yield from *Z.lotus* endocarp with UAE with respect to irradiation time and ethanol percentage(A); solvent-to-solid ratio and ethanol percentage (B); microwave power and ethanol percentage (C); solvent-to-solid ratio and microwave power (D); solvent-to-solid ratioand irradiation time (E); microwave power and irradiation time (F).	***164***
41	Response surface analysis for the total phenolic yield from *Z.lotus* endocarp with MAE with respect to irradiation time and ethanol percentage(A); solvent-to-solid ratio and ethanol percentage (B); microwave power and ethanol percentage (C).	***170***
42	Total phenolic (A), flavonoid (B) and condensed tannin (C) contents of *Zizyphus lotus* extracts obtained by UAE and MAE processes. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).	***172***
43	Heatmap of the chemical profile and of *Z. lotus* endocarps. Mean values refer to colors from minimum displayed in beige to maximum represented with dark brown.	***174***
Chapter IV. Comparison between optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from Zizyphus lotus L using Response Surface Methodology and its biological effects		
44	Total phenolic (A), flavonoid (B) and condensed tannin (C) contents of *Zizyphus lotus* extracts obtained by UAE and MAE processes. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).	***183***
45	Antioxidant activity by DPPH (A) and FRAP assay (B) of *Zizyphus lotus* extracts obtained by UAE and MAE process. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p <$	***185***

	0.05).	
46	Percentage of AChE ihnibitory activity of *Zizyphus lotus* extracts (10 mg/mL of extract). Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).	***188***
47	Cytotoxic effect against HepG2 (A) and MCF-7 cell lines (B) assay of *Zizyphus lotus* extracts obtained by UAE and MAE process. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).	***189***

General Introduction

General Introduction.....1
References 4

Part I (Literature Review)

Chapter I. polyphenol and polysaccharide –Generalities.....5

I.1. Polyphenols -Generalities.....5
I.1.1. Phenolic compounds Chemical structure and classification.....5
I.1.2. Polyphenols and Human Health: Mecanism of action and prevention of Diseases.....10
I.1.2.1. Polyphenols and oxydatif stress.....10
I.1.2.1.1. Mechanism of phenolic compounds action.....11
I.1.2.1.2. Antioxidant effect of polyphenols in food.....15
I.1.2.1.3. Antioxidant effect of polyphenols in humans.....17
I.1.2.2. Polyphenols and cancer 18
I.1.2.3. Polyphenols and inflammation.....19
I.1.2.4. Polyphenols and Alzheimer disease 20
I.1.2.4.1. Anticholinesterase inhibitory.....21
I.1.2.5. Polyphenols and cholesterolemia.....22
I.1.2.5.1. HMGR inhibitory.....24
I.2.Polysaccharides -Generalities.....25
I.2.1. Organization of the plant wall.....25
I.2.2. Structure and polysaccharide composition of the plant wall.....26
I.2.2.1. Pectin 26
I.2.2.2. Cellulose.....30
I.2.2.3. Hemicellulose.....30
I.2.2.4. Protein 31
I.2.3. Cell wall polysaccharide-polyphenol interactions.....32
References 39

Chapter II. Eco-extraction : *Zizyphus lotus* plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality.....41

I. Introduction.....41
II.1. Utrasound process.....42
II.1.1.Physical principles of ultrasonic waves 42
II.1.2. Cavitation mechanism.....42
II.1.3. Application of UAE in food research.....43
II.2. Microwave process.....44
II.2.1. Electromagnetic Fields of microwaves 44
II.2.2. Mechanism of Microwave Heating.....44
II.2.3. Application of MAE in food research.....45
II.3. Botanical description of *Zizyphus lotus L* and its valorization.....46
II.3.1. General Background.....46
II.3.2. Nutritional composition of jujube.....47
II.3.3.Bioactive compounds of jujube.....49
II.3.4. Application to conventional extraction method.....51
II.3.5. Application to ultrasound extraction method.....53
II.3.6. Application to microwave extraction method.....57
References 66

Part II (Material and Methods)

Objectives.....65

Material and Methods.....66

I. *Zizyphus lotus* plant materials.....67
I.1. Source 67
I.2. Drying and Grinding.....67
I.3. Chemical reagents and equipment.....68
II. Experiment Design.....70
II.1. Response surface methodology.....71
II.2. Application to the microwave assisted extraction process.....74
II.3. Application to the ultrasound assisted extraction process.....75
III. Extraction procedure.....77
III.1. Microwave assisted extraction.....77
III.2. Ultrasound assisted extraction.....78
III.3. Conventional solvent extraction.....78
IV. Analytical methodology.....78
IV. 1. Total phenolic content (TPC).....78
IV.2. Total flavonoid content (TFC).....79
IV.3. Total condensed tannins content (TTC).....79
IV.4. Total carbohydrates and proteins content.....80
IV.5. Antioxidant activities.....80
IV.5.1. Scavenging activity against the $DPPH^{\cdot}$ Radical.....80
IV.5.2. Scavenging activity against the $ABTS^{\cdot+}$ radical.....81
IV.5.3. Ferric- reducing antioxidant power (FRAP) activity.....82
IV.5.4. Determination of ferrous ions chelating (FIC) activity 82
IV.6. Anti-inflammatory activities.....83
IV.6. 1. Inhibition of albumin denaturation.....83
IV.6.2. Membrane stabilization assay.....83
IV.6.3. Heat induced haemolysis.....84
IV.7. Acetylcholinesterase inhibition.....84
IV.8. Cytotoxicity studies on HepG2 and MCF-7 cells.....85
IV.9.HMGR Inhibitory activity.....86
IV.10. Infrared spectroscopy.....86
IV.11.High-Performance Liquid Chromatography with Diode Array Detector (HPLC–DAD) Analysis.....87
IV.12.High -performance liquid chromatography (HPLC) and liquid chromatographymass spectrometry (LC-MS) analysis.....87
IV.13.Data analysis.....88
References 91

Part III (Personal contribution)

Results and discussion.....92

Chapter I. Valorization of *Zizyphus lotus* pulp and peel for the recovery of phenolics and polysaccharides: optimization of microwave extraction process and evaluation of some biological activities 92

I.1.Optimization of microwave-assisted phenolic compounds extraction from Algerian jujube (*Zizyphus lotus L.*)pulp and peel.....93

I.1.1. Preliminary study of phenolic compounds.....94
I.1.2.Optimization of MAE conditions.....96
I.1.3. Identification of phenolic compounds from *Z. lotus* pulp and peel.....107
I.1.4. Antioxidant activities of *Z. lotus* pulp and peel extract.....113

I.2. Optimization of microwave-assisted polysaccharide extraction from Algerian jujube (*Zizyphus lotus L.*)pulp and peel.....115

I.2.1. Preliminary study of plysaccharide extraction.....116
I.2.2.Response Surface Methodology Optimization of Polysaccharide Extraction.....118
I.2.3.Chemical analysis of crude polysaccharide.....122
I.2.4.Infrared spectrum analysis.....123
I.2.5.Biological activities of ZLPS 124
I.2.5.1.Antioxidant activity.....124
I.2.5.2.Anti-iflammatory activity.....126

Chapter II. Optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from *Zizyphus lotus* seed using Response Surface Methodology and its biological effects.....129

II.1.Optimization of microwave-assisted phenolic compounds extraction from Algerian jujube (*Zizyphus lotus L.*)seeds.....130

II.1.1. Preliminary study of phenolic compounds extracted by MAE 131
II.1.2. Identification of bioactive compounds from Zls extracts.....138
II.1.3. Effect of MAE on biological ativities.....145
II.1.3.1. Determination of DPPH$^{\cdot}$ and FRAP activities.....145
II.1.3.2. Determination of AChE inhibitory activity.....146
II.1.3.3. Determination of HMGR Inhibitory activity.....147

II.2.Optimization of ultrasound-assisted phenolic compounds extraction from Algerian jujube (*Zizyphus lotus L.*)seeds.....149

II.2.1. Phenolic recoveries and UAE optimized process.....150
II.2.2. Effect of experimental coditions on TPC extraction yield.....152
II.2.3. Biological Activities.....154
II. 2.3.1.Antioxidants activity of Zls extract.....154
II.2.3.2.AChE inhibition of Zls extract.....156
II.2.3.3.HMGR inhibition of Zls extract.....157
II.2.3.4.Anti-proliferative activity of Zls extract.....157

Chapter III. Valorization of *Zizyphus lotus* endocarp for the recovery of Total phenolic Compounds using optimized microwave and ultrasound process : Comparison to *Zizyphus lotus* pulp and seed extracts on their biological properties.....160

III.1. Optimizing phenolic extraction conditions by UAE method.....162
III.1.1. Effect of UAE on biological ativities.....168
III.1.1.1. Antioxidants activity of *Zle* extract.....168
III.1.1.2. Anti-proliferative activity of *Zle* extract.....168

III.2. Optimizing phenolic extraction conditions by MAE method 169
III.2.1. Effect of experimental conditions on TPC extraction yield.....173
III.2.2. Identification of bioactive compounds from *Zle* extracts.....174
III.2.3. Biological Activities.....181
III.2.3.1.Antioxidants activity of *Zle* extract.....181
III.2.3.2.Anti-proliferative activity of *Zle* extract.....182

Chapter IV. Comparison between optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from *Zizyphus lotus* L using Response Surface Methodology and its biological effects.....183

IV.1. Comparison between ZLP, ZLS and ZLE on bioactive compounds.....184
IV.2. Comparison between ZLP, ZLS and ZLE on antioxidant activities.....186
IV.3. Comparison between ZLP, ZLS and ZLE on anticholinesterase inhibition.....188
IV.4.Comparison between ZLP, ZLS and ZLE on antioproliferative activity.....190
IV.5. Phytochemical profile of different jujube parts 192

References 213

General conclusion …….....…….....216

Perspectives…….....…….....217

Part I. Literature Review

General introduction

A positive relationship between foods and health has conduct to several scientific researches to find the significance of foods or food ingredients on specific body functions. The expression “functional food” refers to food with helpful functions. Functional foods are considered as of the most important topics of research and innovation in the food industry. Large quantities of bakery products are consumed all over the world. There are several reasons for this huge popularity including varied tastes, easy availability, extent shelf life and cheap price among other processed foods. Their evolution has experienced various stages of progress, reaching today a rising diversity **[1, 2].**Vegetable plants have always been a source of medicine. In recent years still a majority of the world's population is treated only with traditional herbal remedies, especially in developing countries. Today the pharmaceutical and food industries still rely largely on the diversity of secondary metabolites of plants (polyphenols, alkaloids, terpenes, ...) as primary (polysaccharides, proteins, lipids). Due to these countless beneficial characteristics for human health, researches have been intensified aiming to find fruits, vegetables, plants, agricultural and agro-industrial residues as sources of bioactive phenolic compounds. For the recovery of high-added value compounds from plant materials, they must be firstly separated from their original plant matrix. Obtaining these molecules often requires many long and costly steps, such as extraction, isolation and identification still hampering industrial development **[3, 4].**

Among the fruits, the jujube stands out as a very little valued source despite its wide availability in all the mediterranean region. However, the fruit is the edible and nutritive part of the plant. Several parts of *Zizyphus* have been used in traditional and ancestral medicine, both in North Africa and Middle East, for the treatment of several pathologies including liver complaints, obesity, urinary troubles, diabetes, skin infections, fever, diarrhea, insomnia, inflammation and peptic ulcers. The medicinal properties of this plant depend on the part of the plant concerned (root, leaf stalk, pulp or fruit) and the type of extract.Wehave recently shown that the fruit and pulp of this plant contained higher vitamin A and C contents than the other parts of the plant. The fruit pulp was the richest source of linoleic acid (18:2n−6), a precursor of n−6 fatty acids, and, the leaves were the richest source of vitamin E and linolenic acid (18:3n−3), a precursor of n−3 fatty acids **[5].** *Z. lotus* fruit contains important levels of carbohydrates, minerals, vitamins, fibers, amino acids, fatty acids and phenolic compounds, which are considered the main responsible for its health benefit **[6].**

Various novel extraction techniques have been developed for the extraction of antioxidant secondary metabolites including ultrasound-assisted extraction, supercritical fluid extraction, microwave-assisted extraction, and accelerated solvent extraction. Among these techniques, ultrasound-assisted extraction is a simple, efficient and inexpensive alternative. It is more effective at extracting secondary metabolites due to the acoustic cavitation effect produced in the solvent by the passage of ultrasonic waves which can lead to the destruction of cells and enhance the contact surface area between solid and liquid phases. These effects permit better penetration of the solvent into the sample increasing the extraction yield of secondary metabolites **[7].** Microwave Assisted Extraction (MAE) is a relatively new method by which microwave energy is used to heat polar solvents in contact with solid samples and to partition compounds of interest between the sample and the solvent, reducing both extraction time and solvent consumption. It also produces higher extraction rates and better results with lower costs **[8].**

Within this context, the objective of this thesis work was to set up a strategy of valorization of natural bioactive compounds. This strategy aims scientifically to meet the current demand of the food and nutraceutical industries, which require new formulations suitable for the use of functional food additives or ingredients of natural origin, in particular, to improve the efficiency and the conservation of molecules of interest. This study can have a significant impact on the economic level for the sectors using these new ingredients, as well as on the environmental level. Briefly, to approach the main objective, this thesis aimed to study the effect of extraction conditions on polyphenols and polysaccharides yields from jujube using innovative techniques such as microwaves, ultrasounds and comparison to conventional extraction. Thereafter, to evaluate the antioxidant effect of *Zizyphus* extracts (phenolic and polysaccharide) obtained by three methods by DPPH, ABTS, FIC and FRAP tests. As weel as, the effect of *Zizyphus lotus* on lowering cholesterol level in blood and there by the prevention from Alzheimer disease by studying the inhibition of the key enzyme acetylcholinesterase AChE and HMG-CoA reductase inhibition. Furthermore, to finish by the cytotoxicity effects against breast and liver cells (MCF-7 and HepG2 cell lines) of samples. However, the characterization and identification of the compounds mainly present in all parts of the plant (pulp and peel, seed, endocarp) were studied at the same time then evaluating the biological activity of three parts of jujube plant.

This PhD thesis is organized according to the following structure:

The first part is a bibliographical review which compiles the existing information on the jujube, in particular its fruits. As the aim of this thesis is the promotion of jujube for its application on an industrial scale, as well as to see its biological potential, a brief review of current knowledge on its biological activities is presented and summurized in the first chapter. However, the second chapter consists on a complete review of current knowledge on the green extraction methods and separations of phenolic and polysaccharide products. It describes some details about extraction by microwaves, ultrasounds, the mechanism, some applications, and environmental impacts with comparison to traditional techniques. This chapter explores also all the recent innovative methods applied in *Zizyphus lotus* and summurized in the second paper.

The second part sets out to define the general methodologies used throughout this work for the optimization of extraction parameters based on total polyphenols and polysaccharides through the realization of the experimental design based on response surface methodology either for MAE or UAE.

The third part consists of four distinct under-parts. Initially, demonstrats the results and discussion of optimization procedure of both phenolic and polysaccharide extracts from jujube pulp and peel obtained with comparing the qualitative and quantitative analysis between the studied method. (microwave and ultrasound) and compared to the conventional procedure (chapter I). Thus, followed by the optimization procedure of seeds extract (chapter II) and the optimization procedure of endocarps extract (chapter III). From the acquired knowledge, with these empirical approaches of MAE and UAE optimized methodology, it was possible to make a qualitative and quantitative comparison between these methods (microwave and ultrasound) and from each part of jujube plant. In the meantime the screening of antioxidant, anticancer, antiinflammatory activities and enzyme inhibition (ACh and HMG-CoA reductase) are presented depending on the studied extract (phenolic or polysaccharide) and the part of jujube (pulp and peel, seed, endocarp). It also describes the *in vitro* digestion of jujube with artificial gastric and pancreatic juices. Finally, this part ends with the identification of the main compounds of *Zizyphus lotus* present in all extracts was carried out using UHPLC-MS. As well as, the composition of *Zizyphus lotus* in primary and secondary metabolites is more particularly detailed. The purpose behind this comparison step was, to

offer ultimately a green, simple (one step), fast and effective separation method and giving higher biological effect with protecting quality. This chapter was summarized in the form of articles.

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Chapter I : polyphenol and polysaccharide -Generalities

Part I. Literature Review

Chapter I. Polyphenol and Polysaccharide-Generalities

Diets rich in fruits and vegetables provide unmatched benefits related with health, well-being, and longevity. The latest advances in the field of modern food and nutrition describe bioactive effects attributed to numerous phytochemicals, among which secondary metabolites play a crucial role. In parallel with these advances, worldwide consumers have been influencing the research on this field of knowledge, due to their increasing demands for healthier and biofunctional products **[1].** The protection that fruits and vegetables provide against these maladies has been attributed to the presence of several antioxidants, especially to antioxidative vitamins, including ascorbic acid (vitamin C), alpha-tocopherol (vitamin E) and ß-carotene (provitamin A). Nevertheless, recent studies seem to indicate that (poly) phenolic substances are the main phytochemicals with antioxidant properties found in higher plants **[2].** Polyphenols are commonly present at capricious level in all green plants. Phenolic compounds are low molecular weight molecules, widespread in nature and elaborated by fungi, yeast, algae, lichen, prokaryotes, insects, and mammals. In the producer organism, phenolic compounds are present in small amounts but under stress (physical/chemical/biochemical) these compounds are produced in relatively larger amounts. Thus, their role in the organisms is of defense against predators, infection, inflammation, and allelopathic interactions **[3].**

I.1. Polyphenols -Generalities

I.1.1. Phenolic compounds: chemical structure and classification

Phenolic compounds constitute one of the main classes of secondary metabolites. They display a large range of structures and they are responsible for the major organoleptic characteristics of plant-derived foods and beverages, particularly color and taste properties and they also contribute to the nutritional qualities of fruits and vegetables **[4].** Polyphenols are synthesized as part of the shikimic acid pathway. That pathway is the origin of several phenolic acids (a class of polyphenols) and p-coumaroyl CoA, the latter of which then gives rise to lignans and, by combination with the malonic acid pathway, to stilbenes and flavonoids, the other three most common classes of polyphenols **[5].**

There are three main classes of polyphenols: phenolic acids, flavonoids, other phenolics (e.g., stibenes, lignans, tannins, xanthones, lignins, chromones, and anthraquinones) **[6].** As a

function of the number of phenol rings that they contain and of the structural elements that bind these rings to one another, the classification. Among these compounds, flavonoids constitute one of the most ubiquitous groups of all plant phenolics. So far, over 8,000 varieties of flavonoids have been identified. Until ~50 years ago, information on the working mechanisms of flavonoids was scare. Others demonstarted that carotenoids are the most important natural pigments are which are tetrapyr role derivatives of naturally occurring phenolic compounds ubiquitously distributed in plant kingdom **[4].**

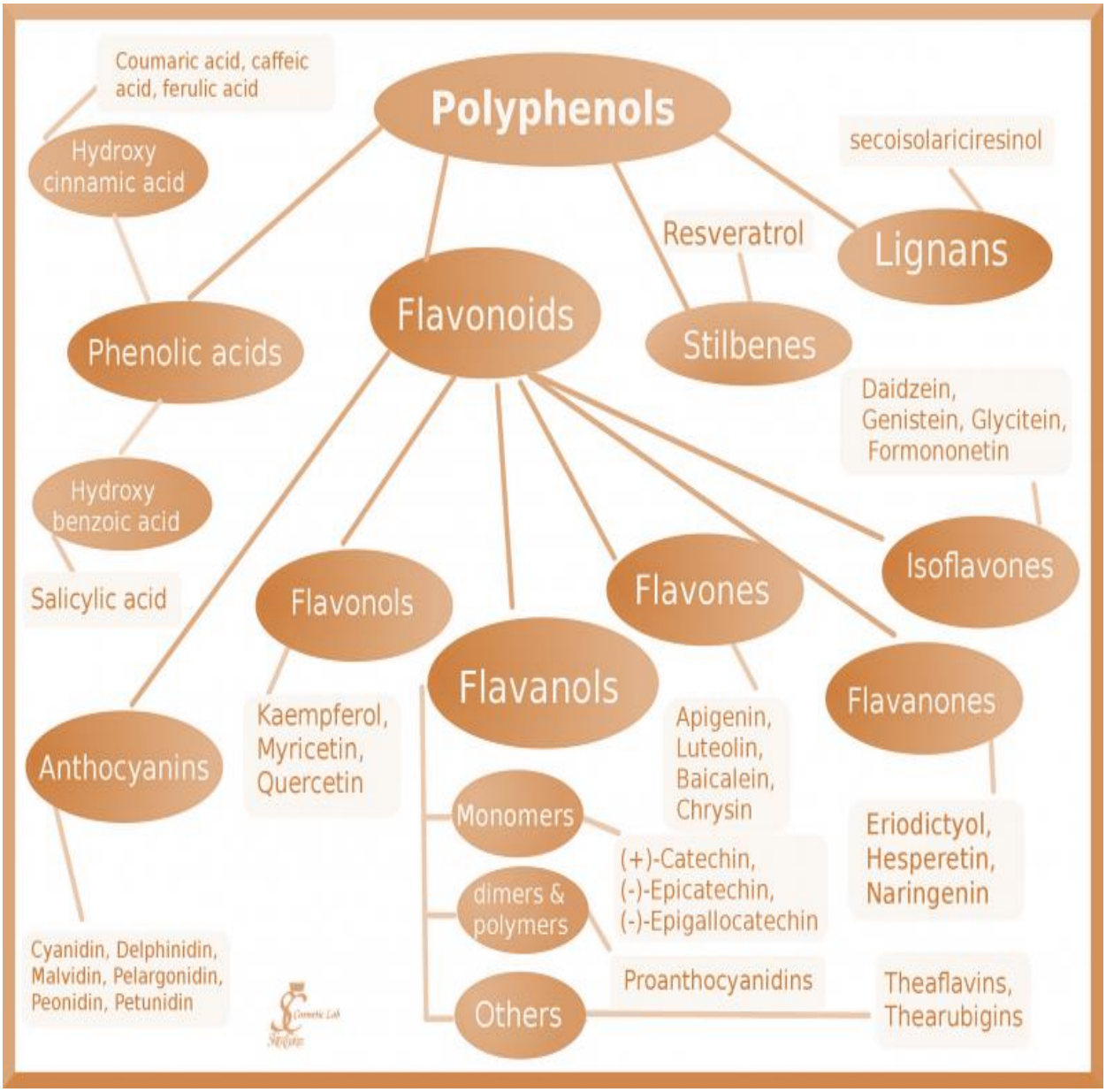


Figure 1 : Phenolic compound classes.

❖ ***Phenolic acid***

The phenolic acids represent approximately 30% of all dietary polyphenols, depending on the geographical location, food-harvesting techniques, processing practices, and cultural considerations inherent to the region of origin **[6].** The two major subclasses of phenolic acids are hydroxybenzoic (C_6C_1) and hydroxycinnamic (C_6C_3) acids. These two classes of compounds possess a common carbon skeleton and differ only in the numbers and positions of the hydroxyl groups on the aromatic ring. Only a minor fraction of phenolic acids exists in the free form. Instead, the majority are linked through ester, ether or acetal bonds either to structural components of the plant, larger polyphenols or smaller organic molecules (e.g., glucose, quinic acid). These linkages give rise to a vast array of derivatives.

Hydroxybenzoic acids are found in the free form as well as combined into esters of glycosides. Some of them are constituents of hydrolysable tannins which are compounds containing a central core of glucose or another polyol esterified with gallic acid or its dimer hexahydrohydiphenic acid, also called gallotannins and ellagitannins **[7, 8].** Hydroxybenzoic acids include ellagic and gallic acids which usullay occur as hydrolyzable tannins.

The major dietary hydroxycinnamic acids, caffeic and ferulic acids are heat sensitive. Caffeic and quinic acids combine to form chlorogenic acids which is widely distributed in fruits and vegetables but notable for its high concentration in seeds **[9].** They are frequently conjugated to organic acids and sugars. In nature, polyphenols are usually water-soluble compounds but can also be volatile materials **[3].**

❖ ***Flavonoids***

Flavonoids comprise the most abundant class of plant polyphenols with more than 6000 structures which have been identified. Its chemical structure comprises two benzoic rings connected with a pyran ring. Depending on its substituents and the most abundant unsaturation they present **[10].** They share a carbon skeleton of diphenyl propanes, two benzene rings (A and B) joined by a linear three carbon chain ($C_6C_3C_6$). This central chain usually forms a closed pyran ring (C) with one of the benzene rings [7]. Based on the variation in the type of heterocycle involved, flavonoids may be are grouped into anthocyanins and anthoxanthins. Anthocyanins are molecules of red, blue, and purple pigment. Which include flavones, isoflavones, flavanones and flavonols, anthoxanthins are color less or white to yellow molecules **[9].**

This subdivision is primarily based on the presence (or absence) of a double bond on position 4 of the C (middle) ring, then a double bond between carbon atoms 2 and 3 of the C ring, and hydroxyl groups in the B ring. Flavones are characterized by the presence of a double bond between C_2 and C_3 in the heterocycle of the flavan skeleton. The B ring is attached to C_2 and usually no substituent is present at C_3. This exactly represents the difference to the flavonols where a hydroxyl group can be found at that C_3 position while flavanones have a saturated three-carbon chain. Flavanols contain a saturated three-carbon chain with a hydroxyl group in the C_3. Anthocyanidins are positively charged at acidic pH and this equilibrium form is called flavylium cation (2phenylbenzopyrylium). In isoflavonoids the substitution is at the 3-position. Individual differences within each group arise from variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation **[8].**

Flavonoids occur both in the free form and as glycosides, most are Oglycosides but a considerable number of flavonoid C-glycosides are also known. The Oglycosides have sugar substituents bound to a hydroxyl group of the aglycone, usually located at position 3 or 7, whereas the C-glycosides have sugar groups bound to a carbon of the aglycone, usually C_6 or C_8. The most common carbohydrates are rhamnose, glucose, galactose and arabinose. Flavonoid-diglycosides are also frequently found. Two very common disaccharides contain glucose and rhamnose, 1→6 linked in neohesperidose and 1→2 linked in rutinose. An interesting combination of flavonoid and lignan structure is found in a group of compounds called flavonolignans. They arise by oxidative coupling process between flavonoid and a phenylpropanoid, usually coniferyl alcohol. Additionally, the flavanols exist as oligomers and polymers referred to as condensed tannins or proanthocyanidins [7].

- ***Tannins***

The definition of tannin has been enlarged to cover a whole mass of constituents which give general phenolic reactions. The essential property of tannin is the ability to combine with proteins and other polymers such as pectin. Generally tannins are water soluble phenolic compounds; they have molecular weight in between 500-3000, giving the usual phenolic reactions and have the special properties such as ability to precipitate alkaloids, gelatin, and other proteins **[11].**

Tannins were once classified into two groups: pyrogallol type tannins and catechol type (or catechin type) tannins, according to the polyphenol groups in their molecules. Then, the developments in tannin chemistry led to the renaming of these two groups to hydrolyzable tannins and condensed tannins **[12].** Condensed tannins are certainly more important than hydrolyables tannins; much less is known about their structure and many aspects are yet to be elucidated. This type of tannin produces "tannin reds" while boiling with acid. Traditionally, most commercial sources of this type are heartwood of quebracho, bark of wattle. These have been used in leather process industries to get better types of quality leather. However, hydrolyables tannins are an ester of sugar and phenolic acids or their derivatives; the sugar is usually glucose, but in some cases polysaccharides have been identified. Acidic, basic or enzymatic hydrolysis often occurs spontaneously during extraction or purification. This type or tannin is further subdivided into ellagitannin and gallotannin **[11].**

Tannins are usually defined as water-soluble polyphenolic substances and have ability bound to proteins that form insoluble or soluble tannin-protein complexes. As a consequence, tannins able to make complex with polysaccharides (cellulose, hemicelluloses and pectin) and nucleic acids, steroids, alkaloids, and saponins. There are some observations with regard to the presence of tannins that deserve some attention. For example, within plant cells, tannins are found in the vacuole and this has been suggested to be a method to preventing inhibition of the cell metabolism by tannins. Also, one must astound about the energetic costs and on the reasons for such a practice, especially when plants devote so much carbon to the production of tannins. It was suggested secondary metabolism serves to maintain primary metabolism in circumstances not propitious for growth. In recent years many researchers demonstrated that tannins have positive effects on animals by anti microbial, anthelmintic, protein bypassed effects in ruminants **[13].**

- ***Stilbenes***

Stilbenes (1,2-diarylethenes) belong to a relatively small group of non-flavonoid class of phenolic compounds found in a wide range of plant sources. Ring A usually carries two hydroxyl groups in the m-position, while ring B may be substituted by hydroxyl and methoxyl groups in various position. Stilbenes exist as stereoisomers and naturally occurring stilbenes are overwhelmingly present in the *trans* form. They occur in free and glycosylated forms and as dimeric, trimeric and polymeric stilbenes, the so-called viniferins. One of the most relevant

and extensively studied stilbene is *trans*-resveratrol for which anticarcinogenic effects have been shown during screening of medicinal plants mainly grapes **[7].**

- ***Lignans***

Lignans also constitute a group of non-flavonoid phenolics that are structurally characterized by the coupling of two phenylpropanoid units by a bond between the β-positions in the propane side chains. Lignans comprise a whole class of compounds with a similar basic skeleton, but with large variations in substitution patterns. They are mostly present in the free form, while their glycoside derivatives are only a minor form **[7].** Lignans are metabolized to enterodiol and enterolactone by the intestinal microflora. The low quantities of secoisolariciresinol and matairesinol that are ingested as part of our normal diet do not account for the concentrations of the metabolites enterodiol and enterolactone that are classically measured in plasma and urine **[14].**

I.2.1. Polyphenols and Human Health: Mecanism of action and prevention of Diseases

I.2.1.1. Polyphenols and oxydatif stress

The production of oxidants in living aerobic species occurs during the respiration process by the reduction of molecular oxygen in sequential steps to produce water. In this process, short-lived reactive intermediate chemical species are produced as byproducts, the so-called reactive oxygen species (ROS), as well as reactive nitrogen species (RNS). ROs are highly reactive oxidants and arise from the reduction of an electron of the molecular oxygen, with the formation of the three primary species: superoxide radical (O2•-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•). In normal physiological conditions, ROS are important in energy production; defense against foreign agents such as viruses and bacteria; intercellular signaling; and programmed cell death (apoptosis). However, ROs are an emerging class in which exposure to pollutants, such as tobacco, smoke, drugs, xenobiotics, radiation, and others, contributes to its overproduction, and consequently to the phenomenon of oxidative stress. Oxidative stress is defined by the imbalance caused by the excessive production of ROS and / or limited antioxidant defense, which implies oxidative damage to structures of biomolecules of DNA, lipids, carbohydrates and proteins, as well as other cellular components and consequently in the development and progression of several pathologies including the degenerative ones like cancer, cardiovascular diseases, cataract, decline of the immune system and cerebral dysfunctions. In order to prevent pathological levels of ROS, the

human organism has an antioxidant defense system formed by endogenous and exogenous antioxidant sources. In the condition of redox equilibrium between the production of oxidant species and the performance of the antioxidant defense, the condition of homeostasis in which the relative regulation of the physiological functioning appears becomes prominent (Figure 2) **[15].**

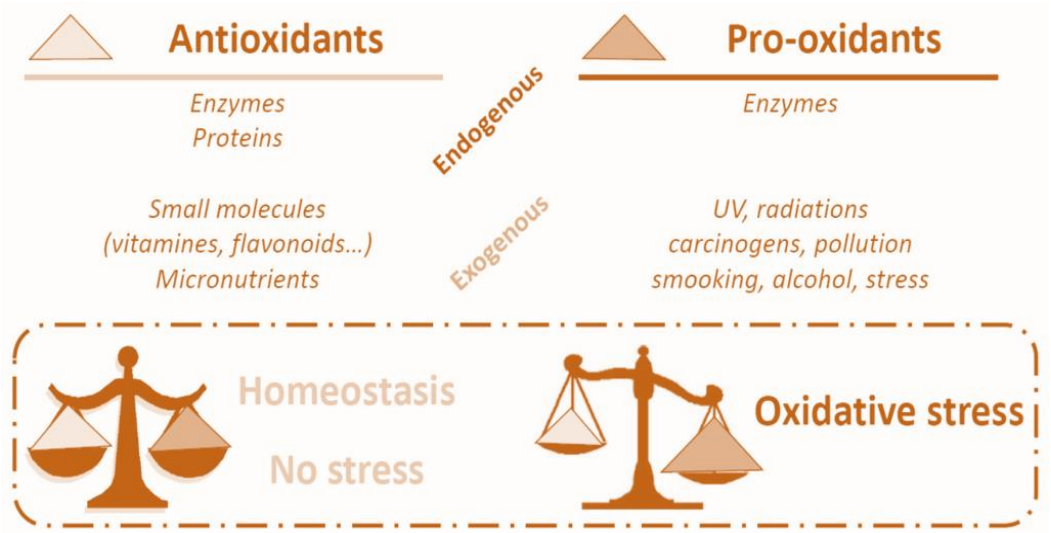


Figure 2 : Equilibrium between antioxidant compounds and pro-oxidants (ROS) **[16].**

Widely distributed in plant kingdom and abundant in our diet plant polyphenols are today among the most talked about concerning the classes of phytochemicals. There are several thousand plant-derived compounds of biogical interest that have more than one phenolic hydroxyl group attached to one or more benzene rings, thus qualifying as polyphenols. In recent years, polyphenols have gained a lot of importance because of their potential use as prophylactic and therapeutic agents in many diseases, and much work has been presented by the scientific community which focuses on their antioxidant effects **[17].**

I.1.2.1.1. Mechanism of phenolic compounds action

The molecular basis for the antioxidant properties of polyphenols is recognised into three main mechanisms, arising from the direct reaction with free radicals, and from the chelation of free metals, the latter involved in reactions finally generating free radicals **[18].**

- ***Inactivate free radicals***

As primary antioxidants, polyphenols inactivate free radicals according to the hydrogen atom transfer (HAT) (1) and to the single electron transfer (SET) (2) mechanisms. In mechanism 1,

the antioxidant, ArOH, reacts with the free radical, R, by transferring to it a hydrogen atom, through homolytic rupture of the O–H bond.The products of the reaction are the harmless RH species and the oxidised ArO radical. Even if the reaction leads to the formation of another radical, it is less reactive with respect to R because stabilized by several factors (see below). The SET mechanism (2) provides for an electron to be donated to the R. The anion R is an energetically stable species with an even number of electrons, while the cation radical ArOH+ is also in this case a less reactive radical species. In particular, the ArO and ArOH+ are aromatic structures in which the odd electron, originated by the reactions with the free radical, has the possibility to be spread over the entire molecule, resulting into a radical stabilization. In the former mechanism, the bond dissociation enthalpy (BDE) of the phenolic O–H bond is an important parameter in evaluating the antioxidant action; the lower the BDE value, the easier the dissociation of the phenolic O–H bond and the reaction with the free radicals. In the SET mechanism, the ionisation potential is the most significant parameter for the scavenging activity evaluation; the lower the IP value, the easier the electron abstraction and the reaction with free radicals **[18].**

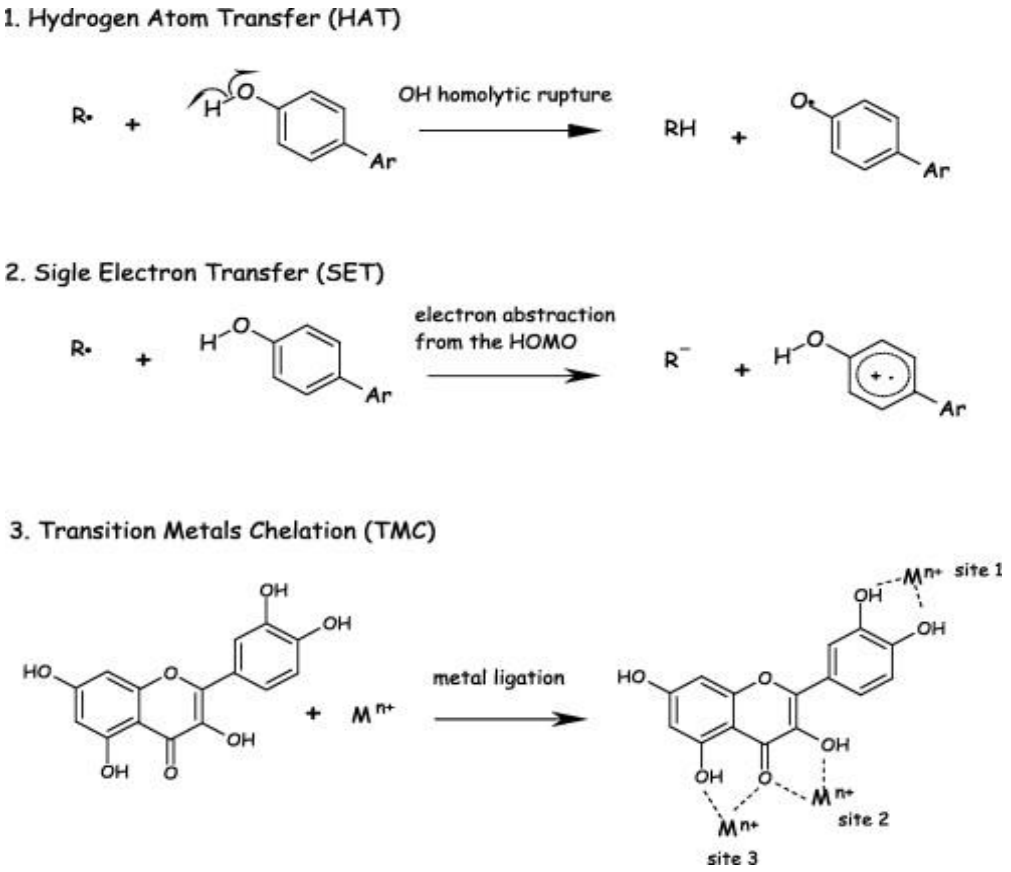


Figure 3 : Mechanisms for the antioxidant activity **[18].**

❖ ***Transition Metals Chelation***

Another antioxidant mechanism (Transition Metals Chelation, TMC, see Scheme 2) arises from the possibility that transition metals ions may be chelated by polyphenols, leading to stable complexed compounds. The latter entrap metals and avoid them to take part in the reactions generating free radicals. In fact, some metals in their low oxidation state (mainly Fe2+) may be involved in Fenton reactions with hydrogen peroxide, from which the very dangerous reactive oxygen species (ROS) OH is formed:

H2O2 + Fe2+ (Cu+) •O H + -OH + Fe3

The OH is generally accepted to be one of the most reactive radicals. It has a very short half-life (around 109 s) and a very high reactivity. With respect to the hydroperoxides that are metabolized by superoxide dismutase, hydroxyl radicals cannot be eliminated by enzymatic reactions. So they will react with every kind of substrate they encounter metals like copper, manganese, cobalt are able to catalyse this reaction, under certain conditions when these metal ions are not bound to proteins or chelators. Fenton-like reaction may take place and cause site specific accumulation of free radicals and initiate biomolecules damage processes **[18].**

❖ ***Inhibition of enzyme***

Polyphenols have an affinity for a wide variety of proteins **[19],** as a consequence of their capacity to develop van der Waals interactions through their aromatic rings and hydrogen bonds through their phenolic groups. For instance, monomeric flavonoid aglycones, usually the planar and polarizable flavones and flavonols, have been reported to bind many globular proteins, including enzymes, receptors and transporters **[20].**

Inhibition of free radical-generating enzymes in biological systems is an important mechanism of antioxidant effect for polyphenols. Several works have reported that polyphenols are the molecules most likely to be involved in this effect by formation of inhibitor-enzyme complex. This double action is clearly demonstrated in the case of Xanthine oxidase (XO), enzyme catalyzing the ultimate step in purine metabolism, i.e. the conversion of hypoxanthine into xanthine and of xanthine in uric acid **[21].** Mammalian 15-lipoxygenase (15-LOX) catalyzes the conversion of arachidonic acid into eicosanoids such as leukotriene B4. LKB4 is produced in human atherosclerotic lesions where it mediates inflammatory responses. Moreover, 15-LOX has been proposed to take part in LDL oxidation in the

vascular wall **[22].** Hence, its inhibition is a potential anti-inflammatory and antioxidant mechanism relevant to the prevention of CVD. Myeloperoxidase (MPO), the most abundant protein in neutrophils and macrophages, is secreted by these leucocytes at the site of inflammation. As such, MPO may be implicated in LDL oxidation during the development of cardiovascular disease. MPO is a heme enzyme that reduces H2O2 into H2O while being converted in a twoelectron oxidized intermediate. In endothelial cells, many dietary flavonoids inhibit superoxide production either by direct scavenging or inhibition of NADPH oxidase. Consequently, the reaction between NO and superoxide is quenched. The subsequent increase in steady-state NO concentration triggers a dilation of arterial vessels and lowers the blood pressure **[20, 23].**

- ***Prooxidant activity***

While the antioxidant properties of flavonoids support a positive role in human nutrition and disease prevention, some focus has involved the prooxidant activity of these compounds *in vitro.* Concentrated extracts of flavonoid-rich plants such as propolis, pine bark, green tea leaves, soy isoflavones and grape seed are widely marketed as nutraceuticals, targeting the aging population and individuals with cardiovascular disease, cancer and chronic inflammatory conditions. Thus, reports of mutagenicity related to flavonoid-mediated oxidative damage raise obvious concerns.

Prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups. In some reports, a series of mono- and dihydroxyflavonoids demonstrated no detectable prooxidant activity, while multiple hydroxyl groups, especially in the B-ring, significantly increased production of hydroxyl radicals in a Fenton system. The latter compounds included myricetin and baicelein, both of which have a pyrogallol structure in the A-ring, which has also been reported to promote hydrogen peroxide production from which Fenton reaction may generate highly reactive hydroxyl radicals.This prooxidant effect is responsible for the cytotoxic and proapoptotic effects of flavonoids isolated from various herbal medicines. In the presence of RNS, flavonoids with A- or B- ring pyrogallol configurations induce DNA single-strand breakage. There is also evidence that the unsaturated 2,3-bond and 4-oxo arrangement of flavones may promote the formation of ROS induced by divalent copper in the presence of oxygen. Collectively, this information suggests that some of the same structural attributes that optimize antioxidant capacity may also exacerbate oxidative stress and damage to functional and structural cellular molecules. However, considering the role of flavonoid radical stability in prooxidant behavior postulated

by Bors and colleagues, structural advantages to radical stability that increase antioxidant activity, such as a 3′4′-catechol, 3-OH, and conjugation between the A- and B-rings, may modulate adverse oxidative effects of flavonoids **[24].**

I.1.2.1.2.Antioxidant effect of polyphenols in food

The process of autoxidation is of the most importance when it comes to food products. Lipid oxidation can occur via three primary mechanisms: autoxidation, photosensitized oxidation and enzyme catalyzed oxidation **[25].** Autoxidation reactions are usually subdivided into initiation, propagation and termination reactions. An antioxidant is a compound capable of stopping the propagation reaction which is a chain reaction. Autoxidation occurs in three stages: initiation (formation of free radicals), propagation (freeradical chain reaction) and termination (formation of nonradical species) **[26].**

- ***Initiation***

One of the most intriguing problems in this area is the source of initial (primordial) free radicals, which initiate the autoxidation chains. Initiation reactions can be triggered by singlet oxygen, $^1O_2$, excited states of photosensitizers, radiation (cosmic and otherwise), and environmental pollutants (ozone and $N0_2$). Two types of initial reactions can take place: abstraction of H atoms from various bonds and addition to unsaturated compounds,

$$X + RH_2 \rightarrow {}^{\cdot}RH + XH \quad (1)$$

$$X + > C = C \leftarrow\rightarrow C - C < \quad (2)$$

Where X can be singlet oxygen, excited state, or a free radical. These reactions are followed by addition of 0_2 to the free radical sites

$$0_2 + {}^{\cdot}RH \rightarrow HROO^{\cdot} \quad (3)$$

$$0_2 + \text{^cx} \rightarrow (4)\ X\ OO$$

- ***Propagation***

Peroxy radicals formed in reactions (3) and (4) can also abstract hydrogen from certain bonds, e.g.

$$HROO\text{-} + RH_2 \rightarrow HROOH + {}^{\cdot}RH \quad (5)$$

Reaction (5)) is followed by reaction (3) and the chain reaction is set. The chain can be quite long (e.g., 100), hence one initial •RH free radical can produce many (100) hydroperoxide molecules, HROOH.

- ***Termination***

Free radical-radical reactions are extremely efficient and lead to disappearance of free radicals, e.g.

$$2\ HROO^{\cdot} \rightarrow HROH + R{=}0 + 0_2 \quad (6)$$

A particular termination reaction is a reaction of a peroxy radical with an antioxidant.

- ***Antioxidants***

Antioxidants are special compounds, highly reactive toward peroxy radicals

$$AH_2 + HROO^{\cdot} \rightarrow {}^{\cdot}AH + HROOH \quad (7)$$

The antioxidant free radical, ·AH, has to have certain properties (a) it should not react with 02, (b) if it does react, the product of that reaction should not engage in the propagation reaction (5). The antioxidant free radicals, ·AH, usually disappear in a reaction with each other.

The inhibition of the oxidative degradation processes caused by the highly reactive oxygen species supposes the action of a control factor namely antioxidants. An antioxidant may be defined as any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate. Antioxidants were used as additives in fats, oils and manufactured food for preventing or delaying the oxidative degradation of food. For this reason, in the latest years the interest of changing the synthetic antioxidants with natural ones has increased. Antioxidant compounds in food play an important role as a health-protecting factor. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radical such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanism **[27, 28].** Morever, secondary metabolites like flavonoids, phenolic acids, saponins and alkaloids are a specific antioxidants compounds that protect plant, animals, food and human from oxidative stress which is a result of the production of reactive oxygen species (ROS), including superoxide anion radical, hydrogen peroxide, hydroxyl radical as well as reactive nitrogen species (RNS) which can cause inflammation and contribute to tissue damage. Besides, in food products, free radicals react with lipids and cause lipid peroxidation which affects the quality of product (taste, color and flavor) **[29].**

I.1.2.1.3. Antioxidant effect of Polyphenols in humans

The effects of polyphenols on health are inseparable from the notion of bioavailability, During the digestion process the food undergoes many changes and comes in contact with different enzymes which may change the components or structure of the food. Bioavailability and bioaccessibility of food ingredients are influenced by the physiochemical and biochemical reactions involved in the digestion process **[30].** Mainly the bioaccessability of polyphenols present in the fruits and vegetables are influenced by the different food processing technologies. Further, breakdown of food through mouth mastication, stomach digestion and intestinal digestion also affect the bioaccessability of poly phenols when the food mixed with gastrointestinal secretions **[31].**

In a general sense, bioavailability is related with the concentration of a given compound or even of its related metabolites that is absorbed and becomes active in the target organ/system. Besides this, the term relative bioavailability is also used in nutrition to describe the bioavailability of a particular compound in relation to another. But overall, in the modern definition of bioavailability, several correlated and strictly linked process are covered, namely, liberation (release of the compound from its matrix), absorption (passage of the compound from the site of administration to the systemic circulation), distribution (passage of the compound from the systemic circulation to the body tissues—extra vascular space), metabolism (biochemical reactions that leads to the compound transformation), and excretion (elimination of the compound, or its derived metabolite from the body, through urinary, dermal, biliary or pulmonary system) **[1].**

One of the main factors influencing bioavailability is the chemical structure of the compound. In foods, most polyphenols exist as polymers or in glycosylated forms—the sugar group is termed as the glycone and the nonsugar group (the actual polyphenol) is termed the aglycone. In these native forms, polyphenols cannot be absorbed and have to be hydrolyzed by the intestinal enzymes or by the colonic microflora before absorption. Anthocyanins represent an exception, because the intact glycosides can be absorbed and detected in the circulation **[32].** The specific chemical structures of polyphenols as well as the type of the sugar in the glycoside determine their rate and extent of intestinal absorption. Aglycones can be absorbed from the small intestine, while glycosides, esters and polymers must be hydrolyzed by intestinal enzymes, or by the colonic microflora, before they can be absorbed. Although this can be considered as a general rule, some exceptions occur like unchanged anthocyanin glycosides detected in human plasma and urine The absorption of plant

flavonoids which are predominantly bound to different sugars mostly occurs in the small intestine **[7].**

The host-related factors that affect bioavailability can be further subdivided into intestinal factors and systemic factors. Intestinal factors are probably the most important among the ones discussed below. Following the ingestion of polyphenols, the absorption of some but not all components occurs in the small intestine where the glycosides are hydrolyzed. The fraction of polyphenols that is not absorbed in the small intestine reaches the colon, where it undergoes substantial structural modifications. In fact, the colonic microflora hydrolyzes glycosides into aglycones and degrades them to simple phenolic acids. This activity is of great importance for the biological action of polyphenols because specific active metabolites are produced in such a way. It is important to underline that there is great inter-individual variability in producing these active metabolites. Once absorbed, and prior to the passage into the blood stream, polyphenols undergo other structural modifications due to conjugation processes, that mainly include methylation, sulfation, and glucuronidation. These modifications could affect the bioavailability of polyphenols and, consequently, their biological activity. The conjugation mechanisms are highly efficient, and free aglycones are generally either absent or present in low concentrations in plasma after consumption of nutritional doses. Therefore, it is clear that, during the course of absorption, polyphenols are extensively modified (Fig. 2). Consequently, the compounds that reach cells and tissues are often chemically, biologically, and in many instances functionally different from the original dietary form **[33].**

Cleavage of oligomeric flavonoids such as procyanidins may occur in the stomach in environments of low pH. All classes of flavonoids undergo extensive metabolism in the jejunum and ileum of the small intestine and resulting metabolites enter the portal vein and undergo further metabolism in the liver. Colonic microflora degrade flavonoids into smaller phenolic acids that may also be absorbed. The fate of most of these metabolites is renal excretion, although, some may enter cells and tissues **[34].**

I.1.2.2. Polyphenols and cancer

Cancer is a major health problem across the globe which refers to a group of diseases caused by abnormal cell growth with invasive potentials. A high proportion of cancer incidence and deaths are due to different environmental and genetic factors such as high body mass index,

low fruit and vegetable intake, lack of physical activity, tobacco use, alcohol consumption, exposure to radiation, chronic infections, and heredity. Introducing novel bioactive components with natural origins, particularly from plant sources, may be considered as a new and reliable therapeutic element to treat different types of human cancers on the basis of their selective molecular targets **[35]**. In addition, oxidative stress plays a crucial role in the pathophysiology of different types of cancer. Therefore, much attention has been paid to antioxidants as novel therapeutic strategy for cancer . During the past two decades, plant-derived bioactive compounds have been reported as novel health-giving agents for prevention and/or mitigation of different human diseases such as cancer, inflammation, cardiovascular, and neurodegenerative diseases **[36]**.

Polyphenols are able to prevent cancer by reducing or blocking the harmful effects of free radicals on cells through their scavenging properties. Their varied chemical structures make them versatile in neutralizing free radical activities thereby preventing or reducing oxidative stress to levels that do not damage cellular DNA and regulatory protein synthesis needed for the coordination of cellular activities **[37].** Experimental confirmations have established that polyphenols also influence cancer propagation, and are able to suppress progression and contribute to the healing of damaged cells. Polyphenols can protect normal cells, have cytotoxic effects on cancerous cells, modulate growth factor-receptor interfaces and cell signaling cascades that regulate survival of normal cells and apoptosis of cancerous cells **[38].** More than 5000 flavonoids have been identified and are distributed in a wide range of plants. On the basis of their chemical structures, these flavonoids have been grouped into 10 categories, 6 of which including flavones, flavanones, anthocyanidins, flavonols, isoflavones, and catechins are commonly present in the human diet. Many of these flavonoids possess documented anticancer activity both in animal and cellular model systems **[36].**

I.1.2.3. Polyphenols and inflammation

Inflammation may be potentially harmful, causing life threatening hypersensitivity reactions and progressive organ damage. Defined as a basic way in which the body reacts to infection, irritation or other injury, the key feature being redness, warmth, swelling and pain. Inflammation is now recognized as a type of nonspecific immune response. In other words, inflammation is the basic process whereby tissues of the body respond to injury. Although we have learned a lot about the signaling pathways that link energy accumulation [adiposity] to chronic inflammation, we know little about the real biological significance of the

inflammation. At present, inflammation is defined by the presence of five macroscopic pathological phenomena, four of them proposed by Celsus as long as 2000 years ago. [39].

In recent years, to contrast the inflammatory pathogenic and chronic diseases, the focus was on traditional remedies with natural compounds derived from herbs, spices and medicinal plants. The renaissance of plants derivatives is due to several reasons such as the higher side effects of the conventional medicine. In particular, the attention is about polyphenols that inhibit the inflammation process due to their antioxidant capability. The polyphenols present in fruits and vegetables such as quercetin, rutin, naringin, naringenin, hesperidin and their derivatives, possess high antioxidant properties and may play a dietary role in reducing the risk of chronic diseases such as cardiovascular pathologies and cancer. Moreover, epidemiological studies have indicated that populations who consume foods rich in polyphenols have lower incidences of inflammatory disease **[40].**

Different targets are involved in the antiinflammatory activities of polyphenols: these can be subdivided into targets related to the arachidonic acid-dependent pathways such as cyclooxygenase (COX) inhibition, lipoxygenase inhibition, and phospholipase A2 inhibition. Within the arachidonic acid-independent pathways, nitrous oxide synthase (NOS), nuclear factor kB (NFkB) and NSAID activated gene-1 (NAG-1) are targets of polyphenols. In addition, polyphenols exert effects on human T helper type 1 and 2 cytokine production. Within the arachidonic-dependent pathway polyphenols partly act in relation to their antioxidative activity. This relationship was investigated more than 20 years ago and a couple of papers presented data on COX-1 and COX-2 inhibition at the transcriptional and enzyme level. Based on recent research, however, it has become clear that polyphenols act on both pathways as antioxidants and as modulators of gene expression. The type of activity can be bearly discriminated. COX inhibition may account for the antiinflammatory effects that reduce prostaglandin synthesis in the arachidonic pathway **[41, 42].**

I.1.2.4. Polyphenols and Alzheimer disease

Alzheimer's disease (AD) is the most common cause of progressive decline of cognitive function in aged humans, and is characterized by the presence of numerous senile plaques and neurofibrillary tangles accompanied by neuronal loss. Alzheimer's disease (AD) is the most common form of senile dementia, accounting for more than 50% of the cases. The prevalence increases logarithmically with age. The incidence of AD increases from 0.1% of the

population at age 60 ± 65 to as high as 47% of the population over age 85. Clinical manifestations of AD are primarily progressive impairments in memory, language, calculation, visuospatial perceptions, judgment, and behavior. Some patients show evidence of psychosis. Activities of daily living become progressively more impaired, and the patients are profoundly demented and often mute, incontinent, and bedridden in the late stage of the disease. The majority of AD cases are late in onset, lack an obvious genetic etiology, and are characterized as sporadic, whereas a small percentage of the cases is early in onset and segregates strongly within families, suggesting a genetic etiology. Some forms of AD are caused by the inheritance of mutant genes, including genes encoding the b-amyloid precursor protein (APP) and presenilin (PS)1 and PS2. There is also an association between AD and the apolipoprotein E (ApoE) gene. aMacroglobulin and endothelial nitric oxide (NO) synthase-3 genes recently were reported to be other genetic risk factors for AD [43].

Although the exact pathogenesis of AD still remains to be fully elucidated, it is currently considered to be a multifactorial disease. As such, different causes have been recognized to be implicated in the course of AD, providing several pharmacological strategies with multiple possible targets, all of them under investigation. Three main approaches have been taken. The first involves the re-establishment of neurotransmitters levels, with the inhibition of cholinesterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), and also monoamine oxidase (MAO) enzymes. The second concerns neuroprotection, wherein oxidative stress is considered to be an early event in the pathological cascade for the disease, thus suggesting the potential use of antioxidants to limit the effects of free radicals on nerve cells. The third approach deals with specific aspects related to AD, including the decrease in the production or aggregation of Ab peptide, and inhibition of g- and b-secretase enzymes, which play a critical role in the amyloidogenic pathway, t-protein, among others. **[44].**

I.1.2.4.1. Anticholinesterase inhibitory

Acetylcholine, the first identified neurotransmitter is a principal neurotransmitter in the peripheral, central, somatic and the autonomic nervous system. It relays the information across the gap (synapse) between the neuron and its neighboring cells. In the absence of acetylcholinesterase enzyme, acetylcholine accumulates at the synapse, thus paralysis occurs and it also stops the heart beat **[45]**. Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7; abbreviated herein AChE') functions in the central and peripheral nervous systems, along with the acetylcholine (ACh) receptor, in the transmission of action potentials across

nervenerve and neuromuscular synapses? The enzyme's physiological task is the hydrolytic destruction of the cationic neurotransmitter ACh. AChE is an extrinsic membrane-hound enzyme that projects into the synapse. The enzyme springs into action when ACh is released from the presynaptic nerve process in response to an action potential. ACh diffuses across the synapse and hinds to the ACh receptor, which among other functions serves as an ion gate for the entrv of K+ into the oostsvnaotic nerve orocess or muscle ;ell. A series of evenis fchows that'results in triggering the action potential in the postsynaptic cell. AChE rapidly terminates the ACh receptor-mediated ion gating by hydrolyzing Ach **[46].**

Treatment of Alzheimer's disease has been dominated by the use of acetylcholinesterase (AChE) inhibitors. These drugs compensate for the death of cholinergic neurons and offer symptomatic relief by inhibiting acetylcholine (ACh) turnover and restoring synaptic levels of this neurotransmitter. However, AChE itself has been implicated in the pathogenesis of Alzheimer's disease (AD). In particular, it appears that AChE may directly interact with amyloid-β in a manner that increases the deposition of this peptide into insoluble plaques **[47].** Though recent intensive efforts have been made to understand the mechanism of neurodegeneration involved in AD and to discover new drugs combating the symptoms, at present there is a deficit in the number of efficient and safe therapeutic agents to treat the disease. No new drugs have been approved by the US Food and Drug Administration (FDA) since 2003, likely because the abnormal brain deposits of Ab and t-proteins still cannot be considered causes or by-products of the disease. The few current drugs available only address the symptoms of cognitive loss, without delaying or modifying the disease progression. In some cases, the medications only work for a limited time and in some patients they do not offer relief at all. Another point to consider is that there is no in-vivo model available that is able to mimic all the cognitive, behavioural, biochemical and histopathological abnormalities observed in the course of AD, making it difficult to investigate new beneficial agents **[43, 48]**.

I.1.2.5. Polyphenols and cholesterolemia

Cholesterol is an unsaturated alcohol of the steroid family of compounds. It is essential for the normal function of all animal cells and is a fundamental element of their cell membranes. It is also the precursor to bile acids, which are necessary for the intestinal absorption of cholesterol, fats and lipophilic vitamins. Cholesterol can be obtained from the diet as well as being endogenously synthesized, the latter beingthe main source in humans

[49]. Cholesterol turn-over and homeostasis in the body involve many pathways: dietary incomes, entero-hepatic cycle with biliary excretion and intestinal reabsorption, reversible fluxes in the bloodstream between liver and peripheral tissues, biosynthesis (mainly in the liver) and steroidogenic tissues, and fecal elimination. The cholesterol homeostasis in the cell is achieved through three different methods: regulation of HMG-CoA reductase, regulation of LDL receptor synthesis, and regulation of the esterification and removal of free cholesterol **[50].** population studies have consistently shown that high-density lipoprotein (HDL) cholesterol levels are a strong, independent inverse predictor of cardiovascular disease. In the Framingham Heart Study, HDL cholesterol level was more potent as a risk factor for coronary heart disease than was the level of low-density lipoprotein (LDL) cholesterol. An analysis of data from four large studies concluded that each increase of 1 mg per deciliter (0.03 mmol per liter) in HDL cholesterol is associated with a decrease of 2 to 3% in the risk of future coronary heart disease **[51].**

Elevated plasma cholesterol levels constitute a major risk factor for atherosclerosis and cardiovascular diseases. Whole body cholesterol balance is regulated by the net effects of dietary cholesterol absorption, de novo cholesterol biosynthesis and biliary excretion from the liver. Available evidence supports the concept that several proteins are involved in mediating intestinal cholesterol transport, biliary secretion and reverse cholesterol transport RCT. Cardiovascular Disease (CVD) is one of the major causes of death in the global human population. In 2016, 17.9 million people died each year from CVD. The risk factors of CVD include hyperlipidemia, which is a condition with elevated levels of low-density lipoprotein (LDL), cholesterol, and triglycerides and with low levels of high-density lipoprotein (HDL) cholesterols. Medications such as statins can be used to lower the cholesterol levels in the blood **[52].**

Cholesterol in the body represents both endogenous sources produced in the liver and peripheral tissues and dietary sources absorbed from the intestine **[53].** Panel 1A shows the normal process in wild type mice. Pancreatic triglyceride lipase (PTL) rapidly digests triglycerides into free fatty acids and monoglycerides while pancreatic phospholipase A2 digests phospholipids into free fatty acid and lysophospholipid. These processes occur in the duodenum and upper jejunum. The digestion products, as well as ingested and biliary cholesterol, are incorporated into bile salt mixed micelles which transit the unstirred water layer to facilitate absorption of both the cholesterol and digested lipids. Absorption occurs throughout the jejunum but only minimally in the ileum. Panel 1B shows how the absence of

PTL affects lipid absorption. In the absence of PTL, carboxyl ester lipase (CEL) and two proteins, pancreatic lipase-related protein-1 and -2 (PTLRP-2) become the primary triglyceride digesting enzymes. However, CEL is less efficient at digesting triglycerides and RP-2 is present at very low levels. As a consequence, digestion is not complete until the distal jejunum. Cholesterol partitions between bile salt micelles and droplets of undigested lipid reducing its absorption in the proximal small intestine. The distal jejunum and the ileum, have less capacity for cholesterol absorption although digested lipids are well absorbed. Bile salts are efficiently absorbed in the ileum for recycling via enterohepatic recirculation, leaving unabsorbed cholesterol without a vehicle for solubilization and absorption in the distal intestinal lumen **[54].**

I.1.2.5.1.HMGR inhibitory

Cholesterol homeostasis is maintained by a series of regulatory pathways to control the synthesis of endogenous cholesterol, the absorption of dietary sterol, and the elimination of cholesterol and its catabolic end products, bile acids. Regulation of the pathway includes sterolmediated feedback of transcription of several genes, including the rate-limiting enzyme HMGR and HMG-CoA synthase, as well as a variety of post-transcriptional mechanisms. Recently, attention has been focused on the regulated degradation of HMGR in the ER. The cellular supply of cholesterol is maintained at a steady level by four distinct mechanisms **[53] :**

✓Regulation of HMGR activity and levels (upstream regulation of cholesterol biosynthesis)
✓ Regulation of squalene mono-oxygenase activity by squalene supernatant protein factor (down -stream biosynthesis of cholesterol).
✓ Regulation of excess intracellular free cholesterol through the activity of acyl-CoA: cholesterol acyltransferase, ACAT (internalization of excessive cholesterol).
✓ Regulation of plasma cholesterol levels via LDL receptor-mediated uptake and HDL mediated reverse transport.

Several reports have demonstrated the effect of Phenolic compounds for promising cholesterollowering agents, which decease the total cholesterol, triglyceride, and LDL-cholesterol concentrations, significant increase of fat excretion and of HMG-CoA reductase inhibitor activity, which was explained as a result of the acceleration of lipid metabolism from extrahepatic tissues to the liver, induced by curcuma consumption, leading to an improvement

of cholesterol excretion via the bile and, then, into the feces. Hypoglycemic effects of natural matrices have been also increasingly clarified and their use as proper blood glucose level promoters was intensified, as part of the daily diet. For example, the in vivo hypoglycemic potential of anthocyanin extracts from Ipomoea (I.) batatas cv. has been observed that markedly inhibited the increase of plasma glucose levels in mice, in a dose-dependent manner and that over time this effect was even more pronounced **[1].**

I.2.Polysaccharides -Generalities

I.2.1. Organization of the plant wall

Plant cells are characterized by the metabolites contained in their cytoplasm but also by the extracellular matrix that surrounds these elements. The plant wall is responsible for the rigidity of cells, the protection of their organelles and the maintenance of the cell and the plant. The polysaccharides organize and interact with each other to form the wall. The wall is composed of a middle lamella, a primary wall and a secondary wall.

The plant wall is a dynamic structure formed of various compounds, particularly polysaccharides, polymers of sugars, grouping together cellulose, hemicelluloses and pectins, and by other non-polysaccharide compounds such as proteins (especially extensin and enzymes) and minor compounds (phenolic and mineral acids). Typical primary plant CWs are composed of cellulose microfibrils (9-25%) and an interpenetrating matrix of hemicelluloses (25-50%), pectins (10-35%) and proteins (10%). It was previously described that the primary CW composition as cellulose fibers bound together by molecules made of sugar units. Approximately 90% of the CW consists of carbohydrates (mostly pentose and hexose units) and the remaining 10% is protein **[55].**

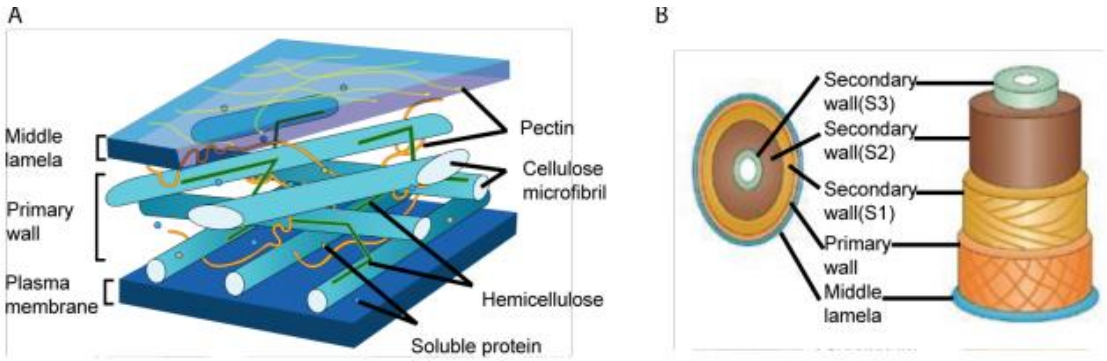


Figure 4: Plant cell wall structures. A, the primary cell wall; B, the secondary cell wall.

The middle lamella represents the outermost structure of the cell and allows cohesion between cells. It is mainly made up of pectins and is formed during the separation of cells. The primary cell wall of dicotyledonous plants contains three broad classes of polysaccharides : cellulose, hemicellulosic xyloglucans, and pectic polysaccharides, as well as structural glycoproteins. Although it is possible to identify typical structures for the hemicelluloses and pectic polysaccharides of the cell wall, there is increasing evidente of structural diversity within each grouping **[56].** The secondary wall is thicker and more rigid than the primary wall and forms at the end of the plant's growth when the cells are no longer developing. As a definition, secondary walls are derived from primary walls by thickening and inclusion of lignin into the CW matrix and occur inside the primary wall**.** They contain over 45% cellulose and up to 35% lignin, which makes them even more rigid **[55].**

I.2.2. Structure and polysaccharide composition of the plant wall

The plant wall is therefore made up of several macromolecules, we generally distinguish :

- Polysaccharides compounds
  - ✓ Pectins
  - ✓ Cellulose
  - ✓ Hemicelluloses
- Non-polysaccharide compounds
  - ✓ Proteins (extensin)

Polysaccharides are a kind of natural macromolecular polymer, which is usually composed of more than 10 monosaccharides through glycosidic linkages in linear or branched chains, with a molecular weight of tens of thousands or even millions. It is widely exist in the plants, microorganism, algae **[57].**

I.2.2.1. Pectin

Pectins are a family of complex polysaccharides that contain 1,4-linked a-d-galactosyluronic acid (GalpA) residues. They are present in the middle lamella and the primary wall of plant cells. Three pectic polysaccharides (homogalacturonan, rhamnogalacturonan-I, and substituted galacturonans) have been isolated from primary cell walls and structurally characterized **[58].** Pectins are a family of covalently linked galacturonic acid-rich plant cell wall polysaccharides. Galacturonic acid comprises approximately 70% of pectin, and all the

pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 position **[59].** The generally accepted model describes pectins as a chain of two major structures, presenting a polygalacturonic acid (PGAs), which are helical homopolymers of (1 -+4)a-~-galactosyluronic acid (GalA), and rhamnogalacturonan I (RG I), which are contorted rod-like heteropolymers of repeating (1+2)a-~-rhamnosyl-(l+4)a-~- GalA disaccharide units. The rhamnosyl units may also interrupt long runs of PGA. An unusual RG II has the richest diversity of sugars and linkage structures known. The molecule is too scarce to be a major structural polymer, but the complex form led the authors to suggest that it is a signal molecule **[60].**

- **Homogalacturonan**

The most abundant pectic polysaccharide is homogalacturonan (HG), a linear homopolymer of a-1,4-linked galacturonic acid that comprises 65% of pectin (Figure) **[59].** Homogalacturonan (HG) is a linear chain of 1,4- linked a-d-galactopyranosyluronic acid (GalpA) residues in which some of the carboxyl groups are methyl esterified. HGs may, depending on the plant source, also be partially O-acetylated at C-3 or C-2 **[58, 61].**

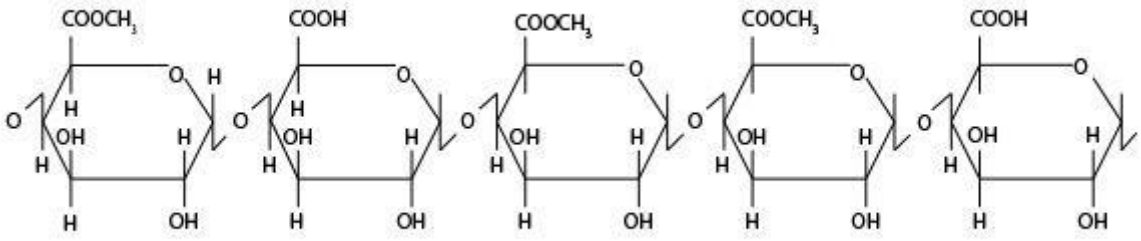


Figure 5: Primary structure of HG **[62].**

The PGAs contain up to about 200 GalA units and are about 100 nm long. The best documented RGs are isolated from the cell walls by enzymic digestion with PGAse, but the length of RG I is unknown because there may be stretches of PGA on their ends. The helical chains of PGAs can condense by cross-linking with Ca2+ to form 'junction zones', linking two antiparallelchains. Just how many contiguous unesterified GalA residues are needed to form stable junction zones and the extent to which several chains can stack to form the multiple 'eggbox' structures are not known in vivo or in vitro. At low Ca2+ concentrations, two chains are thought to form a stable junction with maximum strength at about 14 GalA units. If sufficient Ca2+ is present, some interrupting esterified GalA can be tolerated in the

stable junction zone. With excess Ca2+ available, four-chain or higher-order stacking of PGA chains is possible. Stretches of PGA at the ends or within RG could link these two types of polymers, but the rhamnosyl units of RG I and their sidechains interrupt the Ca2+ junctions. In addition to the Ca2+-binding junction zones, pectins in some species may be cross-linked to other pectins and non-cellulosic polysaccharides by ester linkages with dihydroxycinnamic acids such as diferulic acid **[60].**

- **Rhamnogalacturonan**

Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides that contain a backbone of the repeating disaccharide [-a-D-GalA-1,2-a-L-Rha-1-4-] and represents 20–35% of pectin **[59].** The backbone GalpA residues may be Oacetylated on C-2 and/or C-3. There is no conclusive chemical evidence that the GalpA residues are methyl esterified, however, an enriched RG-I-like wall fraction from flax has been reported to contain methyl esters. The GalpA residues typically are not substituted with monoor oligosaccharide side chains, although a recent study reported that a single b-d-GlcpA residue is linked to C-3 of 2% of the GalpA in the backbone of sugar beet RG-I **[63]**. In contrast, 20–80% of the rhamnosyl (Rhap) residues are, depending on the plant source and method of isolation, substituted at C-4 with neutral and acidic oligosaccharide side chains. The predominant side chains contain linear and branched a-l-arabinofuranosyl (Araf), and/or b-dgalactopyranosyl (Galp) residues, although their relative proportions and chain lengths may differ depending on the plant source . glycosyl residues a-l-fucosyl (Fucp), b-d-glucuronosyl (GlcpA), and 4-O-methyl b-d-glucuronosyl (4-O-Me GlcpA) may also be present, as may ferulic and coumaric acid **[58].**

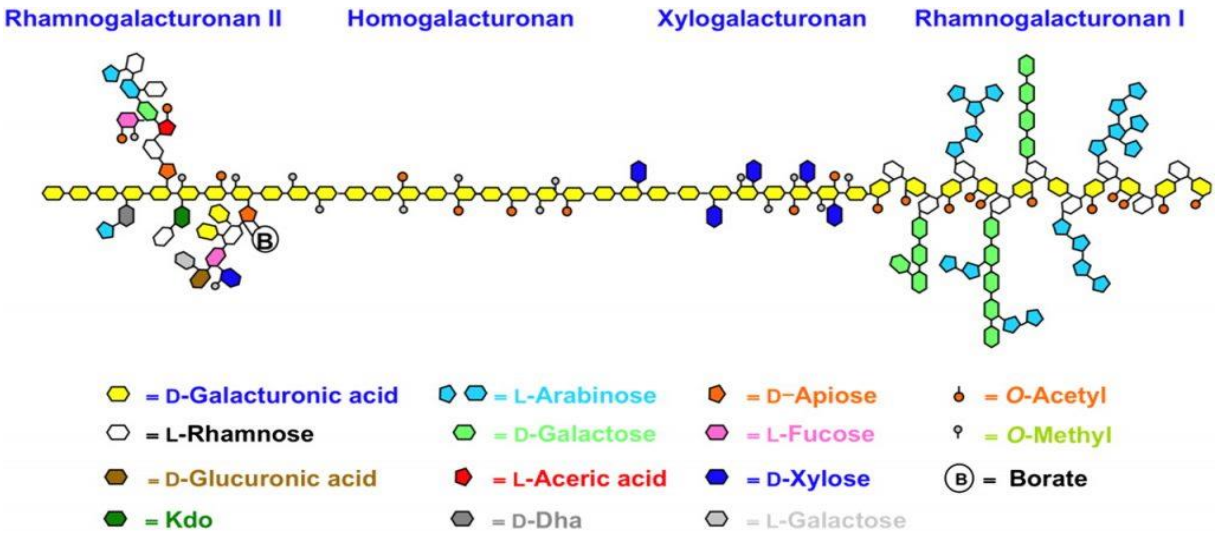


Figure 6: Structure of rhamnogalacturonan I and II **[64].**

- **Substituted galacturonans**

Substituted galacturonans (SG) are a diverse group of polysaccharides that contain a backbone of linear 1,4- linked a-d-GalpA residues. The substituted galacturonan referred to as rhamnogalacturonan II (RG-II) is present in all higher plant primary walls analyzed to date. The most structurally complex pectin, RG-II, makes up 10% of pectin **[59].** The demonstration that wine and other fruit juices contain relatively high amounts (20–150 mg/l) of RG-II, that RG-II binds heavy-metals, and that RG-II has immunomodulating activities has led to a greater interest in the structure of RG-II and to the role of this enigmatic pectic polysaccharide in human nutrition and health. RG-II is not structurally related to RG-I since its backbone is composed of 1,4-linked a-d-GalpA residues rather than the repeating disaccharide. A nonasaccharide (side chain B) and an octasaccharide (side chain A) are attached to C-2 of some of the backbone GalA residues and two structurally different disaccharides (side chains C and D) are attached to C-3 of the backbone. The locations on the backbone of the side chains with respect to one another have not been established with certainty **[58].**

Other substituted galacturonans have been described that are present in the walls of a restricted number of plants. For example, xylogalacturonans (XGA), which contain b-d-xylosyl (Xylp) residues attached to C-3 of the backbone are present in the walls of reproductive plant tissues (e.g. apple, carrot, cotton, and pine). Apiogalacturonans, which are present in the walls of some aquatic monocotyledons (e.g. Lemna and Zostera), contain b-dapiofuranosyl (Apif) residues attached to C-2 and C-3 of the backbone either as a single Apif residue or as the disaccharide **[58, 59].**

I.2.2.2. Cellulose

Cellulose forms the framework of the CW while hemicelluloses cross-link non-cellulosic and cellulosic polymers. Cellulose is composed of approximately $8x10^3$ D-glucopyranose residues linked by β1→4 glycosidic bonds. Hydrogen bonds hold about 40 of these glycan chains together to form a cellulose microfibril. Cellulose microfibril arrangement in the primary wall is random **[55].** It is the most abundant biopolymer on Earth, synthesized by herbs woody plants, many forms of algae, fungi and some species of bacteria, namely Acetobacter xylinum. Bacterial cellulose is identical to plant cellulose in chemical structure, but it can be produced without contaminant molecules, such as lignin and hemicelluloses, and does not require

intensive purification processes **[65].** The cellulosic framework Fundamentally, the type I primary wall is a network of cellulose microfibrils, several dozen linear chains of (144)p-linked D-glucose condensed to form long crystals that wrap around each cell. Microfibrils are 5-15 nm wide and are spaced 2040 nm apart. Although each chain may be just several thousand units long, they begin and end at different places within a microfibril. A microfibril itself is huge relative to the glucan chains, just as a spool of thread is composed of many thousands of cotton fibers each only a few centimeters long **[60].**

I.2.2.3.Hemicellulose

In type I walls, the principal interlocking polysaccharides are xyloglucans (XGs). Other non-cellulosic polysaccharides, such as gluco- and galactoglucomannans, galactomannans, p-~-glucans, and glucuronoarabinoxylans, potentially interlock the microfibrils in some type I primary walls, but are found in much lower amounts. These polysaccharides accumulate in much greater abundance in differentiating tissue, especially in the thickened walls of the seed endosperm or cotyledon **[60, 66].**

The XGs are linear chains of (1+4)p-~-glucan, but, unlike cellulose, they possess numerous xylosyl units added at regular sites to the 0-6 position of the glucosyl units of the chain. Digestion of XG with Trichodenna cellulase, a sequence-dependent endoglucanase whose action is restricted to unbranched 4-linked glucosyl units, yields hepta- and octasaccharides from seed reserve XGs and additional nonasaccharides from the type I primary walls. The basic repeating unit of XG consists of additions of a-o-xylosyl units upon three contiguous glucosyl units of the backbone linked by a single unbranched glucosyl residue. Additional sugars, p-o-galactose and a-Larabinose, are added to the 0-2 of some xylosyl. The nonasaccharide is produced by addition of an a-L-fucose to the 0-2 of a subtending galactosyl unit to produce a trisaccharide sidechain attached to alternate heptasaccharide units. A few of the nonasaccharides are substituted further with another a-L-fucosyl-(l+2)p-o-galactosyl unit to form an undecasaccharide. Further, other side groups sometimes block hydrolysis of many unbranched glucosyl units by the sequence-dependent glucanase, resulting in much larger oligomers whose sequence structure is still not established **[60].**

I.2.2.4.Protein

The primary cell wall comprises three structurally independent but interacting domains. One domain, the fundamental cellulose-xyloglucan framework (about 50% of the wall mass), is

embedded in a second domain of matrix pectic polysaccharides (about 30% of the total mass). The third independent domain consists of the structural proteins. As previously described, the jelly-like pectic polymers are "not just jelly". They are some of the most complex polymers known, and they are thought to perform many functions: to determine wall porosity, to provide charged surfaces that modulate wall pH and ion balance, and to serve as recognition molecules that signal appropriate developmental responses to symbiotic organisms, pathogens, and insects **[60].**

Collagen and elastin provide structural support and tensile properties of animal tissues. In plants, the extensin family of uniquely O-Hyp glycosylated proteins consists of extensinsproline-rich proteins and arabinogalactan proteins **[67-69]**. Extensins are self-assembling amphiphiles that help to form the new cell plate at cytokinesis, crosslinked extensins also contribute to the tensile strength of the cell wall and as a negative regulator of extension growth. Classical AGPs comprise a medium-sized family of large (c. 120 kDa) glycoproteins containing c. 95% carbohydrate. Generally, each AGP consists of a small polypeptide (c. 200 residues) with numerous (12–24) O-Hyp-linked arabinogalactan polysaccharides. AGPs cover the surface of the plasma membrane anchored via a C-terminal glycosylphosphatidylinositol (GPI) lipid. Thus cleavage by phospholipase eventually releases freely soluble AGPs into the periplasm and then into the expanding cell wall matrix where their role as a pectic plasticizer remains a plausible conjecture **[68].**

In plants, the extensin family of uniquely O-Hyp glycosylated proteins consists of extensins **[70],** proline-rich proteins and arabinogalactan proteins. Extensins are self-assembling amphiphiles that help to form the new cell plate at cytokinesis ; crosslinked extensins also contribute to the tensile strength of the cell wall and as a negative regulator of extension growth **[68].** It is characterized by a repeated sequence of Ser-Hyp-Hyp-Hyp-Hyp carrying 50 to 60% carbohydrates in the side chain. Hydroxyprolins are glycosylated by arabinose residues linked by β (1 → 2) bonds, except terminal arabinose which is β (1 → 3) bound, while serine carries a galactose residue **[71, 72].** Proteins other than extensin found in the CW include enzymes such as the cellulose synthases, hydrolases, and oxidases needed for CW thickening, modification, and lignification, respectively, during secondary growth. Reorganization, de novo synthesis, and insertion of new wall polymers lead to rearrangement of the CW during cell growth. This enables inclusion of lignin into the wall and strengthening of the CW matrix. In forage legumes and especially grasses, the order of maximum CW

component deposition is hemicellulose, followed by that of cellulose (1 to 6 days later), and then lignin (up to 14 days after maximum hemicellulose deposition) **[55].**

I.2.3. Cell wall polysaccharide-polyphenol interactions

Several studies have demonstrated the process of interactions between polyphenols and polysaccharides **[73],** namely model polysaccharides such as β-glucan and cyclodextrins , polysaccharides forming a solid support such as dextran gels, as well as than with cell wall polysaccharides. During the destruction of plant cells through certain processes, namely chewing, grinding, heat treatment, etc., the polyphenols contained in the vacuole come into contact with the plant walls or with the walls of yeasts **[74].**

Hydrogen bonds form between the hydroxyl groups of the A and B rings of tannins (or gallic acids) and the oxygen atoms of the glycosidic bonds which link the monosaccharide residues together or the hydroxyl or acetyl groups of polysaccharides **[73].** The presence of hydrogen bonds in these interactions has been studied using urea or by modifying the temperature because increasing the temperature (from 5 ° C to 35 ° C) causes a decrease in the association between procyanidins and cell walls **[75].** Just as the addition of urea partially releases the procyanidins bound to the walls, regardless of the type of procyanidins **[76].**

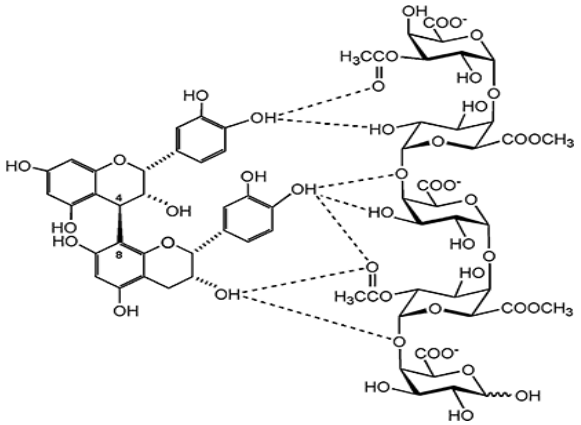


Figure 7: Possible hydrogen bonds between a catechin dimer and a homogalacturonan **[77].**

Hydrophobic interactions also play a role in the associations of polyphenols with polysaccharides as well as with proteins. have shown that the hydrophobic character of procyanidins increases with the degree of polymerization and galloylation. Modulation of ionic strength during interaction analyzes is also used to demonstrate the presence of hydrophobic interactions. The increase in ionic strength (from 0.01 mol.L-1 to 1 mol.L-1) is related to an increase in the level of procyanidins bound to the walls. Likewise, in the

presence of ethanol, the polarity of the aqueous medium decreases, resulting in the breakdown of hydrophobic interactions and the release of procyanidins **[74, 76].**

However, Proteins, on the other hand, interact with procyanidins in several stages. Initially, procyanidins bind to proteins through hydrogen bonds and hydrophobic interactions. Then, by self-association of the protein-procyanidin complexes, aggregates are formed, eventually leading to the formation of colloids by precipitation The phases of precipitation and colloid formation can be slow **[74].**

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Chapter II : Eco-extraction : *Zizyphus lotus* plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality - An overview

Zizyphus lotus plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality : An overview

Abstract

The purpose of this review is to compile the literature published about different aspects of the use of microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE) occurring in jujube worldwide. As a result of the increased consumer demand for natural products, as well as, those of the agri-food, nutraceutical and cosmetic industries, green extraction techniques are today's trend to be mainly potential alternatives. Ultrasounds are very much used method in the extraction of active principles due to their effect of cavitations and microwaves due to their dipolar rotation effect, these two techniques giving an efficiency of extraction while minimizing the time while preserving the quality of the vegetable matrix, overcoming the disadvantages of conventional techniques consuming large quatities of solvents and giving a small quatity of extraction. Jujube, a plant with a high antioxidant potential, thanks to its richness in bioactive substances, there last are affected by various conditions caused by several factors hence the need to use UAE and MAE, this aspect must be taken into consideration to be able not only to value a local plant but also to take advantage of its virtues, to use one of the interesting properties for production which will be the object of industrialization in the future by replacing the synthetic antioxidants manufactured in some industirous formulations which can harm the health of the consumer by natural antioxidants resulting from the jujube which is in abundance that will be able to serve the nutraceuticals and agro-food industries.

Keywords

Microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), antioxidants, *Z. lotus* plant.

I. Introduction

For a number of years, researchers and industrial food companies are increasingly interested in a class of natural antioxidants, polyphenols. Recognition of their antioxidant properties and their abundance in food and their place in the prevention of diseases associated with oxidative stress, the consumer's demand for a natural diet to counteract synthetic antioxidants, are the main reasons for this craze **[1-4]**. In general, the first process of treatment of severals plant materials is the extraction of their crude pigments. Extraction of natural products can be done by various extraction techniques. It has been used probably since the discovery of fire. Egyptians and Phoenicians, Jews and Arabs, Indians and Chinese, Greeks and Romans, and even the Mayans and Aztecs, all possessed innovative extraction processes using conventional methods, such as maceration, solvent extraction, soxhlet extraction and alembic distillation, etc., used even for perfume, medicine, or food **[5].** More recently, however, many non-conventional methods such as ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE), extraction and enzyme-assisted extraction (EAE) have been proposed due to their enhanced extraction efficiency and environmental friendliness **[6].**

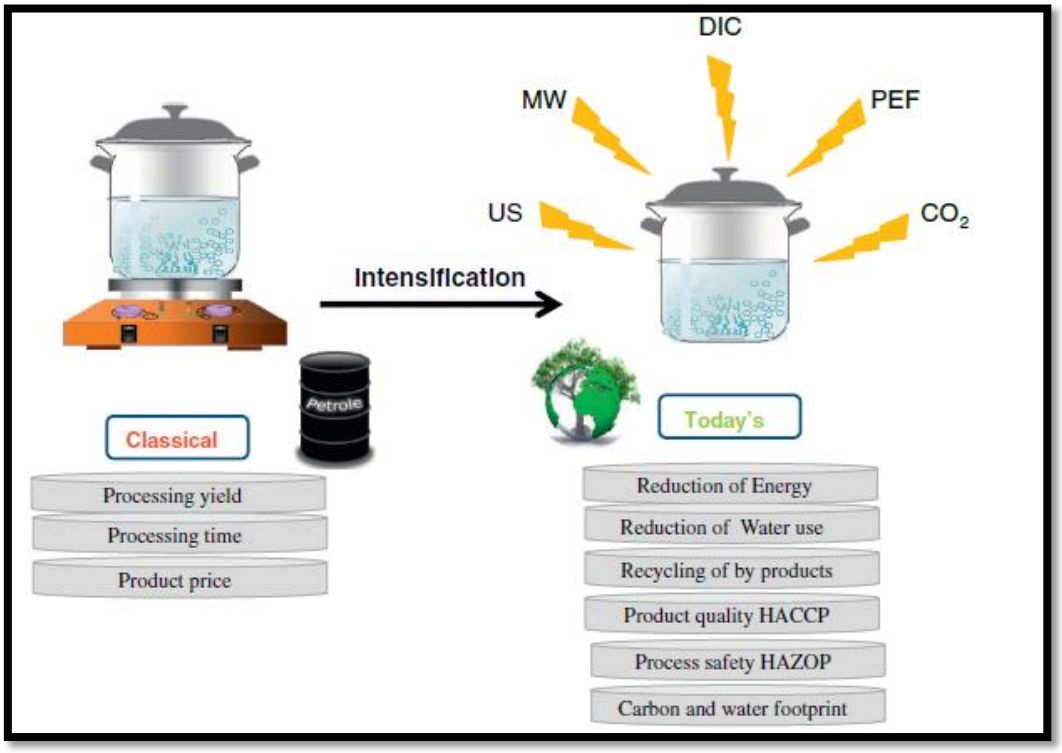


Figure 8 : Green Food Processing: evolution or revolution.

II.1. Utrasound process

II.1.1.Physical principles of ultrasonic waves

Ultrasound is a mechanical wave, with frequencies higher than that of the ear **[7]** which can propagating in a material and causing cycles of expansion and compression in the middle environment can create bubbles that surround themselves in a liquid, the cavity collapse produces a jet of liquid at high speed, so-called phenomenon cavitation **[8].** Ultrasound can also be broadly classified as low-intensity sonication (<1 W/cm^2) and high-intensity sonication (10–1000 W/cm^2) **[9].** According to **Hielscher [10],** ultrasound shows a very important expansion in medicine because of their effectiveness; Thus in medical imaging, ultrasound has been much more interesting compared to other imaging methods **[7]**. It provide access to quantities such as blood flow mapping as well as their positive impact on human health, and all economic, which is justified in the place of ultrasound in medical diagnostic and therapeutic applications. There are two types of ultrasonic equipment used in laboratories, one is called ultrasound probe, which confirmed a direct contact with the sample to be analyzed, used for the extraction of bioactive compounds of plants to accelerate the maceration. Unlike the ultrasonic probe, we find the the second, ultrasonic bath, used for homogenization, dispersion, degassing and cleaning, generally based for the indirect contact and used for enrichement **[11, 12].**

II.1.2. Cavitation mechanism

Ultrasounds in general are based on heating, it is the phenomenon of ultrasonic cavitation which is due to the cycles of compression and decompression of water molecules. The mechanical effect of ultrasound at high acoustic pressure form cavitation bubbles as shown in **Figure 9** and allows the acceleration and release of bioactive principles of the plant, via the disruption of cell walls and the intensification of mass transfer. When ultrasound wave passes through the medium, longitudinal displacements are formed with a series of compressions and rarefactions in the particles of the medium. In these areas of varying pressure, cavitation effect and the formation of gas bubbles is observed. The bubbles generated during the sonication process wave are able to change their size during the rarefaction and compression cycles. These bubbles grow over the period of a few cycles to reach a critical size, then they collapse violently and release large amounts of energy **[13].** The size of the cavitation bubble is dependent on the frequency of ultrasound. This cavity can absorb ultrasonic energy more efficiently by expanding rapidly until it can no longer absorb energy when liquid rushes in

and the cavity implodes. The cavity containing gas and vapors allows to generate enormous local temperatures and pressures creating an environment for a chemical reaction **[14].**

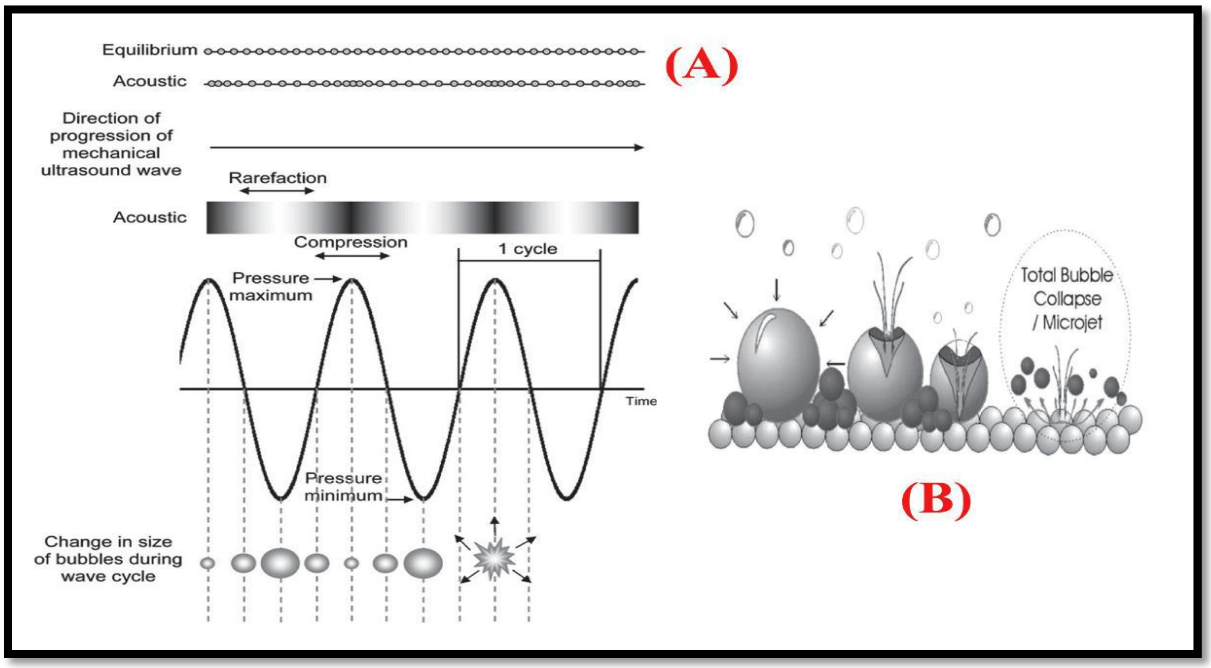


Figure 9: (A) Development and collapse of cavitation bubbles, and (B) schematic depicting classically thought bubble collapse at the solid surface

II.1.3. Application of UAE in food research

For their significant effects in the extraction of oils, herbs and bioactive components from plant plants, improve the extraction time and yield **[5, 15, 16]**. Ultrasound is used for plant dehydration **[17]**, drying **[11, 18]**, emulsification, extraction of bioactive substances **[19, 20]**, recently **Koubaa, Mhemdi [21]** suggests a supercritical fluid extraction system of matrices assisted by ultrasonic waves for the purpose of inactivation of microorganisms, another study proves that the heat treatment of fruits such as cactus not only allows to guarantee the good quality screw inactivation of micro organisms but also gives a better functional feature **[22]** ; both in the heat treatment on the sensory quality of alcoholic beverages **[23]**. For years, many researchers have been drawn to the importance of ultrasound in the development of agro-food industries **[12, 24].** Others suggested that in its place, the economic advantages proven by ultrasonic treatments in the extraction and encapsulation of materials while preserving the quality of the plant matrix **[15]**. In addition, **Dalvi-Isfahan, Hamdami [25]** showed that ultra-violet is a much more innovative alternative preservation technique in place of the freezing of foodstuffs made in the past, a technique that can alter the nutritional and hygienic quality of the food.

II.2. Microwave process

II.2.1. Electromagnetic Fields of microwaves

The uses of microwaves began to appear in the 1950s, the litterature suggested that Tappan was introduced the first microwave oven in 1955 but the widespread use of domestic microwave ovens occurred during the 1970s and 1980s **[26].** Its first application was in chemical synthesis and it was published in 1986 **[27]**, it's used in several subjects, such as food processing and drying on industrial process and domestic house **[28].** Microwaves are a non ionising electromagnetic energy **[29]**, with frequencies ranging from 0.3 to 300 GHz. They can be transmitted, absorbed and reflected, thanks to the laws of optics. Domestic microwave units generally operate at a frequency of 2450 MHz while for industrial applications are 915 MHz. **[30].**

II.2.2. Mechanism of Microwave Heating

The capacity of microwave to convert a part of plant materials of the absorbed electromagnetic energy to heat energy. Microwave heating of plant materials is mainely caracterised by the rotation dipole and the ionic conduction **[31]**. The mechanism of dipole rotation is based on the principle that any molecule under microwave irradiation that generates heat must have a dipole moment where it has two ends, one is negatively charged and the other is positively, just like water, made up of two hydrogen atoms and one oxygen atom, which try to align with the electromagnetic field by rotation according to the polarity of the field. The latter causes friction heat **[29, 32].** When an electromagnetic field is applied, ionic compounds move at an accelerated rate producing ionic polarization. As the movement of the ions increases, kinetic energy is converted into thermal energy of the solution quickly **[29].** The ability of a given material to interact with an electric field by converting energy into heat depends largely on its dielectric properties. Dielectric constant and dielectric loss factor are the parts of dielectric properties. Dielectric constant (ε'), meaning the ability to store electrical energy, while dielectric loss (ε"), describes the material's ability to convert electrical energy into heat **[33, 34]** , according to the following equation:

$$\varepsilon' = \varepsilon'' \tan \delta \quad \text{Eq (1)}$$

The dissipation factor (tan δ) is an indicator of the efficiency of the dissipation or absorption of electrical energy in the form of heat by microwave which is described by :

$$Pv = kf\varepsilon' E2 \tan \delta \text{ Eq (2)}$$

Where

P = absorbed microwave power (W/cm3)

f = microwave frequency (GHz)

ε" = dielectric loss factor of material

k = a constant

E = electric field intensity for a given volume (volts/cm)

Numerous studies have been published by several autors for microwave applications acquired and tested in food industry, as shown by **Smith and Arsenault (1996),** including drying, moisture determinations, safety guidelines, economy, automation and robotics. **Routray and Orsat [29]** showed that several factors can affect microwaves extraction of bioactive compounds (gives an example of flavonoids especially), as suggested by authors, both polar and non polar solvents can be used for extraction with respecting substances nature of extraction in each used solvent . In the other hand, the power level, temperature and time of extraction may affect positively the extraction process and cause the increasing solubility due to the interaction molecules with opening cell matrix and the liberation of bioactive compounds. More recently, **Sadeghi, Hakimzadeh [35]** demonstrated the extraction mechanism of MAE which was supposed to involve several steps that are based on the effect of microwave radiation, which increases the temperature and the pressure of the microwaves during the extraction, these will allow the diffusion of the solvent in the sample matrix and will thus release the active ingredients of the this last. Thanks to these effects, the advantage of using microwaves is very important because it not only guarantees an efficient extraction allowing the recovery of a maximum of bioactive compounds more quickly compared to the conventional extraction processes, but also considered as green technology because it does not consume a lot of organic solvents **[36].** Several studies demonstrated the efficiency of the MAE process compared to UAE and the feasibility of using MAE process at industrial scale has been very used for green extraction of bioactive compounds from plants and industrial by-products **[37].**

II.2.3. Application of MAE in food research

At the opposit of MAE, the conventional extravtion methods in which the mass transfer occurs from inside to the outside, it occurs from the outside to the inside of the substrate (**Figure 10**) and the heat is not tranferred as the same. During this extraction, there is no

instable conduction because the concentration of solute in interaction with the solid varies, and this according to the solvent penetrated into the matrix, the solubilization of the components and the migration solute from the outside to the solution as well as. Extraction efficiency is not a linear function of extraction time **[38].**

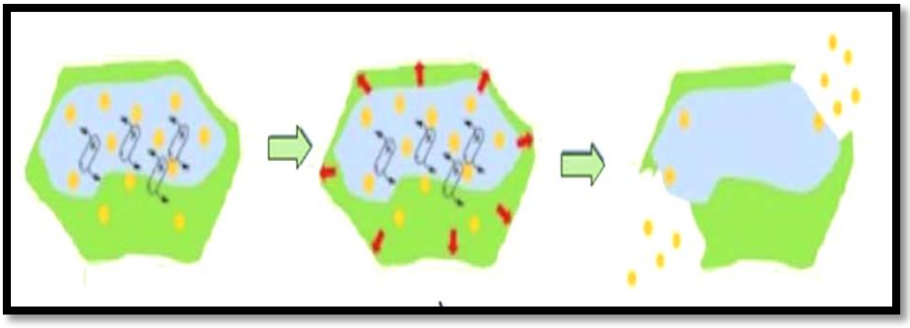


Figure 10 : A brief description of phenomenon in the cell generated by microwave irradiation.

A number of authors have evaluated and optimized conditions of extraction of bioactive contents using MAE. **Dahmoune, Boulekbache [39]** used MAE of *Citrus limon* peels and compared to UAE and CSE for the recovery of total phenolic compounds. The optimized result for MAE was 48%, 28 :1 mL/g, 123s and 400 W for ethanol as extraction solvent, solvent: solid ratio, irradiation time and power respectively. Results shows that maximum predicted TPC recoveries under the optimized conditions for MAE was 15.74 mg GAE/g model. In comparison to UAE and CSE, MAE is showed better that others in terms of yeild and antioxidant activities against DPPH and reducing power. **Dahmoune, Spigno [40]** used the MAE of total phenolic compounds from the leaves of *Pistacia lentiscus L*. which gives better extraction yeild (185.69 ± 18.35 mgGAE/gdw) with higher antioxidant activites in comparison to UAE and CSE with optimal conditions as 46% ethanol, extraction time 60 s, potency density 17.86 W/mL, and liquid/solid ratio 28:1. This is due to the rapid energy-saving heating rates which deep penetration of organic solvent in raw material, leadings to very short extraction times.

II.3. Botanical description of *Zizyphus lotus L* and its valorization

II.3.1. General Background

Interest in jujubes (Zizyphus lotus) dates back many thousands of years. The utilization of the medicinal plant was recorded in in tropical regions such as Asia, America, South of Europe and in all North Africa as in Algeria. There are several species of this genus (*Zizyphus*

vulgaris Lam, *Zizyphus Lotus Lam*, *Zizyphus Spina-Christi* (L.) Wild, *Zizyphus Mauritiana Lam* ,.) Its forms vary with the soil and climate. **Danthu, Touré [41], [42].**



Figure 11: *Zizyphus lotus* plant.

In Algeria, the *Z. lotus* (L.) specie is so recovered **[43]**. It is known as 'Sedra'. The fruit, called 'Nbag'**[44].** Several botanists have been described the various compartments of jujube plant (*Zizyphus lotus Tourn*.) which have a perianth pentamer; the fruit is a drupe the size of a pea or an olive. The leaves are alternate, coriaceous and accompanied, each of two spines straight or crooked. In the most common species, the leaves are small (15 x 10 mm). It is a shrub or a tree frequent in the hot countries, it is cultivated for its fruits, on all in Algeria such as the regions of Kabylia (Bejaia, Tizi ouzou, Boumerdes and Bouira). Also in south of Algeria (Djelfa, Biskra and Msila), as well as other Mediterranean countries such as Morocco and Tunisia **[45]**.

Table 1: Classification and vernacular names of *Zizyphus lotus* plant.

Classification	Vernacular names
Branch: Spermatophytes.	- English : Lotus jujube, jujube, ber.
Sub branch: Angiosperms.	- French : Jujubier, jujubier sauvage, jujubier de Berberie.
Subclass: Dicotyledon.	- Arabic : Sedra, N'Beg
Order: Celastrales.	-Kabyle :Thazguarth, Azougar.
Family: Rhamnaceae.	
Genus: *Zizyphus.*	
Species: *Zizyphus lotus*	

II.3.2. Nutritional composition of jujube

In recent years, the physiological function of foods including fruits, vegetables, legumes and grains, and food components such as phytochemicals has received much attention. Possible correlations between the biologically active compounds and human health have generated

interest in in-vitro and in-vivo studies. The major class of phytochemicals found in plants is constitued of phenolic compounds which contain a large variety of derivatives including simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, tannins, lignans and lignins **[46].** On the other hand, from these compounds, it has been found that polysaccharides represented a vital category as they exhibit numerous pharmacological and biological potentiel such as antitumor, anticancer and antioxidant **[47].** The classification of bioactive compounds from plant materials according to **Croteau, Kutchan [48]** are divided into terpenes and terpenoids, alkaloids and phenolic compounds. These categories contain in minimum 8000 types approximately. Morever, the pathway for synthesis of bioactive compounds is found to be different on fonction of nature of this contents. **Azmir, Zaidul [49]** suggested that shikimic acid and malonic acid are the pathway of synthesis of phenolic compounds. While, alkaloids and terpenes come from mevalonic acid and non-mevalonate pathways, respectively.

The *Zizyphus lotus* specie is known for its richness in primary metabolites mainly, protein 19.11%, carbohydrate 40.87% and lipids 32.92% **[50, 51]**. While for secondary metabolites (Table 2), several scientific reports have been carried out the extraction of bioactive compound and demonstrated the presence of many biological active molecules from jujubes **[52]**, such as polyphenols (flavonoids, tannins), triterpenes, anthraquinones, alkaloids (cyclopeptides and isoquinolides) and saponosides, everything depends on parts of the vegetable matrix (leaf, root, fruit and seeds) **[53, 54]**. The leaves are a source of flavonoids, tannins, alkaloids and saponins **[55-57]**. The fruits contain flavonoids, tannins and saponins **[58]**. Roots are a source of flavonoids, tannins and alkaloids **[59]**. Besides containing higher amount of secondary metabolites, both seed and fruit revealed the presence of important minerals such as magnesium, calcium and potassium **[52]**. These compounds are valued for their contribution to healthy diet and also as ingredients for designing new foods **[60, 61]**.

Table 2: Chemical composition of *Z. lotus* in deferent part of jujube.

Fraction	Fruits	Pulps	Seeds	leaves	Root bark	References
Moisture content(%)	-	12.27	6.05	-	9.11	
Carbohydrates(%)	-	65.90	40.87	8720 (mg/100 g)	8.71	
Crude protein(%)	-	3.80	19.11	-	3.18	
crude fat(%)	-	1.32	-	-	-	
crudefibe(%)	-	8.41	-	-	47.90	
Ash(%)	-	3.28	1.05	-	2.69	**[44]**
Pectin(%)	-	3.78	-	-	-	**[50]**
Vitamin C	5.67	190.65	31.24 –170.84	63.40	47.20	**[62]**
calorific valuesKj/g	-	16.341	-	-	-	**[63]**
oleic acid(%)	-	88.12	61.93	-	-	**[64]**
elaidic acid (%)	-	7.88	-	-	-	**[52]**
linolenic acid(%)	-	-	-	9.15	-	
Saponins (mg/100 g)	-	-	-	340	219	
Polyphenols (mg/100 g)	297–4078.2	325	14.68	664	2009	
Total flavonoids (mg/100 g)	122	173	-	133-199	120	
Total tannins (mg/100 g)	33	929	-	39	156 (Proanthocyanidins)	

II.3.3.Bioactive compounds of jujube

Apart from the nutritional potential, several *in vitro* and *in vivo* studies on phytochemical and pharmacological effects have clearly revealed that some biological substances from *Zl* are the main active molecules responsible for its benefical effects depending on the part of the plant concerned (root, leaf, seed, pulp, or fruit) mainly as antifungal, antibacterial, antiulcer, anti-inflammatory, antioxidant and immunostimulant properties, jujube has been a dietary food that appears in the list A of the medicinal plants of French pharmacopeia **[65].** Based on litteratures, flavonoid, polysaccharide, protein and triterpenic acid are the main active molecule responsible for its biological effects. Both flavonoids and polysaccharides which are found in both seed and pulp are known for exhibiting antioxidant and antimicrobial, immunomodulatory properties **[52].** Triterpenic acids which are abundant in leaves were proposed to be main active ingredients for the effect on anti-inflammatory and anticancer activities **[66]**. While, proteins are found in seeds and pulps knowing by their functionnal properties such us protein solubility, emulsifying activity, emulsion stability, and water holding capacity **[60].** Most isolated compounds from *Zl* plant are the phenolic acids mainly

by the presence of considerable amounts of caffeic acid, gallic acid, rutin, epicatechin, taxifolin and catechin from the various fractions of the ethanol extract in all parts of the jujube plant. Elsewhere, these compounds may well explain the biological activity, manufactured as compounds by pharmaceutical companies and used as control drugs in most laboratory experimental setups **[67].** Table 3 mentioned some isolated compounds from *Zizyphus* extracts.

Table 3 : Most classes of polyphenols isolated from *Zizyphus* species.

Bioactive compounds	*Zizyphus* species	Fruit	Pulp & peel	Seed	Leave	Stem bark	Branche	References
Phenolic acids								
Gallic acid	*Z. lotus* *Z. jujuba*	+	+	+	-	-	-	Current study, Benabderrahim, Elfalleh [68], Zhao, Zhang [69]
Methyl gallate	*Z. lotus*	-	+	-	-	-	-	Our study
Protocatechic aldehyde	*Z. lotus*	-	+	-	-	-	-	Our study
p-Hydroxybenzoic acid	*Z. jujuba* M	-	+	-	+	-	-	Our study, San and Yildirim [55]
Vanillic acid	*Z. lotus*	-	+	-	-	-	-	Our study
Syringic acid	*Z. jujuba* M *Z. lotus*	+	+	-	+	-	-	Our study, San and Yildirim [55], Benabderrahim, Elfalleh [68]
p-coumaric	*Z. jujuba* M *Z. lotus*	+	+	+	-	-	-	Our study, San and Yildirim [55], Benabderrahim, Elfalleh [68]
Succinic acid	*Z. lotus*	-	+	-	-	-	-	Our study
Cinnamic acid	*Z. lotus*	-	+	-	-	-	-	Our study
Ferulic acid	*Z. jujuba* M *Z. lotus* *Z. jujuba*	+	+	+	+	-	-	Our study, San and Yildirim [55], Benabderrahim, Elfalleh [68], [69]
Caffeic acid	*Z. jujuba* M *Z. jujuba*	+	+	+	+	-	-	Our study, San and Yildirim [55], Zhao, Zhang [69]
Sinapic acid	*Z. lotus*	-	+	-	-	-	-	Our study
Acylated quinic acid derivatives								
Quinic acid	*Z. lotus*	+	+	-	-	-	-	Our study, Benabderrahim, Elfalleh [68]
1-O-caffeoylquinic acid	*Z. lotus*	-	+	-	-	-	-	Our study
3-O-caffeoylquinic acid (cholorogenic acid)	*Z. jujuba* *Z. jujuba* M	+	+	+	+	-	-	Our study, San and Yildirim [55], Zhao, Zhang [69]
Esters								
Epigallocatechin gallate	*Z. lotus*	-	+	-	-	-	-	Our study
Hydrolysable tannin derivatives								
Digallic acid	*Z. lotus*	-	+	-	-	-	-	Our study
O-galloylnorbergenin i	*Z. lotus*	-	+	-	-	-	-	Our study
O-galloylnorbergenin ii	*Z. lotus*	-	+	-	-	-	-	Our study
Trigalloyllevoglucosan	*Z. lotus*	-	+	-	-	-	-	Our study
Coumarins								
Umbelliferone	*Z. lotus*	-	+	-	-	-	-	Our study
Lignans								
Secoisolariciresinol	*Z. lotus*	-	+	-	-	-	-	Our study
Xanthones								
Tri-hydroxy-dimeto-xyxanthone	*Z. lotus*	-	+	-	-	-	-	Our study
Flavonoid aglycones								
Luteolin	*Z.lotus*	+	-	-	-	-	-	Our study, Benabderrahim, Elfalleh [68]

Quercetin	*Z.jujuba* *Z. jujuba* M *Z.lotus* *Z.jujube* M *Z. mauritania* *Z. mistol*	+	+	+	+	-	-	San and Yildirim [55], Benabderrahim, Elfalleh [68], Zhao, Zhang [69], Rached, Barros [70], Zozio, Servent [71], Orqueda, Zampini [72], Wojdyło, Carbonell-Barrachina [73]
Catechin	*Z.jujuba* *Z. jujuba* M *Z.lotus* *Z. jujube* M *Z. mauritania* *Z.joazeiro*	+		+	-	-	-	Our study, San and Yildirim [55], Benabderrahim, Elfalleh [68], Zhao, Zhang [69], Rached, Barros [70], Zozio, Servent [71], Wojdyło, Carbonell-Barrachina [73], Andrade, Silva [74]
Vicenin -2	*Z.lotus*	-	+	-	-	-	-	Our study
Myricetin	*Z.lotus*	-	+	-	-	-	-	Our study
Chrysoeriol	*Z.lotus*	-	+	-	-	-	-	Our study
Amentoflavone	*Z.lotus*	-	+	-	-	-	-	Our study
2,3- Dihydroamentoflavone	*Z.lotus*	-	+	-	-	-	-	Our study
Procyanidin trimer	*Z. jujube* M	+	+	-	-	-	-	Our study and Wojdyło, Carbonell-Barrachina [73]
Flavonoid glycosides								
Naringenin-7-rhamnoglucosie	*Z. lotus*	-	+	-	-	-	-	Our study
Isorhamnetin hexoside	*Z. lotus*	-	+	-	-	-	-	Our study
Glycitein 7-O-glucoside	*Z. lotus*	-	+	-	-	-	-	Our study
Luteolin 7-*O*-glucoside	*Z. lotus*	-	+	-	-	-	-	Our study
Chrysoeriol-6-O-acetyl-40-b-d-glucoside.	*Z. lotus*	-	+	-	-	-	-	Our study
Kaempferol-3-O-glucoside	*Z. lotus* *Z. jujuba* M	+	+	-	-	-	-	Our study a nd Wojdyło, Carbonell-Barrachina [73]
Kaempferol-3-O-robinobioside	*Z. lotus* *Z. jujuba* M	+	+	-	-	-	-	Our study a nd Wojdyło, Carbonell-Barrachina [73]
Vitexin	*Z. jujuba M.*	-	+	-	-	-	-	Our study and Choi, Ahn [75]
Quercetin -3-O-glucoside	*Z. mauritania* *Z. lotus*	+	+	-	-	-	-	Our study and [76]
Quercetin-3-O-robinobioside	*Z. jujuba* M *Z. mauritania*	+	-	-	-	-	-	Our study, [73, 76]
Quercetin-3-O-rutinoside	*Z. jujuba* M *Z. jujuba* *Z. mistol* *Z. mauritania* *Z. lotus*	+	+	+	+	-	-	Our study, San and Yildirim [55], Zhao, Zhang [69], Rached, Barros [70], Orqueda, Zampini [72], Memon, Memon [76]
Quercetin-3-O-rutinoside-7-O-pentoside	*Z. jujuba* M	+	+	-	-	-	-	Our study and Wojdyło, Carbonell-Barrachina [73]
Terpenonid and derivatives								
oxoglycyrrhetinic acid	*Z. lotus*	-	+	-	-	-	-	Our study

II.3.4. Application to conventional extraction method

Several studies have been showed the large choice of traditionnal extraction methods of antioxidants compounds from plant materials, such as maceration, hydrodistillation and soxhlet extraction **[49].** Generally, this area based on the application of temperature treatment and the use of different solvents depending on the compound to remove to improve extraction. The most common processes used for the extraction of compounds from plants are either

physical or chemical **[77].** In addition, the heat is transferred from the heating medium to the interior of the sample when we applicate the conventional extraction, which is no the case for MAE, the heat is dissipated volumetrically inside the irradiated medium **[78].** Maceration is very used in home made preparation of tonic from a long time, which is inexpensive, based on the mixture of solvent with the surface area to get bioactive compounds. Hydrodistillation and soxhlet extraction are generally used for extraction of essential oil, very little for bioactive compounds, this are based on the condensed mixture flows from condenser to a separator, where the compounds were separated automatically from the water **[79].**

Many reports have been studied on the extraction of secondary metabolites from *Zl* plant using conventional methods. **Borgi, Ghedira [53]** reported the extraction of antioxydants in root barks from *Zl* with different solvents. **Borgi, Recio [80]** used soxhlet for extraction of flavonoids and saponin fractions from the leaves and root bark of *Zl*. **Borgi and Chouchane [43]** extracted bioactive compounds from the leaves and root barks of *Zl* using maceration method. The effect of *Zl* root barks extracts on antiulcerogenic activity using soxhlet extractor as demonstrated by **Wahida, Abderrahman [81]**. Similarly, **Naili, Alghazeer [82]** studied the antimicrobial and antioxidant activities of *Zl* plants growing in south part of Libya, which showed a high contents of polyphenols and alkaloids using differents solvents extracts, and considered as source of phenolic antioxidants and antimicrobials which show a minimal inhibitory for gram. **Benammar, Hichami [83]** used the antioxidant effect of *Zl* root, leaf, stem, fruit pulp and seed extracted with decoction and the role of different crude extracts plant on human T-lymphocyte proliferation and expression of IL-2 mRNA, results showed that extracts frim seed exerted the most potent immunosuppressive effects on T cell proliferation and IL-2 mRNA expression. **Bakhtaoui, Lakmichi [84]** suggested the uses of *Zl* fruit extracted with soxhlet using methanol from Morocco and results demonstrated their gastro-protective, anti helicobacter pylori and, antioxidant properties. More recently, **Marmouzi, Kharbach [85]** studied the effect of phenolic compounds extracted from *Z.lotus* fruit and leave by infusing, it show a very important antioxidant, antidiabetic and dermaprotective potential, the identification of this compounds using HPLC-DAD-QTOF-MS show a highest yeild in gallic acid with 2715 mg/kg in leaves and 15,640 mg/kg in fruits. In another study conducted by **Ghalem, Merghache [86]** the antioxydant activity of *Z. lotus* roots from Algeria extract using soxhlet method with application of beta carotene bleaching methods confirm the antioxydant capacity of this extracts.

Other jujube species have been studied using conventionnal technique. *Z. mauritania* plant from south Algeria was used for extraction of alkaloids using chloroform and for the first time, the identification was made using gas chromatography-mass spectrometry (GC-MS) analysis **[87].** The soxhlet apparatus was used for extraction of total phenols and flavonoids content, including antioxidant activity with evaluation of polarities of crude extracts from the leaves and fruit of Omani *Z. jujuba* **[88]**. The study of effect of different extraction solvent using soxhlet on yield of active metabolites extracted from *Z. jujuba* leaves was made by **Al-Saeedi, Al-Ghafri [89]** which confirm their highest yeild of extraction and their antimicrobial activities, this results should be used on pharmacology. Similarly, the extraction of phenolic compounds from APPLE KUL pulp (*Z. mauritania*) were made with methanolic extract by a soxhlet extractor for 6 h which found was found to be a rich source of polyphenols (52.19 ± 2.38 mg gallic acid equivalents/100 g), flavonoids (13.19 ± 1.31 mg catechin equivalents/100 g), ascorbic acid (48.17 ± 2.04 mg ascorbate equivalent/100 g) and tannins (50.20 ± 3.61 mg tannic acid equivalents/ 100 g) **[90]**. Morever, the *Z. jujuba* seeds were studied using conventional method with ethanol/water extracts and analyzed for their bioactive phytochemicals using chromatographic techniques which revealed the presence of many bioactive compounds in which 20 were identified **[91]**. Similarly, **Abdulla, Abdel-Samie [58]** used the same method for extraction of polyphenols from *Zizyphus* leaves. More recently, **Najafabadi, Sahari [92]** demonstrated the high content of phytochemical compounds mainly total phenolic, total monomeric anthocyanin and vitamin C contents in *Z. jujuba var vulgaris* fruit extract using maceration method at different extraction conditions giving maximum of phytochemical compounds at ethanol concentration of 60%, pH of 3, extraction time of 180 min, extraction temperature of 25°C, with values of TPC, TMAC, and vitamin C content for 164.51 mg GAE/g DW, 52.94 mg cy-3-glu 100 g-1 DW, and 137.12 mg LAA 100 g-1 DW, respectively.

Finaly, the conventional extraction methods are characterized by high volumes of solvents and longer extraction times, with a low extraction yields of bioactive compounds. To overcome the limitations of these kind of methods, non-conventional extraction methods have been introduced such us microwave and ultrasound assisted extraction.

II.3.5. Application to ultrasound extraction method

As seen in last paragraph, the application of UAE have been wiedly used for high-added value compounds extraction recovery from plant materials. It seems to be an effective extraction method of antioxidants from jujube fruit. A number of authors have more recently evaluated

and optimized ultrasound extraction conditions, **Boulanouar, Abdelaziz [93]** showed extraction efficiency of phenolic compounds from several plants, from which *Zl* hydro-alcoholic extracts that contains total phenol, hydroxycinnamic acid derivatives, flavone/ flavonol and flavanones/ dihydroflavonol under sonication found to have an extraction yield of 81.44 ± 5.64 mg/g, dry weight which exhibited a good antioxidant effect against ABTS, chelating, DPPH, inhibiting lipoxygenase, reducing, superoxide radicals and ORAC assays with an IC_{50} values of 0.049 ± 0.002, 1.406 ± 0.023, 0.042 ± 0.018, 0.138 ± 0.005, 0.001 ± 0.006, 0.129 ± 0.011 mg/ml and 110.64 ± 39.71 µmol TE g^{-1} (d.w.), respectively. **Hammi, Jdey [94]** studied the effect of the independent variables under ultrasound extraction, including ethanol concentration (0-100%), sonication time (5-45 min), ratio of solvent to solid (10-70 mL/g) and sonication temperature with varying from ambient temperature to 65°C, were investigated. The authors reported that the use of ultrasound with high intensity, improves significantly the phenolic extraction yield from *Zl* pulp and peel. The results showed that increasing the amplitude and the extraction time increased the extraction yield, with minor effect of temperature. The optimum extraction conditions were found using ethanol concentration of 50%, ratio of solvent to solid of 67 g/mL at 25 min and 63ºC. Using these conditions an extraction yield of about 40.782 mg GAE/g DM with significant antioxidant properties by DPPH (IC_{50} of 0.289 mg/mL) and TAA (IC_{50} of 75.981 mg GAE/g DM) in a shorter working time. Morever, the effects of UAE (20 kHz, 80-95°C, 1-4 h, 20-40 g/mL) on polysaccharide recovery with its antioxidant activities from *Zl* pulp and peel were evaluated by **Hammi, Hammami [95]**. The authors reported that direct UAE process led to the highest yield of polysaccharide (18.88%) and six polysaccharides with an average molecular weight of 2720 kDa were identified (arabinose, rhamnose, glucose, fructose, galactose and xylose). However, at the optimal conditions sach as 3h 15min, 91.2°C and water to solid ratio of 39 mL/g, the polysaccharide exract showed a significant DPPH (IC_{50} of 0.518 mg/ml), FRAP (614.39 µmol/L) and anti-lipid peroxidation effects at 50% of 2.417 mg/mL. Similarly, **Adeli and Samavati [96]** investigated the effect of UAE on yield of water-soluble polysaccharide extracted from *Zl* fruit while obtaining a maximum yield of 13.398 ± 0.019% under optimized condition as follows: 88.77 W, 29.96 min, 77.73°C and water to raw material ratio 24.44 mL/g with highest antioxidant activites for DPPH (78%) and hydroxyl radical-scavenging (91%).

There are few reports on extraction of bioactive compounds from *Zl* plant using UAE (Table 3). While, other *Zizyphus* species have also been considered as source of bioactive comounds

for which their extraction using innovative techniques interested several authors. **Qu, Yu [97]** studied the application of UAE in polysaccharide extraction from *Z. jujuba Mill* using differents solvents and compared to MAE and hot water extraction methods on antioxidant effects. Results showed that UAE is considered the best technique that gives a higher yield of extraction with better antioxidants activity againt $^{\cdot}OH$ scavenging assay with 68% in comparaison to other methods hot water extraction and microwave extraction with 54 and 52%, respectively. Moreover, UAE enhanced the extraction of polysaccharides from *Z. jujube cv. Muzao* (ZMP) by UAE with using both 29% ethanol and 15%$(NH_4)2SO_4$, the autors used jujube powder with liquid-to-solid ratio (mL/g) of 30 under a power of 70 W for 38 min at 48°C. Following these conditions, the experimental extraction yield of ZMP was for 8.18% with a high antioxidant potential against DPPH (29.68%) and ABTS radical scavenging (21.45%) at a concentration of 2.5 mg/mL **[98].** In another study conducted by **Lin, Liu [99]** that utilized UAE for the recovery of polysaccharides from *Z. jujuba Mill* var. spinosa seeds showed a higher yield of polysaccharide (1.05 ± 0.08%) at 52.5 °C, 21.2 min, 134.9W and ratio of liquid to solid 26.3 mL/g as applied conditions. These results are significantly equated to that 0.93 ± 0.14% of 6 h using heating water extraction method. The seeds extract scavenged more rates of ABTS (33.41%), superoxide anion (41.72%), and hydroxyl radicals (69.78%), while it's chelating capacity of Ferrous ion was up to 42.70%. Similarly, **[100]** extracted phenolic compounds using UAE from jujube leaves and evaluated its antioxidant activity. RSM study has been used under some extraction conditions including solvent concentration (25-100%), solid/solvent ratio (1/50-1/300), extraction time (1-15 min) and ultrasound intensity (25-100%). Authors demonstrated the positive use of UAE giving 6 g GAE/100g for total phenolic content, under methanol 60%, 75% intensity, time of 10 min and ratio of 1/200. The extract showed a positive correlation with the antioxidant activities against DPPH (3.886 g ascorbic acid equivalents/100g) and FRAP (2.587 g ascorbic acid equivalents/100g).

The extraction of antioxidants mainly polyphenols and polysaccharides from different part of jujube plant by conventional and non conventional method has been the subject of several studies, the following Table 4 represents most of them.

Table 4 : Different green extraction techniques of antioxidants from *Zizyphus* plant.

Extraction method	Category	Vital Products	Matrix	Conditions	Model	Yield.	Antioxidant effect	References
UAE	Polysaccharides	Fruit	*Z. jujuba Mill*	15 min 40 °C 80 W	·OH scavenging assay.	ND	The extract revealed 68% inhibition ·OH scavenging.	**[97]**
	Polyphenols	Fruit	*Z. lotus*	6 min 20 kHz 1g/ 7 mL of a hydro-alcoholic solution (70%)	In vitro ABTS, ORAC, DPPH, chelating, superoxide radicals and inhibiting lipoxygenase were assayed	81.44 ± 5.64 mg/g, dry weight	*Zl* extract exhibited IC_{50} values of 0.049 ± 0.002, 1.406 ± 0.023, 0.042 ± 0.018, 0.138 ± 0.005, 0.001 ± 0.006, 0.129 ± 0.011 mg/ml and 110.64 ± 39.71 µmol TE g^{-1} (d.w.) against ABTS, chelating, DPPH, inhibiting lipoxygenase, reducing, superoxide radicals and ORAC assays	**[93]**
	Polyphenols	Pulp and peel	*Z. lotus*	Ethanol 50% 25 min 63°C 67 mL/g	In vitro DPPH and TAA assays.	40.782 mg gallic acid equivalents/g dry matter	The extract revealed IC_{50} values of 0.289 mg/mL and 75.981 mg GAE/g DM for DPPH and TAA tests, respectively.	**[94]**
	Polysaccharides	Pulp and peel	*Z. lotus*	3h 15min 91.2°C 39 mL/g	DPPH scavenging ability, reducing power and anti-lipid peroxidation assays.	18.88%	The *Zl* extract revealed potent IC_{50} values of 0.518 mg/ml), 614.39 µmol/L and 2.417 mg/mL at 50% for DPPH, FRAP and anti-lipid peroxidation tests.	**[95]**
	Polysaccharides	Fruit	*Z. lotus*	88.77 W 29.96 min 77.73◦C 24.44 mL/g	DPPH and hydroxyl radical-scavenging activities.	13.39 ±0.019%	The polysaccharide extract revealed an antioxidant effect of 78 and 91% for DPPH and hydroxyl radical-scavenging tests, respectively.	**[96]**
	Polyphenols	leave	*Z. jujuba Mill*	methanol 60% 75% intensity 10 min 1/200 mg/mL	FRAP and DPPH assays.	6 g GAE/100g	Antioxidant activities against DPPH and FRAP were for 3.886 and 2.587 g ascorbic acid equivalents/100g , respectively.	**[100]**
	Polysaccharides	Fruit	*Z. jujube cv. Muzao*	29% ethanol 15% $(NH_4)2SO_4$ 30 mL/g 70 W 38 min 48°C.	ABTS and DPPH assays.	8.18%	The extract revealed to have a moderate antioxidant activity for both DPPH (29.68%) and ABTS (21.45%).	**[98]**
	Polysaccharides	Seeds	*Z. jujuba Mill* var. spinosa	52.5 °C 21.2 min 134.9W 26.3 mL/g	ABTS, superoxide anion, hydroxyl radicals and chelating capacity of Ferrous ion	1.05 ± 0.08%	The extract showed an ABTS, superoxide anion, hydroxyl radicals and chelating capacity of Ferrous ion of 33.41, 41.72, 69.78 and up to 42.70%, respectively.	**[99]**

MAE	Polysaccharides	Pulp and peel	*Z. lotus*	600 W 40 min 26.69 mL/g	ABTS, DPPH and FRAP tests	13.98 ± 1.55%	The Zl extract revealed a good scavenging capacity against ABTS.+ (70.45%), DPPH*.(66.02%), and FRAP (A = 0.63).	**[101]**
	Polysaccharides	Fruit	*Z. jujuba Mill*	4 min 300 W	·OH scavenging assay.	ND	The extract revealed 52 % inhibition ·OH scavenging test.	**[97]**
	Polysaccharides	Peels	*Z. jujube Mill*	400W 75°C 60 min 30 g water/g powdered jujube	FRAP and DPPH assays.	9.02%	Jujube extract showed a scavenger effect against DPPH arround 65 to 75% and FRAP (A= 0.63).	**[102]**

II.3.6. Application to microwave extraction method

More recently, there are few studies about MAE of jujubes. The influence of microwave heat treatment on jujube plant in terms of storability and quality has been studied. The response surface methodology (RSM) was used to evaluate and optimize MAE in polysaccharide recovery from *Z. jujube Mill* peels. For this purpose, jujube fruits were treated by MAE until reaching a temperature level of 45-85°C, microwave powers (250-450 W), extraction time (30-70 min) and ratio of solvent to solid (10-70 mL/g). The authors reported that the use of microwave with high intensity, improves significantly the yeild of polysaccharides from *Z. jujube. Mill* fruit (9.02% of polysaccharide) at 400 W, 75°C, 60 min, using 30 g water/g powdered jujube with a good antioxidant effect against DPPH (arround 65 to 75%) and FRAP (A= 0.63) **[102]**. For instance, there is no report about the use of MAE to enhance extraction of phenolic compounds from the *Zl* species. However, only our previous work focused on polysaccharide extract from *Zl* pulp and peel using MAE under RSM study investigating the effect of the independent variables, including microwave power (200-600 W, X_1), irradiation time (20-40 min, X_2), and a liquid/solid ratio (20-40 mL/g, X_3) have been recently published. Our results improved significantly the polysaccharide extraction yield from *Zl* pulp and peel at 600 W, 40 min, and 26.69 mL/g. Under these conditions, the *Zl* extract exhibited a good antioxidants capacity against ABTS+ (70.45%), DPPH· (66.02%), and FRAP (A = 0.63) **[101].**

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Part II. Material and Methods

Part II : Personal contribution

Objectives

Nowadays, consumers are highly aware of the close relationship between nutrition and health and they want to include health-promoting ingredients in their diets. Therefore, natural ingredients recovered from vascular plants have specific dietary and func-tional properties and can be utilized effectively to develop food additives or supplements with high nutritional value **[1].** Published data indicates that natural compounds mainly polysaccharides and polyphenols are abundant in jujube fruits which could be used as a natural source of antioxidants to prevent the progression of some diseases. Secondary metabolites like flavonoids, phenolic acids, saponins and alkaloids are a specific antioxidants compounds that protect plant, animals, food and human from oxidative stress which is a result of the production of reactive oxygen species (ROS) which can cause inflammation and contribute to tissue damage. In the meantime, free radicals react with lipids and cause lipid peroxidation which affects the quality of product (taste, color and flavor). In humans, oxidative stress causes many current diseases including inflammation, cataract, cancer, arteriosclerosis, autoimmune, Parkinson and neurodegenerative syndromes. Besides, it is well known that polyphenols and polysaccharides of jujube genus could preserve the quality of food products from chemical oxidation due to exposure to air (oxygen) or to the effects of light and for their potential pharmacological and biological activities including antitumour, immunostimulant, anticancer, anticomplementary, anti-inflammatory, anticoagulant hypoglycaemic, antiviral and immunological activities **[2, 3].**

The present study explores such attractive biological features of *Z. lotus* pulp and peel (ZLP), seed (ZLS) and endocarp (ZLE) from Djelfa (Algeria). The first step for both analysis and exploitation of medicinal plant bioactive constituents is their extraction from the cellular matrix. The “ideal” extraction method should be quantitative, non-destructive, and time saving. The efficiency of an extraction process is influenced by many factors such as solvent composition, extraction temperature, extraction time, and solvent to solid ratio. Therefore, the objectives of the current chapter were to: (i) investigate the effects of different parameters on the extraction efficiency (in terms of recovery and biological activity of total phenolic compounds and polysaccharides) by MAE and UAE processes; (ii) optimize the MAE and UAE conditions by response surface methodology (RSM); (iii) compare the optimized MAE and UAE results with a reference Conventional Solvent Extraction procedure (CSE).

I. *Zizyphus lotus* plant materials

I.1. Source

The vegetable material were harvested from Djelfa regions in 2017 and 2019 as represented in figure below.



Figure 12: Map representing the areas of harvesting of the jujubes.

I.2. Drying and Grinding

The jujube fruits have been well washed with tap water followed by bidistilled water to remove all impurities, drying was carried out in two stages, first a pre-drying in the open air to remove excess water. Jujube fruits were manually separated from the pulp, seed and endocarp, followed by drying of each part of fruit in a ventillated oven at < 40°C for about 24 to 48 h to protect the active compounds content from light oxidation. Representative samples of 10 g (triplicate) are brought to 102 ± 3 °C for 4 hours to calculate the moisture content. After confirmation of the humidity test after this time, dried samples were ground with an electrical grinder, the powders were passed through standard 250 µm sieve and only the fraction with particle size < 250 µm was used. They were stored in airtight bags until use. One portion of the different powders was used for the extraction of phenolics and polysaccahrides by conventional, microwave and ultrasonic mechanical agitation; the remaining materials were stored in the glass jars, sealed and stored in the dark conditions for other use.



Figure 13: Parts of *Z. lotus* fruits **[4].**



Figure 14: Parts of *Z. lotus* plant used during experimental work (Photos from BBBS-Lab).

I.3. Chemical reagents and equipment

All chemicals used were of analytical grades. The reagents used in the experiments are collected in the following tables represented in the appendix 1 and 2.

II. Experiment Design

The influences of the process parameters using both UAE and MAE process were firstly separately investigated in single-factor experiments to limit the total experimental work. When one variable was not studied, it was kept constant. In the MAE trials, the constant values for irradiation time, solvent-to-solid ratio and ethanol concentration were 150 s, 30 mL/g and 50%, respectively. While, microwave power was set at 500 W in the trials to investigate the influence of solvent type, solvent-to-solid ratio.and ethanol concentration. In the UAE trials, the constant values of sonication time, solvent-to-solid ratio and ethanol concentration were 15 min, 35 mL/g and 50%. Sonication temperature was set at 20°C in the trials to investigate the influence of ethanol concentration and sonication time, and at 30°C in the trials to investigate the influence of solvent-to-solid ratio.

Major influence factors were selected by taking into account the results of single-factor experimental. Then, an RSM based on a Box–Behnken Design (BBD) and Central Composite Rotatable Design (CCRD) was conducted to optimize both UAE and MAE processes depending of the part of jujube plant. Regression analysis of the data to fit a second-order polynomial equation (quadratic model) was carried out according to the following general equation (Eq. 1) which was, then, used to predict the optimum conditions of extraction process.

$$Y = B_0 + \sum_{i=1}^{k} B_i X_i + \sum_{i=1}^{k} B_{ii} X_i^2 + \sum_{i>1}^{k} B_{ii} X_i X_j + E \quad (1)$$

Where Y represents the response function (in our case the TPC yield); B_0 is a constant coefficient; B_i, B_{ii} and B_{ij} are the coefficients of the linear, quadratic and interactive terms, respectively, and x_i and x_j represent the coded independent variables. According to the analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were determined. In order to visualize the relationship between the response and experimental levels of each factor and to deduce the optimum conditions, the regression coefficients were used to generate 3D surface and contour plots from the fitted polynomial equation. The factor levels were coded as −1 (low), 0 (central point or middle) and 1 (high), respectively. The variables were coded according to the following equation (Eq. 2):

$$X_i = (A_i - A_o) / \ dA \quad (2)$$

Where xi is the (dimensionless) coded value of the variable Xi; X0 is the value of X at the center point and ΔX is the step change.

II.1. Response surface methodology

In order to evaluate relationships between a group of independent variables and one or more responses, a collection of mathematical and statistical methods defined as Response surface methodology (RSM), this is enables to evaluate operation variables that could or not have significant effect in the main response on the basis of a set of statistical methods that can be used to develop, improve, or optimize the processing conditions. RSM typically is used in situations where several factors influence one or more performance characteristics, or responses of the system **[5].**

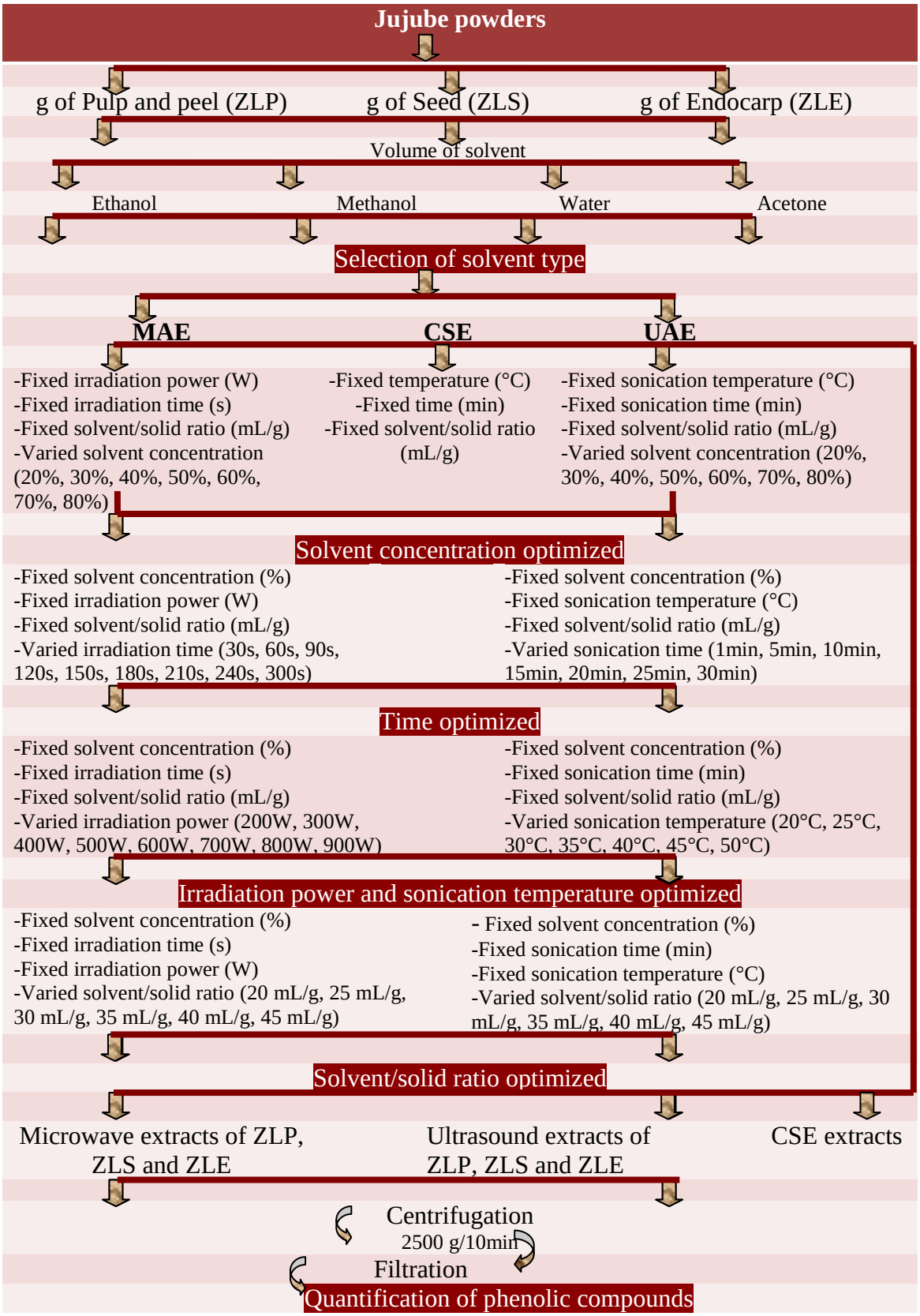


Figure 15 : Single-factor experimental procedure of phenolics for both MAE and UAE of different jujube parts.

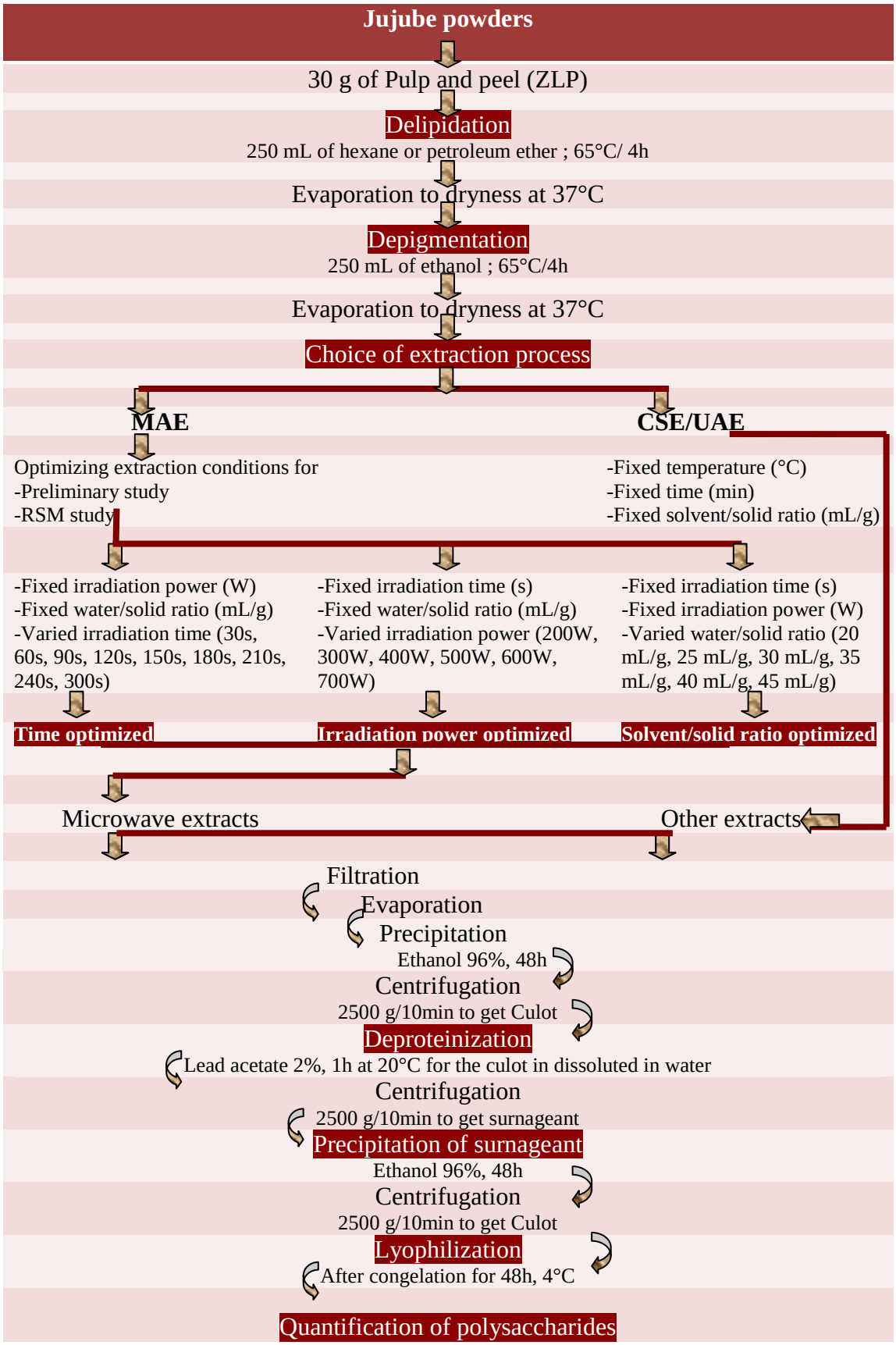


Figure 16: Preliminary study of polysaccharides extraction using MAE process and other extraction process of ZLP extracts.

II.2. Application to the microwave assisted extraction process

After knowing the range of factors that obtain maximum extraction of jujube extracts from the sreening study. A central composite rotatable design (CCD) was performed using RSM in order to study the cumulative effect of the independent variables previously choosed, to evaluate the interaction effect between the factors and to obtain the optimal conditions of extraction of the studied response under microwaves. In response surface experimentation, it is desirable to estimate a pure or experimental error term and lack-of-fit error term. The variation experienced when repeating a run at the same design point determines the pure error.

The multifactorial model based on CCD has been constructed, in which all factors were kept invariable and one factor is changed. Otherwise, the independent factors namely solvent concentration (%), microwave power (W), irradiation time (s) and liquid/solid ratio (mL/g) were evaluated for ZLP extract, while for both ZLS and ZLE extracts only liquid/solid ratio (mL/g) was not studied (it wasn't significant and it was keep constant) to investigate their effects on amount of total phenolics. As well as linear effect, mutual interaction and quadratic effect. Otherwise, the effect of microwave power (W), irradiation time (s) and liquid/solid ratio (mL/g) were evaluated on amount of polysaccharides for only ZLP extracts.

The CCD is used to build a second order experimental model and composed of a factorial design, a set of central points, and axial points equidistant to the center point. The geometric representation of a factorial is a cube in which each corner represents an interaction of the factors (**Figure 17**). In this perspective, 8 interactions are to be evaluated when 3 processing variables are selected to determine their significance in the final response. The lowest and the highest values of each parameter is coded as (-α) and (+α), respectively. Specification of the lowest and the highest values determines the remaining part of the design:

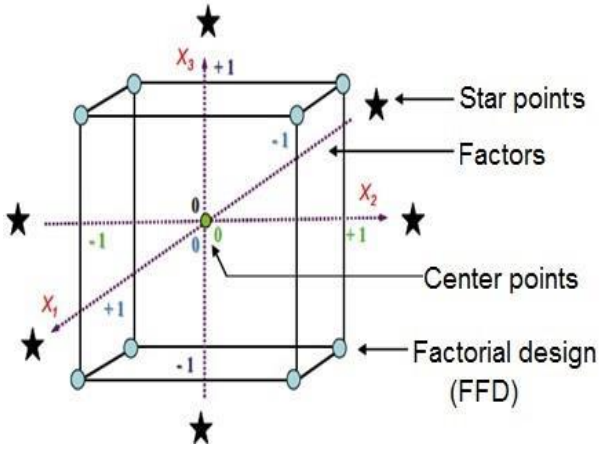


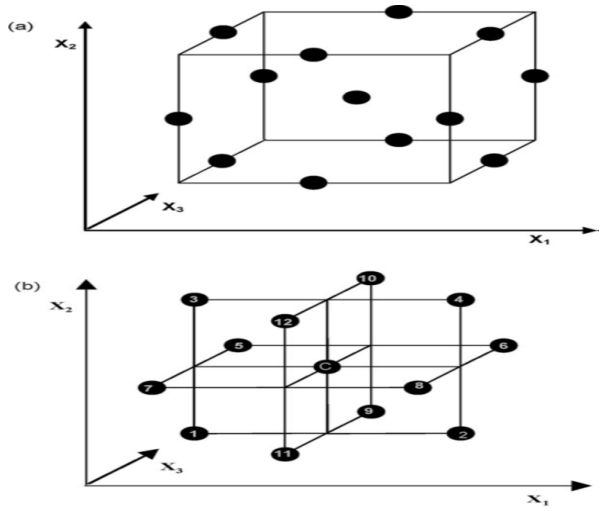
Figure 17: Layout of the Central Composite Design (CDD) for 2 variables at 5 levels.

The points of the Full Factorial Design (FFD) with 02 levels (n_f) (coded values ± 1): $n_f = 2^k$. are located at the vertices of a square, cube, hypercube or a fraction of hypercube. The coded

independent variable levels of these points are ± 1 **[6].** In the mean time, experimental points belonging to this group represent the replication at center point conditions. These points have the coordinates (0,…,0), n_0 = number of experiments that can be a function of the experimenter. These experiments are used to assess the repeatability, they are used to ensure that there is no slip between the FFD and plan star, they are involved in the calculation of α, these points provide also a mean for estimation of the experimental error and provide a measure of lack-of-fit and finally they are used to test the validity of the model. The "star points" (coded ± α), n_α = 2 x k axial experiences or star points on the axes of the cube, in consequence, there is a positive axial value (+α) and a negative axial value (-α). The axial points add two more levels in each variable. These experimental points will define the parameters of the quadratic-mathematical model (effect may involve the curvature of the response surface). However, the α value is calculated to obtain a near-orthogonality. Frequently the value of α is selected to make the design rotatable. A rotatable design has uniform variance at any given radius rom the center of design. The value of α is a function of the number of point in the center, the number of points in the factorial design and the number of point star.

II.3. Application to the ultrasound assisted extraction process

The BBD is a model of second degree with several variables, it is easy to implement, different to the CCD model by the advantageous of sequentiality compared to the factors. We can undertake the study of the first k factors while reserving the option to add new without losing the results of tests already carried out **[6, 7].** The main characteristics of BBD are:



- The number of experiments (N) required for the development of BBD is defined as $N = 2k(k-1) + C0(3)$, with k is the number of factors and Pc is the number of central points;
- All levels of factors must be adjusted only at three levels (-1, 0, +1) with regular intervals

The experimental points are placed in the middle of the edges of the cube as shown in Figure 18.

Figure 18: (a) the cube for BBD and three interlocking 2_2 factorial design (b) **[8].**

A cube has 12 edges; there will be 12 trials and following the advice of Box and Behnken, adding three points in the center of the study area. BBD plans for three factors contain 15 experimental points which are on a sphere, the criterion isovariance by rotation will be respected. Noting, when we place 4 points in the center of the study area, instead of three points, we get a plan that meets the near-orthogonality test **[8].**

The BBD model for four factors in the case of ZLS and ZLE extracts was constructed as like the four factors, the experimental points are located in the middle of the edges of the hypercube which has four dimensions and an adding points to the center give the matrix representation **[7].** In this study, four process variables, namely composition of solvent as volume percentage of EtOH (X_1), irradiation time (X_2), microwave power (X_3) and liquid-to-solid ratio (X_4) (**Table 7**), were studied on three levels to investigate their effects on amount of total phenolics in the extract by 2_{nd} order model with 24 points, and 3 replications of the center points.

Table 5: The independent variables and its levels of factors influencing microwave and ultrasound assisted extraction for jujube phenolic extracts.

Extraction process	Independent variables	ZLP			ZLS			ZLE		
MAE	Solvent concentration (%)	-1	0	+1	-1	0	+1	-1	0	+1
	Irradiation time (s)	-1	0	+1	-1	0	+1	-1	0	+1
	Irradiation power (W)	-1	0	+1	-1	0	+1	-1	0	+1
	Solvent solid/ratio (mL/ g)	-1	0	+1	-1	0	+1	-	-	-
UAE	Solvent concentration (%)				-1	0	+1	-1	0	+1
	Sonication time (min)		-		-1	0	+1	-1	0	+1
	Sonication temperature (°C)				-1	0	+1	-1	0	+1
	Solvent solid/ratio (mL/ g)				-1	0	+1	-1	0	+1

Table 6: The independent variables and its levels of factors influencing microwave assisted extraction for jujube polysaccharide extract.

Extraction process	Independent variables	ZLP		
MAE	Solvent concentration (%)	-	-	-
	Irradiation time (s)	-1	0	+1
	Irradiation power (W)	-1	0	+1
	Solvent solid/ratio (mL/ g)	-1	0	+1

III.Extraction procedure

Throughout the project, different types of extraction were performed which depends on the part of the studied plant and also on the molecule to be extracted. To reduce inter-individual variation an equal amount of vegetable powder is used for extraction.

III. 1. Microwave assisted extraction

A domestic microwave oven with cavity dimensions of 22.5 cm × 37.5 cm × 38.6 cm and 2450 kHz working frequency is used. The apparatus was equipped with a digital control system for irradiation time and microwave power (adjustable from 100 to 900 W). The oven was modified in order to condensate into the sample the vapors generated during extraction giving a constant sample volume (**Figure 19).** For the extraction conditions, in a 250 mL volumetric flask containing a known volume of the extraction solvent, one gram of the sample powder is placed. The suspension is irradiated at regular intervals according to oven operation. Depending on the trial, a different solvents, irradiation time, microwave power and solvent/solid ratio are used. At the end of microwave irradiation, the volumetric flask was allowed to cool to room temperature. After extraction, the extract was recovered by filtration in a Büchner funnel through Millipor 45 Micron filter paper, and collected in a volumetric flask. The extract was stored at 4◦C until use and analyzed for the TPC, TFC and TTC contents for phenolic extracts and for polysaccharide extracts, some studied biological activities were measured for the extract obtained under the optimum conditions by RSM.

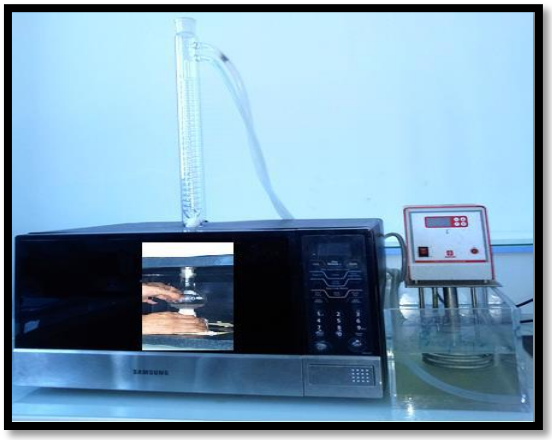


Figure 19: MAE apparatus used during extraction of both polysaccharides and phenolic compounds (Photo from BBBS-Lab).

III.3.2. Ultrasound assisted extraction

Extraction of phenolic compounds using ultrasound bath with working frequency fixed at 20 kHz has to improve the efficiency and/or speed of this step. For the extraction, one gram of both ZLS and ZLE powders were placed in a 250 mL amber glass bottle (Ø x H: 45 mm × 140 mm and cap size of 28 mm) containing water–ethanol mixture; the obtained suspension was exposed to acoustic waves for 15 min (**Figure 20**). The temperature was controlled continuously by circulating external cold water and checking the temperature using a T-type thermocouple. After the extraction, the extract was recovered and analyzed as reported in section of the optimized MAE extract.



Figure 20: Ultrasonic equipment used during extraction of phenolic compounds (Photo from GVRNAQ-Lab).

III.3.3. Conventional solvent extraction

For the conventional extraction, one grams of each powder were placed in a conical flask of 250 mL (Ø x H: 51 x 150 mm and cap size of 38 mm), and 50 mL of 50% (v/v) ethanol were added. The mixture was kept in a thermostatic water bath with shaking speed of 110 strokes perminute, at 60◦C for 2 h, according to the method recommended by **Dahmoune, Nayak [1]**. The extract was then recovered and analyzed as reported for MAE in Section.

IV. Analytical methodology

IV. 1. Total phenolic content (TPC)

The Folin-Ciocalteu method was used to assay total phenolics in this study. The Folin-Ciocalteu Reagent is an oxidizing agent consisting of heteropolyphosphotungstatemolybdate. It oxidizes the phenolates, reducing the heteropoly acids to a blue Mo-W. The blue colored

product is a mixture of 1-, 2-, 4-, and 6- electron reduction products in tungstate series $(P_2W_{18}O_{62})^{-7}$ to $(H_4P_2W_{18}O_{62})^{-8}$ and 2-, 4-, and 6-electron reduction products in the molybdate series $(H_2P_2Mo_{18}O_{62})^{-6}$ to $(H_6P_2Mo_{18}O_{62})^{-7}$ **[9].**

Total phenolic content (TPC) of different extracts were determined using Folin–Ciocalteu reagent slightly modified by **[10]**, using gallic acid as a standard. Briefly, 125 µL of each suitable diluted extract was added to 500 µl of distilled water and 125 µl of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before addition of 1250 µl of Na2CO3 (70 g. L-1185). The solution was then adjusted with distilled water to a final volume of 3 mL and mixed thoroughly. After 90 min of incubation at room temperature (20°C), the absorbance was measured at 760 nm using a UV–vis spectrophotometer. The calibration curve was performed with gallic acid (concentrations ranging from 50 to 200 µg/mL) and total phenolic content was expressed as milligram of gallic acid equivalents (GAE) per gram of dry weight material (mg GAE/g *Z.lotus*). Measurements were performed in triplicate.

IV. 2. Total flavonoid content (TFC)

The total flavonoid content was determined according to the **Ghafar, Prasad [11]** method with slight modifications. Briefly, 300 µl of diluted extracts were added to 300 µl of 2% (w/v) methanolic aluminium chloride ($AlCl_3$) and then mixed thoroughly. The absorbance of the mixture was measured at 430 nm after 15 min versus the prepared blank and positive control. The TFC results were expressed as mg quercetin equivalents/100g of dry matter (mg QE/100g). Samples were measured in triplicate.

IV. 3. Total condensed tannins content

The total condensed tannins content was determined using the method of **Hagerman [12]**. Briefly, 625 µl of analytical reagent (315 µl of vanillin solution (1%) mixed with 315 µl of HCl solution (8%)) were added to 125 µl of diluted extracts (prepared with methanol) or catechin (positive control). After 30 s; 625 µl of HCl solution (4%) was added and the mixture flask was incubated for 20 min at 30 °C. The absorbance was readed at 500 nm and the results were expressed as mg catechin equivalent per 100 g of dry matter (mg CE/100g). Samples were measured in triplicate.

IV. 4. Total carbohydrates and proteins content

The total carbohydrate content of *ZLPS* was estimated using method of **Dubois, Gilles [13]** with minor modifications. It uses the complexe of sulfuric acid with phenol at 490 nm which showed a color after absorption resulting from reaction between phenol and carbone hydrate. Briefly, 5 mg of *Z.lotus* powder was mixed with 25mL of distilled water, under agitation. Then, 250µL of phenol (5%) and 1250µL of sulfuric acid was added, and mixed with 50µL of extract. The mixture was incubated at 95°C for 5min, then absorbance was measured at 480 nm. Total carbohydrate concentration was calculated from a calibration curve, using D-glucoseas a standard, obtained in the same conditions. Thus the results were expressed in mg of D-glucose per 100g of polysaccharide dry matter. The protein content of *Z.lotus* polysaccharides was analysed by the colorimetric method of **Bradford [14],** using bovine serum albumin as the standard.

IV. 5. Antioxidant activity

IV. 5.1. Scavenging activity against the DPPH· radical

The DPPH • (1,1-diphenyl-2-picrylhydrazyl) test consists of a measurement of the ability of an antioxidant compound to reduce the persistent free radical DPPH• (0.19 mM) by visible spectrophotometry **[15].** DPPH• is a stable radical with a purple coloration, due to the presence of an unpaired electron on the nitrogen atom, having a maximum absorption in the visible range at 517 nm in alcoholic solution. The absorbance of the DPPH solution • decreases giving a yellow color, even colorless, when the single electron on the nitrogen atom of DPPH• is reduced by one atom hydrogen from the antioxidant product (**Figure 21**). Antioxidant capacity increases as the substance reduces DPPH• :

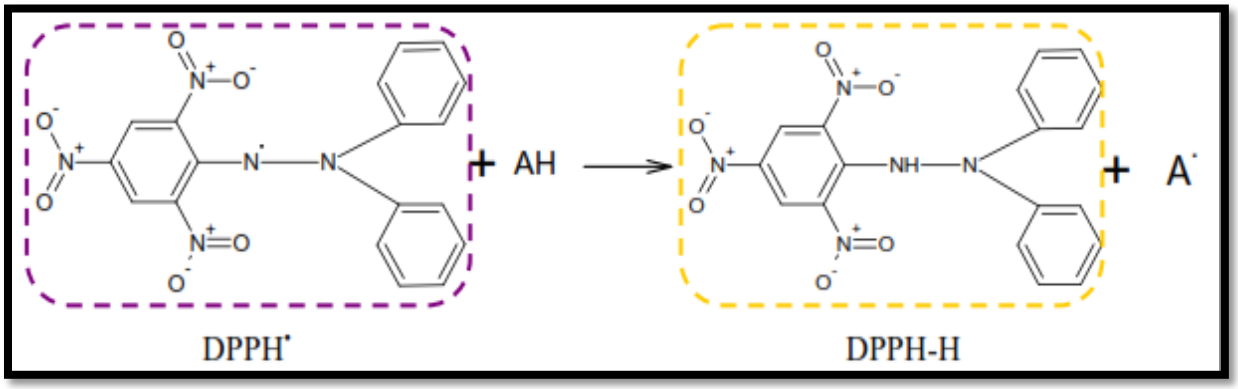


Figure 21 : Reaction of reduction of DPPH radical.

The free radical-scavenging activity of extracts were measured using method of **Achat, Tomao [16]** with some modifications. Briefly, 0.5mL of *ZLPS* extract at different concentrations (25-200 µg/mL) was added to 1 mL of freshly $DPPH^{\cdot}$ solution (0.2 mM in methanol, 99%). The mixture was incubated in the dark at 25 °C for 30 min and the absorbance was measured at 517 nm using a UV–vis spectrophotometer (Perkin Elmer Lambda 40 UV/Vis Spectrophotometer). Results were expressed as the percentage of inhibition of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$), calculated according to the equation 4 (Eq. 4), against ascorbic acid as a standard :

$$\%\text{Inhibition} = \left[(\text{Abs}_b - \text{Abs}_s) / \text{Abs}_{DPPH}\right] * 100 \quad (4)$$

Where: A_b is the absorbance value of the blank ($DPPH^{\cdot}$solution without extract); A_s is the absorbance of the diluted extract with $DPPH^{\cdot}$solution; A_{DPPH} is the absorbance of the $DPPH^{\cdot}$solution.

IV. 5.2. Scavenging activity against the $ABTS^{\cdot+}$ radical

The $ABTS^{\cdot+}$(2,2'-azino-di [3-ethylbenzthiazoline sulphonate]) assay was performed following the method of **Dahmoune, Nayak [1].** This assay is based on the ability of interaction of bioactives substances with $ABTS^{\cdot+}$ radical, which is used for both lipophilic and hydrophilic antioxidants, decreasing its absorbance at 734 nm **[17].** The radical solution was prepared in ethanol with 7 mM $ABTS^{\cdot+}$ and 2.45 mM of potassium persulfate and stored in the dark at 27 °C for 16 h then diluted to an absorbance of 0.7 at 734 nm, with ethanol. About 120 µL of *ZLPS* extract in various concentrations (25-200 µg/mL) was added to 480 µL of radical solution. The percentage inhibition of $ABTS^{\cdot+}$ was calculated (Eq. 5) and compared to ascorbic acid as a reference standard.

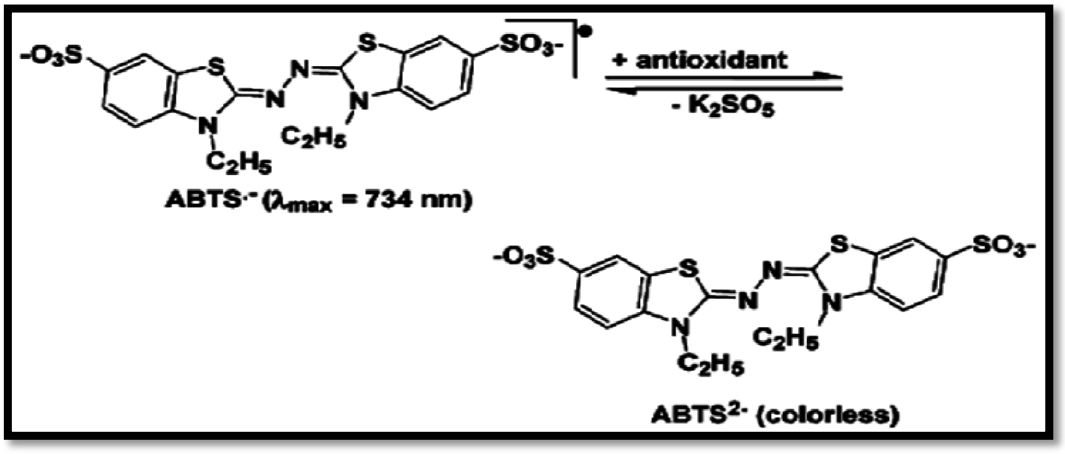


Figure 22 : Reaction between ABTS radical and antioxidant compound **[18].**

$$\% \text{ Inhibition} = [(\text{Abs}_{b(t=6)} - \text{Abs}_{s(t=6)}) / \text{Abs}_{ABTS(t=0)}]*100 (5)$$

Where : $Abs_{b,}$ is the absorbance value of the blank ($ABTS^{\cdot+}$solution without extract); Abs_s, is the absorbance of the diluted extract with $ABTS^{\cdot+}$solution; Abs_{ABTS}, is absorbance of the radical solution at t=0; t, is the time in min at which absorbance was read.

IV.5.3. Ferric- reducing antioxidant power (FRAP) activity

The FRAP assay was determined according to the method described by **Hammi, Hammami [2]** with some modifications. Briefly, 625 µL of $K_3Fe(CN)_6$ (1%) and 625 µL of sodium phosphate buffer (0.2 M, pH6.6), were mixed with 250 µL of each *ZLPS* extract in distillated water solution at different concentrations (25-200 µg/mL) then incubated at 50°C for 20 min. After cooling, the reaction was stoped before addition of 625 µL of trichloroacetic acid (TCA, 10%), then mixtures were centrifuged at 2000×*g* for 10 min. After that, 625 µL of this mixture was added to 125 µL of $FeCl_3$ (1%) and 625 µL of distilled water and kept for 10 min. The absorbances were readed at 700 nm against a blank using ascorbic acid as a reference standard. The greater ferric reducing capability showed the highest absorbance.

IV.5.4. Total antioxidant power by PAOT technology

The total antioxidant activity of *Zlp* was determined according to **Kaci, Belhaffef [19]** by using PAOT (Pouvoir AntiOxydant Total) Liquid® technology (WO 2020/109736A1). The measurement was carried out in a reaction medium (1 mL physiological solution at pH ranging from 6.7 to 7.2, temperature 24- 27°C) containing a molecule in a free radical state called mediator (M•). Two microelectrodes, one is the working electrode and the second is the reference electrode, were then immersed in the medium. After addition of 20 µL of pure antioxidants (1 mM final) or studied supplements samples, PAOT-liquid® activity was estimated by registering electrochemical potential modifications in the reaction medium. Results were expressed as PAOT Score per gram of extract (PAOT Score/g) **[20]**, according to the following formula:

$$\text{Antioxidant activity} = \left(EP_{product10} - EP_{control0} / EP_{control0}\right) \times 100 \ (Eq.6)$$

Where: $EP_{control0}$ is the electrochemical potential at time 0. $EP_{product10}$ is the electrochemical potential obtained after 10 min registration in presence of tested antioxidants or studied supplements samples.

IV.6. Anti-inflammatory activities

IV.6. 1. Inhibition of albumin denaturation

The anti-inflammatory activity of *ZLPS* extracts was investigated using inhibition of albumin denaturation technique according to **Karthik [21], Rani, Punitha [22]** methods with some modifications. Briefly, the reaction mixture was containing 1 mL of each extract at different concentrations (25-200 µg/mL) using distillated water as solvent and 1 mL of 0.2% aqueous solution of bovin serum albumin (BSA) prepared in Tris-HCL (0.05M, pH 6.8). The standard consists of 100 µg/mL of profenidin solvent with 5mL of BSA solution (0.2%). The control consists of 5mL of BSA solution (0.2%) with 50 µL of distillated water (obtained results correspond to the total denaturation of BSA, in the absence of inhibitrice substances). All samples were incubated at 37 ºC for 15min and then heated to 72 ºC for 10 min. After 10 min of cooling, the turbidity was measured at 660 nm using a UV-visible Spectrophotometer. The percentage inhibition of protein denaturation was determined using the following formula :

$$\% \text{ Inhibition} = [(Abs_1 - Abs_2) / Abs_1] * 100 (7)$$

Where : Abs_1: absorbance of control, Abs_2: absorbance of the sample.

IV.6.2. Membrane stabilization assay

a- Preparation of red blood cells (RBC)

The red blood cells suspension (RBCs) were prepared after collection of blood from healthy human volunteers in heparinic tubes **[23].** After centrifugation at 3000 rpm for 10 min, the tubes were washed three times with 2 volumes of normal saline (10 mM, 154 mM NaCl, pH 7,4). After washing, the volume of blood was reconstituted as 10% of erythrocyte suspension with normal saline (PBS iso-salin) **[22, 24].**

b-Hypotonicity-induced haemolysis

As described by **Oyedapo, Akinpelu [25] ; Rani, Punitha [22]**, the reaction mixture consisted of 1 mL of *ZLPS* extract at different concentrations (25-200 µg/mL), 1 mL of isosaline (10 mM phosphate buffer, 154 mM NaCl, pH 7,4), 2 mL of hyposaline (10 mM phosphate buffer, 50 mM NaCl, pH 7,4), 0.5 mL of 10% RBCs prepared in isosaline. Profenid (100 µg/mL) was used as standard anti-inflammatory drug. 1mL of isosaline was used as a control test instead of extracts while drug control tests lacked red blood cells. All prepared tubes were incubated for 30 min in water bath at 37 ºC and centrifuged at 2500 g for 5 min.

The absorbance of supernatants were read at 560 nm. The percentage of inhibition of haemolysis was calculated as follows:

$$\% \text{ Inhibition} = [(Abs_1 - Abs_2) / Abs_1] * 100 (7)$$

Where : Abs_1: absorbance of positive control and Abs_2: absorbance of extract.

IV.6.3. Heat induced haemolysis

The test of heat induced haemolysis was carried out using method of **Rani, Punitha [22].** Briefly, 500 µL of RBCs suspension (10%) was added to 500 µL of extract at different concentrations (25-200µg/mL). The control was measured with 500 µL of RBCs suspension (10%) with saline solution. Profenid was used as a standard drug. The mixture was incubated at 56 ºC for 30 min in water bath, after cooling the temperature, all tubes containing reaction mixture were centrifuged at 2500 rpm for 5 min and the absorbance was measured at 560 nm. The percentage of inhibition of Haemolysis was calculated according the equation below (Eq.7):

$$\% \text{ Inhibition} = [(Abs_1 - Abs_2) / Abs_1] * 100 (8)$$

Where : Abs_1: absorbance of positive control and Abs_2: absorbance of extract.

IV.7. Acetylcholinesterase inhibition

Acetylcholinesterase enzymatic activity was measured using an adaptation of the method described by **[26].** Briefly, 325 µl of 50mM HEPES buffer pH 8, 100 µl of sample (plant dried extract, dissolved in water or an aliquot coming from the enzymatic digestions) and 2.5 µl acetylcholinesterase solution containing 0.26 U/ml were mixed in a spectrophotometer cuvette and left to incubate for 15 min at 25°C. Subsequently, 75 µl of a solution of AchI (0.023 mg/ml) and 475 µl of 3 mM DTNB were added. The absorbance at 405 nm was read during the first 5 min of the reaction and the initial velocity was calculated (Eq.9). A control reaction was carried out using water, which was considered to possess 100% activity.

$$I\ (\%) = 100 - (Vsample\ /\ Vcontrol) \times 100 (9)$$

Where I is the percent inhibition of acetylcholinesterase, Vsample is the initial velocity of the extract containing reaction and Vcontrol is the initial velocity of the control reaction. Tests were carried out in triplicate and a blank with HEPES buffer instead of enzyme solution was used.

IV.8. Cytotoxicity studies on HepG2 and MCF-7 cells

The MTT test is a powerful cell viability test that measures the activity living cells via the activity of mitochondrial metabolism. This is the most widely used test in vitro (resistance and sensitivity cancer drugs, glial cell toxicity) **[27].** This test is based to the capacity of cell viability to capture and reduce the tetrazolium sel of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) soluble with a yelow color with presence of deshydrogenase mitochondriale succinate into a pricipitate of formazan MTT insoluble having a blue-magenta color (**Figure 23**). Crystals of formazan are then dissolved in DMSO. The absorption of dissolved formazan takes place at 550 nm in the visible region. It is correlated with the number of living cells intact **[28].**

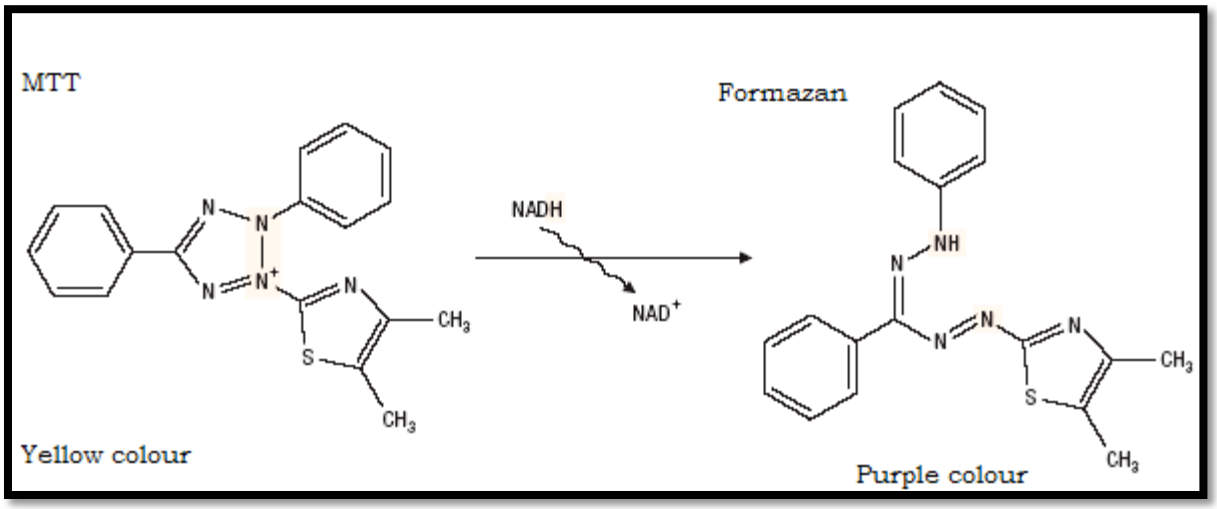


Figure 23 : Principle of MTT assay **[29].**

The cytotoxicity of *Zls* extracts were performed using the MTT viability test as described by **Ressaissi, Attia [30]** against the human tumor cell lines $HepG_2$ from human hepatocellular liver carcinoma cell lines, and MCF-7 from breast cancer, were cultured in DMEM supplemented with 10% FBS, 100 U/mL Pen-Strep, and 2 mM L-glutamine at 37 °C in an atmosphere containing 5% CO_2 every 48 to 72 h before reaching confluence, the medium was changed. Briefly, 2×10^3 cells well were seeded in 96-well plates and incubated at 37 °C for 48 h in an atmosphere containing 5% CO_2. The medium was replaced by new medium without FBS. Before applying the MTT reagent, different concentrations of *Zls* extracts were prepared and incubated for 24 h under the same conditions. The medium was discarded and replaced by MTT according to the method described in **Mosmann [31].** Then, 100 µL of MTT solution (0,5 mg/mL) was added to each well after removing the medium. After 2 h of

incubation cells, the dissolution of insoluble purple formazan crystals have been achieved in 200 µL methanol/well after removing the MTT solution. The absorbance was determined at 595 nm and 630 nm by using a Tecan Sunrise microplate reader. For each concentration of extract, the assays were done in 8×12 replicates and the cell viability percentage was calculated by the following equation (Eq. 10):

$$Viability\ (\%) = [(Abs\ 595 - Abs\ 630\ of\ experimental\ wells) / (Abs\ 595 - Abs\ 630\ of\ control\ wells)] \times 100 \quad (10)$$

IV.9.HMGR Inhibitory activity

The quantification of cholesterol and phenolic compounds during the permeation study was performed by means of RP-HPLC-DAD. The permeation (nmol h-1cm-2) was determined as the amount of cholesterol in the basolateral compartment (nmol)per hour.The percentage of permeation (%) was calculated as the proportion of the original amount that permeated through the monolayer, which was calculated as the amount transported (mol) divided by the initial amount in the apical chamber (mol).

To determine HMGR activity by HPLC, the enzymatic reaction was performed as suggested by the supplier, with minor modifications. The quantification of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) was performed by using the method described by **[32].** Briefly, aliquots were removed at 0, 1, 2, 4, and 6 min. The reaction was stopped by adding 50% methanol, and the amount of NADPH was measured by HPLC-DAD.The assays were performed in triplicate. Several concentrations of inhibitors (plant extracts) were studied. The activity value without inhibitor was considered to be 100%, and the inhibition values were determined by the percentage of decrease in the activity relative to that without inhibitor. The IC50 values were determined from regression curves by plotting inhibition as a function of the concentration of inhibitor.

IV.10.Infrared spectroscopy

The polysaccharide fractions of *ZLPS* were analyzed using Fourier transform infrared spectrophotometer (FTIR) according to method of **Abbou, Kadri [33].** A mass of 2 mg of each extract was mixed with dried potassium bromide (KBr) and compressed into a salt disc which is subjected to FT-IR analysis between 400 and 4000 cm^{-1}.

IV.11. High-Performance Liquid Chromatography with Diode Array Detector (HPLC–DAD) Analysis

The chromatographic analysis of the decoctions, the xanthones fraction collection and the enzymatic assay of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR) were carried out in VWR-Hitachi Elite LaChrom®, equipped with a LiChroCART®RP-18, 5*m, 250 *4 mm, 100 Å column from Merck, autosampler L-2200, column oven L-2300 and diode array detector (DAD) L-2455. The software for data acquisition was EZChrom Elite®, Hitachi Japan. For extract analysis 1 mg/mL of each extract was used and for XF isolation 10 mg/mL of DMf was used. The flow rate was 0.8 mL/min and the detection was carried out between 200 and 500 nm using DAD. For decoction analysis and XF collection the mobile phase consisted of 0.05% (v/v) of TFA in water (A) and acetonitrile (B). The elution conditions were as follows: 0 min, 92% A, 8% B; 20 min, 82% A, 18% B; 25 min, 45% A, 55% B; 28 min, 92% A, 8% B and 30 min, 92% A, 8% B. For HMGR inhibition assay the mobile phase consisted of KH_2PO_4 100 mM in water (A) and MeOH (B), the gradient is described in **[32].** The standard gentiopicroside was used to confirm the identification of the peak with higher intensity.

IV.12. High -performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) analysis

The LC-MS and LC-MS/MS analysis were carried out on a liquid chromatography Surveyor Plus Modular LC system connected to a LCQ Duo ion trap mass spectrometer equipped with an electrospray ionisation (ESI) source, from Thermo Scientific (Bremen, Germany). The column used was a LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 µm) column (Merck, Darmstadt, Germany).

Samples were analyzed by liquid chromatography-high resolution tandem mass spectrometry (LC–MS-MS) using an Ultimate 3000 RSLCnano system (Thermo Fischer Scientific, Idstein, Germany) interfaced with a quadrupole time-of-flight (QqToF) Impact II mass spectrometer equipped with an electrospray source (Bruker Daltonics, Bremen, German). Chromatography separationwas carried out on a Kinetex 1.7 µmC18 100Å, LCcolumn 150*2.1 mm (Phenomenex, California, USA), at flow rate of 150 µL/min. Mobile phase consisted of 0.1% (v/v) of acid formic in water (A) and 0.1% (v/v) of acid formic in acetonitrile (B), the elution conditions were described in **Guedes, Reis [34].** The column and the sampler were maintained at 35°C and 10°C, respectively. The high-resolution mass spectra were acquired in

the electrospray ionization (ESI) positive/negative modes. The optimized parameters were set as follows: ion spray voltage, +4.5/-2.5 kV; end plate offset, 500 V, nebulizer gas (N2), 2.8 bars; dry gas (N2), 8 L/min; dry heater, 200°C. Internal calibration was performed on the high-precision calibration mode (HPC) with a solution of sodium formate 10 mM introduced to the ion source via a 20 µL loop at the beginning of each analysis using a six-port valve. Acquisition was performed in full scan mode in the m/z 50–1300 range, and in a data depending MS/MS mode, with an acquisition of 3 Hz using a dynamic method with a fixed cycle time of 3 s. Dynamic exclusion duration was 0.4 min. The acquired data were processed by Data Analysis 4.1 software (Bruker Daltoniks).

IV.13. Data analysis

All data were expressed as mean ± standard deviation of three replicates. Additional analysis of variance (ANOVA) was performed with $\alpha = 0.1$ for the cholesterol permeation studies and $\alpha = 0.05$ for the other studies. The software SPSS, version 24, was used to accomplish this analysis. THE JMP Version 7 and 13 were used for the RSM study and ANOVA analysis.

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Part III. Results and discussion

Chapter I. Valorization of *Zizyphus lotus* pulp and peel for the recovery of phenolics and polysaccharides: optimization of microwave extraction process and evaluation of some biological activities

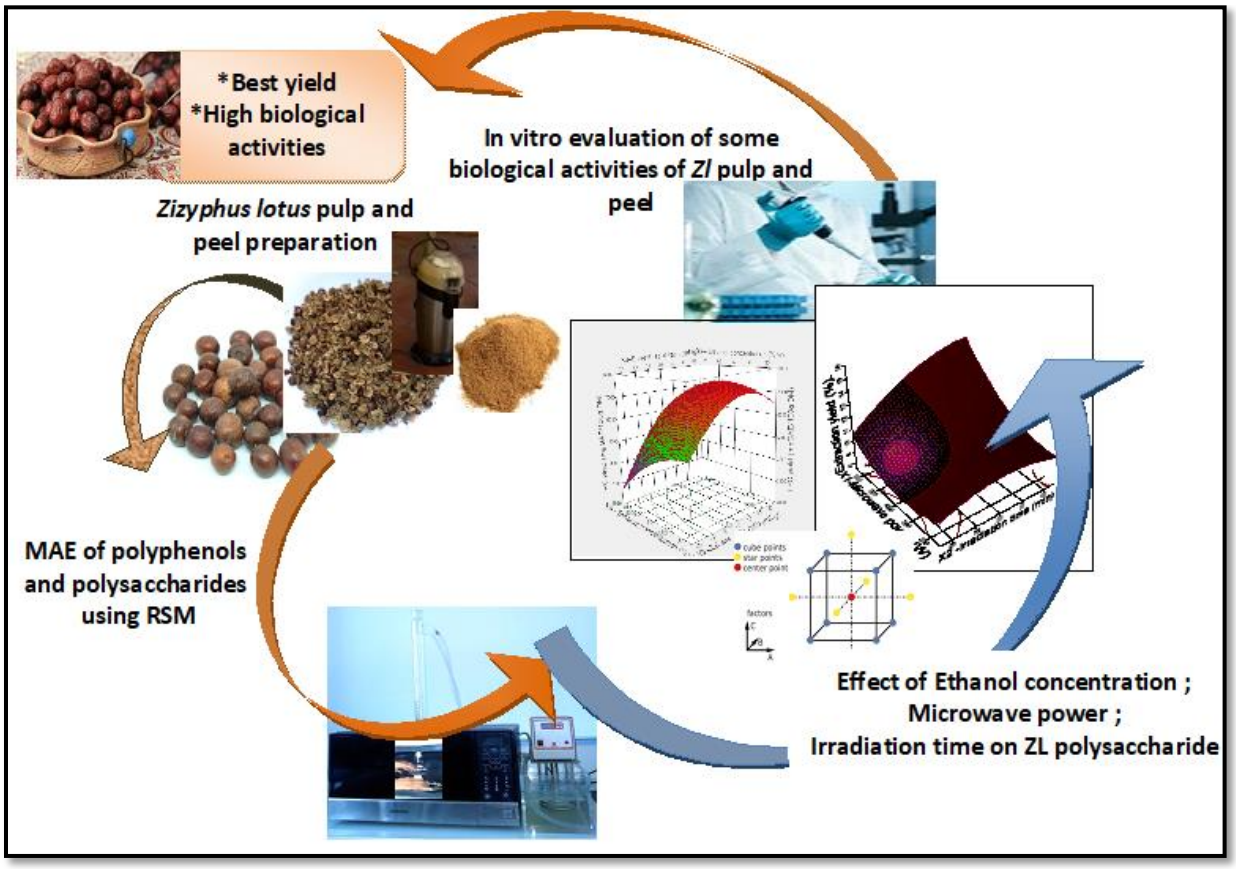


Figure 24: Graphical abstract of polysaccharides and phenolics extracted from Algerian jujube pulp and peel using microwave process.

Optimization of microwave-assisted phenolic compounds extraction from Algerian jujube (*Zizyphus lotus L.*)pulp and peel

Abstract

Zizyphus lotus fruit is a good source of antioxidants, which could be used as ingredients against several diseases. The microwave-assisted extraction (MAE) optimization of bioactive compounds from *Z. lotus* pulp and peel was achieved using response surface methodology (RSM). The effect of the extraction parameters (microwave power "MW", extraction time, ethanol/water solvent and liquid-solid ratio) on the total phenolic compounds (TPC), total flavonoids (TFC) and total condensed tannins (TTC) yields were well described by second-degree regression model. The extracts were also analyzed using liquid chromatography-high resolution tandem mass spectrometry (LC-MS-MS) to identify some bioactive compounds including primary and secondary metabolites. From these compounds, 10 phenolic compounds have never been priviously identified from *Zizyphus* plant. The predicted MAE optimal conditions were: 600 W MW power, 180 s irradiation time, 51% ethanol concentration and 47:1 mL/g solvent-to solid ratio which having given TPC of 7473.38 ± 740.55 mg GAE/100 g, TFC of 1019.96 ± 75.03 mg CE/100 g, TTC of 14253.11 ± 2453.86 mg CE/100 g and antioxidant activities based on $DPPH^{\cdot}$(0.42 ± 0.23 mg GAE/mL), $ABTS^{\cdot+}$ (14573.85 ± 7.03 TE/100 g), FRAP (3305.18 ± 9.75 mg GAE/100 g) and FIC (137.41 ± 6.49 mg EED/100 g). A comparison in terms of compounds and antioxidants activities was made under the optimal conditions with those obtained by ultrasound-assisted extraction (UAE) and conventional solvent method (CSE). The *Z. lotus* pulp and peel were studied and the results obtained were found to satisfy both bioactive compounds and antioxidant activities. This research revealed that MAE is faster and an effective method to extract TPC, TFC and TTC.

Keywords: *Zizyphus lotus*, bioactive compounds, microwave assisted extraction, RSM optimization, antioxidant activities.

I.1.1. Preliminary study of phenolic compounds

In this study, the effects of extraction parameters such as nature of solvent, microwave power, extraction time, concentration of solvent and solvent-to-solid ratio were systematically studied separately in single factor experiment in order to determine the experimental intervals of the studied factors in the optimization step of TPC, TFC and TTC yields from pulp and peel jujube. The choice of extraction solvent is considered to be important for the phenolic compound recovery from plant and leads to know the type of existing compounds and their yields. In the present work, we have observed that the type of solvent significantly influences the yield of phenolic compounds (**Table 9**). Ethanolic extract showed a higher TPC (6553.51 ± 3.57 mg GAE/100 g) than acetone (5682.57 ± 0.59 mg GAE/100 g), water (5531.68 ± 5.02 mg GAE/100 g) and methanol (5039.29 ± 7.03 mg GAE/100 g), the difference observed is due to the dielectric property of the solvent. The ethanol / water system has a higher boiling point because the water present has the highest dielectric constant (ε = 80.4) therefore, the absorption rate of the ethanol / water mixture by microwaves is much higher than the rate at which the system can dissipate heat. This mechanism increases the temperature inside the cells of the plant matrix, thus breaking the cell membranes and liberating the compounds in interaction with the solvent **[1-3].** These observations were in agreement with other literature results reported that ethanol is the appropriate solvent for the extraction of phenolic compounds since it is the least toxic solvent **[4-6].** Therefore, ethanol was used in the optimization study.

Table 9. Results of single-factor experiments for MAE from *Zizyphus lotus* pulp and peel.

Solvent		Ethanol concentration		Irradiation time		Microwave power		Solvent-to-solid ratio	
Type	TPC yield (mg GAE/100 g)	(% v/v)	TPC yield (mg GAE/100 g)	(s)	TPC yield (mg GAE/100 g)	(W)	TPC yield (mg GAE/100 g)	(mL/g)	TPC yield (mg GAE/100 g)
Water	5531.68 ± 5.02^{d}	10	4691.52± 2.91^{f}	30	4857.29± 0.11^{g}	300	4306.66 ± 0.72^{e}	20 :1	6278.86 ± 2.47^{c}
50%	5039.29 ± 7.03^{b}	20	5123.50 ± 8.30^{e}	60	4894.35± 4.75^{f}	400	4698.45± 5.12^{d}	30 :1	6478.86 ± 2.47^{b}
MeOH	6553.51 ± 3.57^{a}	30	4316.67± 4.81^{g}	90	6056.49± 9.32^{b}	500	6286.80± 2.26^{a}	40 :1	6697.12 ± 4.31^{a}
50%	5682.57 ± 0.59^{c}	40	5430.63± 3.94^{c}	120	5516.45± 4.58^{e}	600	5407.91± 6.52^{c}	50 :1	5161.06 ± 3.78^{d}
EtOH		50	6553.51± 0.22^{a}	150	6758.01± 2.81^{a}	700	5444.97± 1.67^{b}		
50%		60	5403.23± 4.85^{d}	180	6032.66 ± 7.62 ^{c}				
Acetone		70	5543.01± 12.37^{b}	300	5699.11± 3.93^{d}				

It has been observed that the extraction yield tends to increase with an increase of extraction time when others factors were fixed: 50% ethanol (v/v), 500 W microwave power level, and 30 mL/g solvent-to-solid ratio and with extraction times varied from 30 to 300 s. The results

indicate that phenolic, flavonoids and condensed tannins yields increased with the increase of irradiation time at the beginning of extraction and reached the maximal concentration with irradiation time of 150 s. This may be due to the dissolution of the solute in contact to the surface of samples **[7].** However, the TPC decreased significantly from 6758.01 ± 2.81 to 5699.11 ± 3.93 mg GAE/100 g by increasing irradiation time from 150 to 300 s. This could be explained by the thermal degradation of different compounds after exposing to long microwave irradiation time **[8].** In addition, it is also associated with an intensification of the MAE process following the disruption of the plant cell wall in a very short irradiation time **[7].** These results are in agreement with those obtained by Dahmoune, Boulekbache **[9].** Based on these results, 150 s was selected for the next single-factor trials, while the range 120–180 s was selected for the RSM trials. As shown in the Table 9, the extraction yield increased with the increase of microwave power from 100 to 500 W, due to its heating effect **[10].** Morever, the extraction at higher powers (after 500 W) caused a decrease of the extraction yield, which may be due to a possible degradation of the bioactive substances by both temperature and power effects that are interrelated. Thus, by the free radicals formed within the liquid medium which may cause oxidation phenomena. The rupture of the cell wall of the vegetable matrix could take place gradually which allows preserving the molecules without altering its quality **[1, 3, 7]**. These results were in agreement with those found by Surati, Jauhari **[11],** Nayak, Dahmoune **[12].** The range 400–600 W was selected for the RSM study, while the 500 W power was used for the remainig trials.

The TPC, TFC and TTC recoveries were extracted at various solvent-to-solid ratios (20, 30, 40 and 50 mL/g) during the preliminary study and fixed microwave power, extraction time and ethanol proportion. The TPC content was maximized at 40 : 1 (mL/g) solvent-to-solid ratio and decreased when the ratio is much higher than this value. This result is probably due to the increase of the solvent volume allowing the dissolution of the matrix, but at a certain point where heating microwaves will be lower because of the reduced penetration of the solvent into the sample will reduce the yield. Moreover, the volume of the solvent must allow the vegetable matrix powder to be sufficiently immersed to ensure a good yield of bioactive compounds **[13].** Our results are in agreement with those obtained by Belwal, Bhatt **[14]** using microwave extraction technique for improving polyphenolic antioxidants in *Berberis asiatica* leaves which show a solvent-to-solid ratio of 45:1 (mL/g) was the best one for extraction of total phenolics, total flavonoids, total tannins and for several antioxidants activities. The range of the solvent-to-solid ratio chosen for the RSM was 20, 30 and 50 mL/g, respectively. A

range of 10 to 70% ethanol in water was tested in the single factor experiment in order to understand its effect on TPC, TFC and TTC yields. It can be noticed that increasing ethanol concentration from 10 to 50% increase the TPC, TFC and TTC yields and then, a slightly decreased was observed until 70% (**Table 9).** The percentage of ethanol in the solvent extraction play an important role because it modifies the dielectric properties of the final solvent mixture but also offers a better medium solubility for molecules target i.e.polarity of mixture solvent close to phenolic compound present in the vegetable matrix which accelerate their release **[15].** The ethanol solvent concentration of 50% absorbs microwave energy relatively well and was then selected for the determination of optimal microwave power, extraction time and solvent-to-solid ratio.

I.1.2.Optimization of MAE conditions

Modeling and fitting the model using response surface methodology

In this study, the extraction efficiency of the microwave process was estimated by measuring bioactive compounds. The experimental design matrix and the response, namely TPC, TFC and TTC yields in *Z. lotus* extracts obtained in the trials of the CCD from 27 experiments are presented in Table 10. The regression coefficients were determined using least squares as a technique of individual linear, quadratic and model interaction **[16].**

As shown in the table 9, for the three responses studied, ethanol concentration and solvent-to-solid ratio have significant linear effects ($p < 0.05$), as well as the microwave power was only observed for TTC with a positive effect, while no significant effect of irradiation time (X_1) was found on all responses. However, all responses show significant quadratic effects for the solvent-to-solid ratio (X_3^2) with a negative effect for all responses. Also ethanol concentration (X_4^2) showed the positive effect for TFC and TTC. On the other hand, the irradiation power (X_2^2) showed no significant effect on all the responses, indeed the irradiation time (X_1^2) showed a single positive quadratic effect for the TFC. Regarding the effect of interaction between the variables, it was observed only for the TPC and TTC with a significant effect, between the solvent-to-solid ratio (X_3) with ethanol concentration (X_4). In addition, the interaction between ethanol concentration and both extraction time and microwave power, also between extraction time and microwave power showed a significant effect on TTC response (Table 10).

Table 10. Central composite design with the observed responses for yield of phenolic compounds (TPC, TFC, TTC) from *Z. lotus* pulp and peel using MAE.

Run	Independent Factors				Response Variables					
	X_1 – Irradiation time (s)	X_2 – Microwave power (W)	X_3 –Solvent-to- solid ratio (mL/g)	X_4 – Ethanol concentration (% v/v)	TPC yield (mg GAE/100 g DM)		TFC yield (mg QE/100 g DM)		TTC yield (mg CE/100 g DM)	
					Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	180 (+1)	300 (-1)	50 (+1)	30 (-1)	7094.41	7054.59	909.71	904.18	5663,58	4894.31
2	120 (-1)	700 (+1)	20 (-1)	30 (-1)	5816.37	5914.58	568.88	562.53	16666.6	15669.56
3	150 (0)	700 (+1)	35 (0)	50 (0)	8215.96	7654.08	777.54	744.76	12962.96	12541.28
4	120 (-1)	300 (-1)	20 (-1)	70 (+1)	3896.71	3918.11	422.89	404.70	15432.09	16861.26
5	120 (-1)	700 (+1)	50 (+1)	30 (-1)	7198.74	7410.62	917.36	911.20	13888.88	14195.58
6	120 (-1)	700 (+1)	50 (+1)	70 (+1)	7355.24	7498.36	797.16	774.57	47839.50	49628.75
7	180 (+1)	700 (+1)	50 (+1)	70 (+1)	7094.41	7059.09	808.09	857.92	50925.92	49295.03
8	120 (-1)	700 (+1)	20 (-1)	70 (+1)	4267.08	4158.29	405.41	418.58	31481.48	30875.11
9	120 (-1)	300 (-1)	50 (+1)	70 (+1)	6729.26	6636.12	761.10	758.50	35493.82	33828.64
10	120 (-1)	300 (-1)	20 (-1)	30 (-1)	5252.99	5139.71	568.45	526.26	8024.69	8279.94
11	120 (-1)	300 (-1)	50 (+1)	30 (-1)	6168.49	6013.69	860.54	872.73	4629.62	5019.70
12	150 (0)	500 (0)	50 (+1)	50 (0)	7381.32	7294.14	891.13	852.82	11574.07	10495.67
13	180 (+1)	300 (-1)	50 (+1)	70 (+1)	7003.12	6992.36	814.64	815.31	41512.34	44054.33
14	120 (-1)	500 (0)	35 (0)	50 (0)	6846.63	6842.05	827.26	899.97	12962.96	12061.17
15	150 (0)	500 (0)	20 (-1)	50 (0)	4689.61	5021.49	456.55	487.00	3703.703	4105.07
16	180 (+1)	500 (0)	35 (0)	50 (0)	6727.96	6977.24	1023.36	942.79	12962.96	13187.72
17	180 (+1)	700 (+1)	20 (-1)	30 (-1)	5884.19	5828.72	581.12	591.37	7407.40	7696.95
18	150 (0)	500 (0)	35 (0)	50 (0)	6965.31	7114.52	740.83	781.51	9722.22	10665.91
19	150 (0)	500 (0)	35 (0)	50 (0)	6919.66	7114.52	747.71	781.51	9722.22	10665.91
20	150 (0)	500 (0)	35 (0)	70 (+1)	6079.81	6281.56	661.27	645.03	39969.13	38638.68
21	150 (0)	500 (0)	35 (0)	30 (-1)	7147.88	7190.83	753.07	761.45	10802.46	11455.89
22	150 (0)	300 (-1)	35 (0)	50 (0)	6426.70	7233.28	680.40	705.32	6481.48	6226.13
23	180 (+1)	700 (+1)	20 (-1)	70 (+1)	3145.53	3387.77	490.64	472.76	32098.76	33253.58
24	150 (0)	500 (0)	35 (0)	50 (0)	8192.69	7114.52	832.41	781.51	10522.22	10665.91
25	180 (+1)	300 (-1)	20 (-1)	70 (+1)	4303.59	3943.11	418.52	432.33	31481.48	29799.15
26	180 (+1)	300 (-1)	20 (-1)	30 (-1)	5905.05	5849.37	511.62	528.53	11111.11	10866.75
27	180 (+1)	700 (+1)	50 (+1)	30 (-1)	7589.98	7656.01	956.70	969.20	3395.06	3510.78

Three second order polynomial models were fitted to generated data to describe the significance and adequacy of the model and the empirical relationship between the TPC, TFC and TTC with operational conditions. The below predictive equations in term of significant independent variables were obtained (Eq. 11 (A-C)). Table 10 reports the statistical analysis of the regression model.

$$Y(TPC) = 7114.52 + 1136.32X_3 - 454.63X_4 - 956.7013X_3^{\,2} + 461X_3X_4 \quad \text{Eq. (A.11)}$$

$$Y(TFC) = 781.51 + 182.90X_3 - 58.20X_4 + 139.87X_1^2 - 111.59X_3^2 - 78.26X_4^2 \quad \text{Eq. (B.11)}$$

$$Y(TTC) = 10665.91 + 3157.57X_2 + 3195.30X_3 + 13591.39X_4 - 3365.53X_3^2 + 14381.37X_4^2 - 2639.85X_1X_2 + 2587.77X_1X_4 + 1656.05X_2X_4 + 5056.90X_3X_4 \quad \text{Eq. (C.11)}$$

Table 11. Estimated regression coefficients for the quadratic polynomial model and the analysis of variance (ANOVA) for the experimental results of TPC, TFC and TTC from *Z. lotus* pulp and peels.

Parameter	TPC				TFC				TTC			
		a				b				c		
	Estimated coefficients	Sum of squares	F-value	Prob>F	Estimated coefficients	Sum of squares	F-value	Prob> F	Estimated coefficients	Sum of squares	F-value	Prob> F
Model B_0	7114.5237	41407285	12.9234	<0.0001*	781.51502	809205.50	24.6027	<0.0001*	10665.912	5370342536	152.6562	<0.0001*
Linear												
X_1	67.59694	82248	0.3594	0.5600	21.40885	8250.10	3.5116	0.0855	563.2716	5710948,21	2.2727	0.1575
X_2	210.39819	796813	3.4817	0.0867	19.724189	7002.79	2.9807	0.1099	3157.5789	179465478	71.4202	<0.0001*
X_3	1136.3241	23242185	101.5561	<0.0001*	182.90862	602200.12	256.3260	<0.0001*	3195.3018	183779163	73.1369	<0.0001*
X_4	-454.6311	3720410	16.2562	0.0017*	-58.2073	60985.62	25.9585	0.0003*	13591.392	3325067012	1323.247	<0.0001*
Quadratic												
X_1^2	-204.8755	107933	0.4716	0.5053	139.87087	50307.07	21.4132	0.0006*	1958.5391	9863679.55	3.9254	0.0709
X_2^2	329.16208	278608	1.2174	0.2915	-56.4695	8199.78	3.4902	0.0863	-1282.202	4227534.16	1.6824	0.2190
X_3^2	-956.7013	2353570	10.2839	0.0075*	-111.5989	32025.37	13.6316	0.0031*	-3365.535	29126123.2	11.5911	0.0052*
X_4^2	-378.3236	368045	1.6082	0.2288	-78.26992	15753.04	6.7053	0.0237*	14381.379	531833273	211.6489	<0.0001*
Interaction												
X_1X_2	-198.8785	632842	2.7652	0.1222	6.6384736	705.11	0.3001	0.5938	-2639.853	111501215	44.3731	<0.0001*
X_1X_3	82.811685	109724	0.4794	0.5019	0.5463764	851.27	0.3623	0.5584	-678.0478	7355981.94	2274	0.1128
X_1X_4	-171.1659	468764	2.0483	0.1779	6.3379666	642.72	0.2736	0.6105	2587701	107144862	42.6395	<0.0001*
X_2X_3	155.51643	386966	1.6908	0.2179	7.2941254	4.78	0.0020	0.9648	446.56636	3190744.2	1.2698	0.2818
X_2X_4	-133.6724	285893	1.2492	0.2856	-5.600358	501.82	0.2136	0.6522	1656.0571	43880401.8	17.4627	0.0013*
X_3X_4	461.0678	3400436	14.8581	0.0023*	1.8303611	53.60	0.0228	0.8824	5056.9059	409156751	162.8284	<0.0001*
Lack Of Fit		1703270.2	0.3266	0.9082		22989.004	0.8836	0.6395		29727047	13.9346	0.0688
Pure Error		1043056.2				5203.230				426667		
Total Error		2746326.3				28192.234				30153714		
R^2			0.937801				0.96633				0.994416	
R^2Adjusted			0.865235				0.92705				0.987902	
CV%	7.58				6.82				8.58			
Corr.Total		44153611				837397.73				5413814110		

Very high F-value and a very low *p*-value ($p < 0.0001$), indicating that these models were highly significant and it can be used to optimize the extraction variables (Table 11). In

addition, the determination coefficients for TPC, TFC and TTC yields are R^2 = 0.93; R^2 = 0.96; R^2 = 0.99 respectively and adjusted determination coefficient (Adj. R^2 = 0.86 ; Adj. R^2 = 0.92 ; Adj. R^2 = 0.98), were reasonably close to 1, with low value of CV (< 10%) (Table 11). This indicates a high degree of correlation between the observed and predicted values, which implied that only 3, 6 and 1% respectively of these variations could not be justified by the models. Also, other parameters were insignificants as the lack of fit (p > 0.05) for the three responses which provide also the validity of the experimental model. The coefficient of variation (CV) for all responses were within the acceptable range (< 10%) **[17, 18].** This indicates that variation in the mean value issmall and satisfactorily develop an adequate response model.

The response surfaces of regression models were plotted by their three-dimensional profile in order to study the effects of interaction between independent variables on the TPC, TFC and TTC extraction yields (Fig 25). According to Hayat, Hussain **[19],** maintaining two independent variables at their zero levels and using the z-axis. against two independent variables, a response can be drawn. The surface response (3D) is considered as the diagram permitting to represent the regression equation.

Response surface analysis of TPC yield

The effect of ethanol concentration on each of the factors (solvent-to-solid ratio, microwave power and irradiation time) was shown in Figure 25. Fig. 25A shows that the TPC could be greater than 6000 mg GAE/100 g for a low solvent-to-solid ratio with ethanol concentration between 30 and 40%. At 50% ethanol concentration, the TPC yield increased up to 7000 mg GAE/100 g with the significant increase in extraction time up to 180 s, where as, above this extraction time with additional ethanol concentration, the extraction yield decrease gradually (Fig. 25B). As shown in the Fig. 25B, the TPC content increases considerably and could reach more than 8000 mg GAE/100 g at higher power with an increase in ethanol concentration up to 50%. But TPC decreases progressively when the microwave power decreased (500 W) and ethanol concentration increases over 50%. Indeed, as shown in Table 11 (a), the yield of TPC depends mainly on ethanol concentration as its linear and interaction effect (positive effect) with solvent-to-solid ratio were highly significant (p < 0.01). Figure 25 (D-E) describe the interactions between the irradiation power and each of the other two factors (irradiation time and solvent-to-solid ratio) on the TPC recovery. Fig.25D shows that the amount of TPC increased with increasing microwave power and irradiation time during the initial extraction

followed by a decrease at mean values. The graphs suggested that the irradiation power has an interaction effect with the extraction time and linear effect ($p < 0.01$) on the TPC yield (Table 11-a). Our results are in agreement with those found by different authors who underlined that all factors influencing the output of phenolic compounds extraction from medicinal plant tissus **[20, 21].**

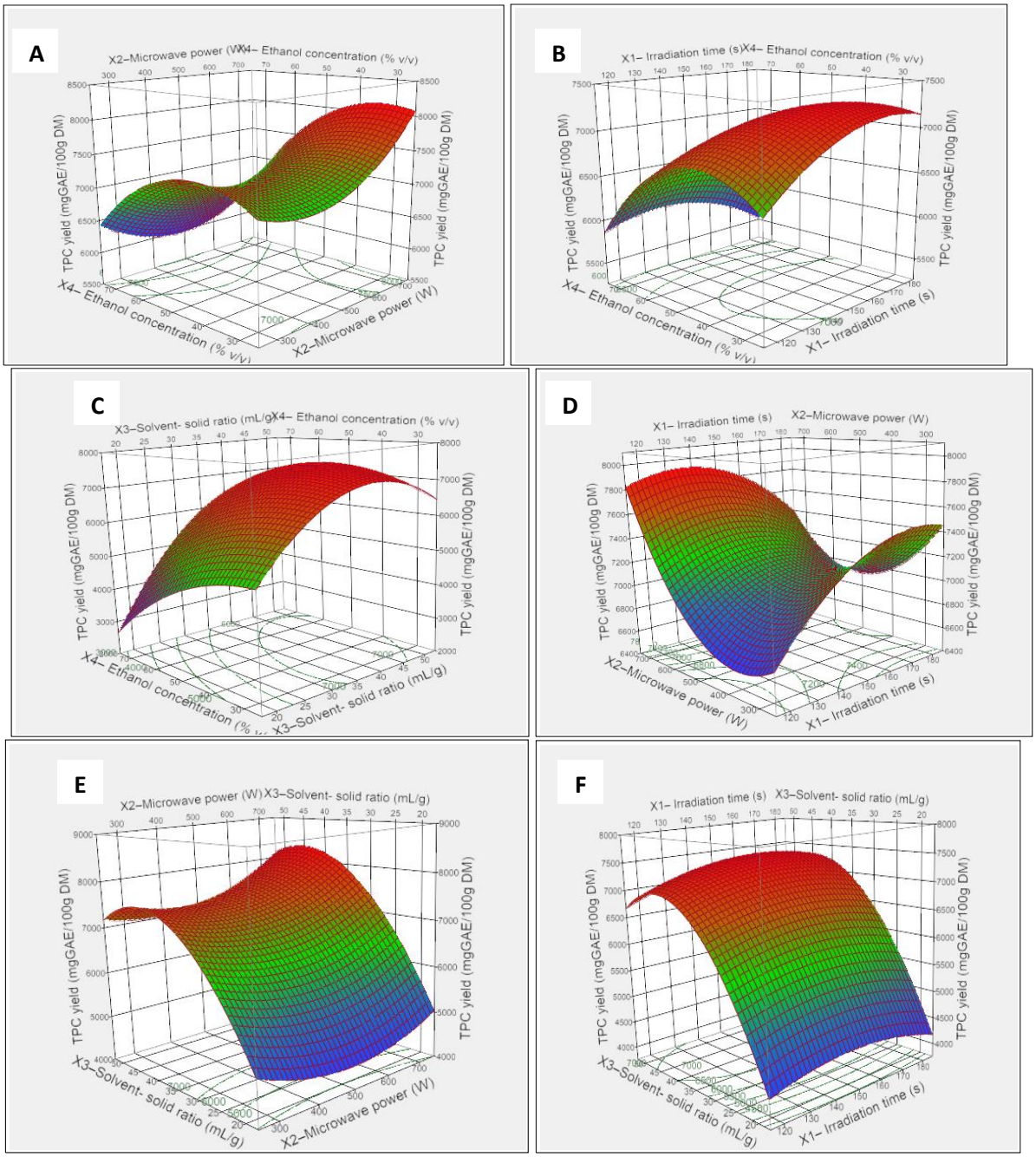


Figure 25 : Response surface analysis for the total phenolic compounds from *Z. lotus* pulp and peel with microwave assisted extraction with respect to ethanol concentration and solvent-to-solid ratio (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and microwave power (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).

Fig. 25E clearly shows that with a significant increase in the solvent-to-solid ratio from 30 to 47 mL/g, the extraction yields increased approximately to 7000 mg GAE/100 g with a slight decrease when power increased. This trend could be explained by the total absence of inertia effect between the extraction power and the solvent-to-solid ratio, but we can say that the yield of TPC from *Z. lotus* depends mainly on the solvent-to-solid ratio, since its quadratic and linear effects were very significant ($p < 0.01$). Fig. 25F depicts the effect of the solvent-solid ratio with the irradiation time, the TPC content is maximized (6500 mg GAE/100 g) at a high solvent-to-solid ratio with an extraction time of less than 150 s, but it decreases considerably when the solid to liquid ratio begins to decrease.

Response surface analysis of TFC

Response surfaces for total flavonoids extraction yield from *Z. lotus* pulp and peel are shown in Figure 26. Fig. 26A shows the profiles obtained on the effects of ethanol concentration and microwave power on the yield of TFC. An increase in TFC content was noted with increasing microwave power and ethanol concentration. On the other hand, just after a few seconds of exposure of the *Zizyphus* powder to a high concentration of ethanol, it was noticed that the yield of TFC decreased. The results found correspond to those explained by Alara, Abdurahman **[8]** concerning the ability of microwaves to better extract total flavonoids from *Vernonia amygdalina* leaf using the RSM while evaluating the effectiveness of its antioxidant activities. Fig.26B indicates the interaction effect of the solvent-to-solid ratio and the ethanol concentration on the TFC yield. TFC reached the maximum value of 800 mg QE/100 g when the solvent to solid ratio is 50 mL/g and by increasing ethanol concentration from 40% to 50%. It was also observed that the extraction yield decreased with increasing ethanol concentration beyond 50% and decreasing the solvent-to-solid ratio. These results suggest that no interaction between the solvent-solid ratio and the ethanol concentration is observed on TFC extraction, which is clearly shown in Table 11 (b) where these factors had negative effects. Fig. 26C shows the effects of ethanol concentration and microwave extraction time on TFC of *Z. lotus* pulp and peel. By increasing the irradiation time during the extraction from 120 to 160 s and ethanol concentration from 40 to 55%, the TFC increased and reached 1000 mg QE/100 g. However, the TFC decreased from 1000 to 700 mg QE/100 g, the value has decreased over increase in ethanol concentration to 70% and 180 s.

The effect of extraction time and microwave power on TFC yield was observed in Fig. 26D. The TFC content increased significantly until reaching a certain value when the power of the

microwaves is 700 W, however at this stage, the time is increased to 150 s however the TFC has decreased . Further, after 150 s it started resuming the higher yield which was maximum at 180 s.

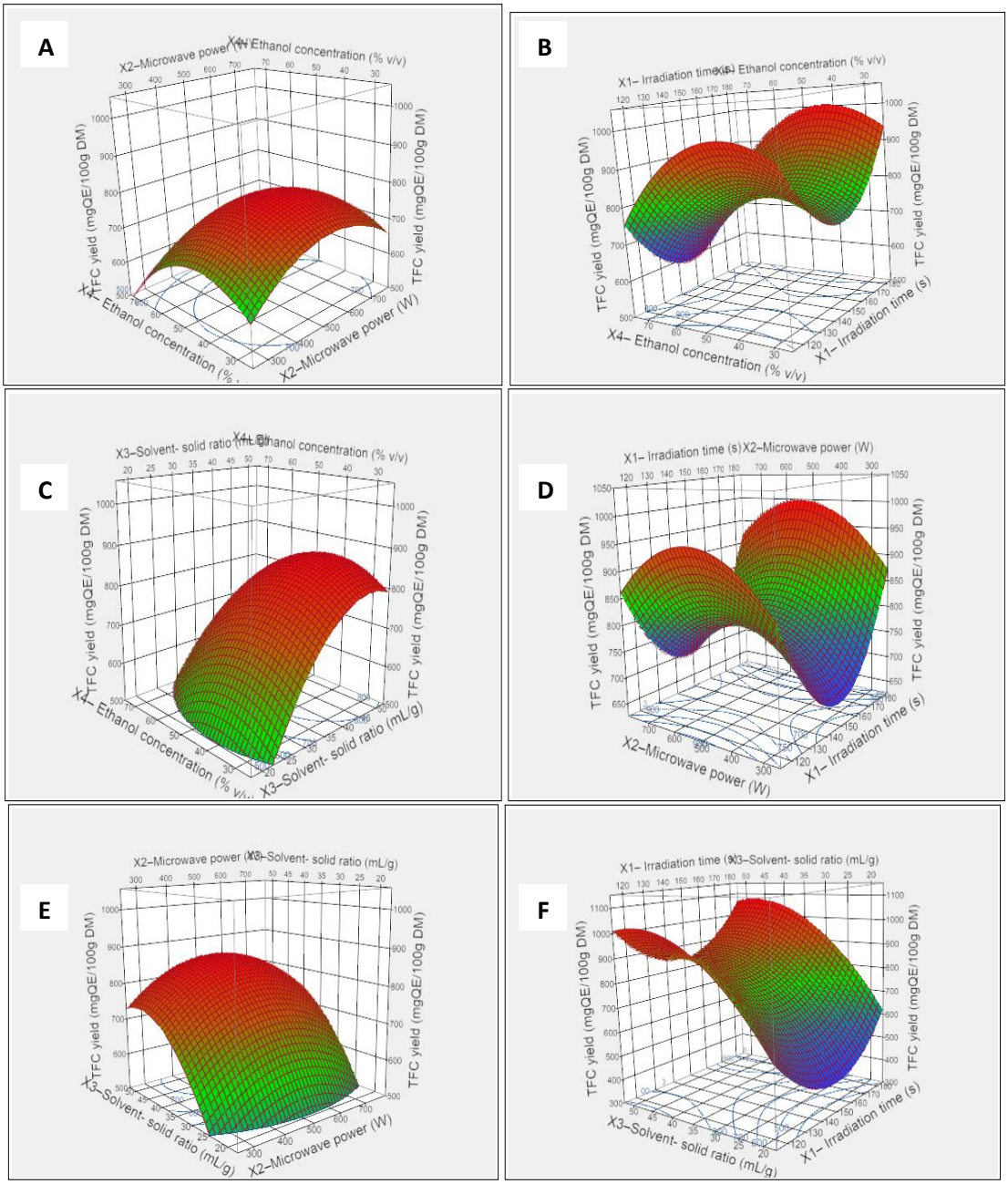


Figure 26: Response surface analysis for the total flavonoids from *Z. lotus* pulp and peel with microwave assisted extraction with respect to ethanol concentration and solvent-to-solid ratio (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and microwave power (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).

Fig. 26E-F describes the interaction between the solvent-to-solid ratio with two independent variables, irradiation time and microwave power. Fig. 26E shows that the increase in the solvent-to-solid ratio and microwave power, maximized the TFC up to 700 mg QE/100 g. However, increase in power showed a significant decrease in TFC yield as shown in response curve, which was also noticed during our preliminary study. This is due to thermal degradation of TFC extract at high power. Fig. 26F shows that the increase in TFC extraction yield to a maximum value with simultaneous increase of the solvent-to-solid ratio and the extraction time up to 150 s. But a prolonged duration leads to significant decrease of yield (quadratic and linear effect). This result is in agreement to the work of Lu, Fu **[22]** that showed the efficiency of MAE to extract flavonoids from *Cryptotaenia japonica Hassk.*

Response surface analysis of TTC

The response surface of microwave extraction yield in TTC condensed tannins from *Z. lotus* pulp and peel was shown in Figure 27. From fig. 27A-C, it clearly shows the effect of ethanol concentration on the yield of TTC with the other variables, namely, irradiation time, microwave power and solvent-to-solid ratio. It has been observed from the three figures 27 (a, b and c) that ethanol concentration has a positive influence on the yield of TTC, hence its linear, quadratic and interaction effect with the solvent-to-solid ratio and the irradiation time were significant (Table 11 c).

In fact, the increase in the concentration of the ethanol solvent with the simultaneous slight increase in irradiation time (Fig. 27A), microwave power (Fig. 27B) and solvent-to-solid ratio (Fig. 27C) induces an increase in TTC up to 10000 mg CE/100 g. This is one of the most important factors in this study, which suggests that it may be possible to absorb the effective function of microwave power **[23].** Fig. 27D showed that the simultaneous increase in irradiation time and microwave power significantly increased the TTC yield up to about 18000 mg CE/100 g. Fig. 3E clearly shows that the increase in irradiation time with a decrease in the ratio results in a significant increase in the content of condensed tannins. However, the interaction between microwave and solvent-to-solid ratio showed that TTC increased up to a certain value of microwave power and decreased at higher solvent-to-solid ratio (Fig. 27F). It is clear that the extraction yield of bioactive compounds, such as TPC, TFC and TTC from jujube pulp and peel was well influenced by the microwave irradiation. The optimised parameters offer a good heating conditions allowing to break the cell wall, increase the solubility of the compounds and reduce the viscosity of the solvent which accelerate the

release of phenolic compounds from jujube plant. In addition, the increase in the solvent-to-solid ratio can lead to rised phenolic compounds yield.

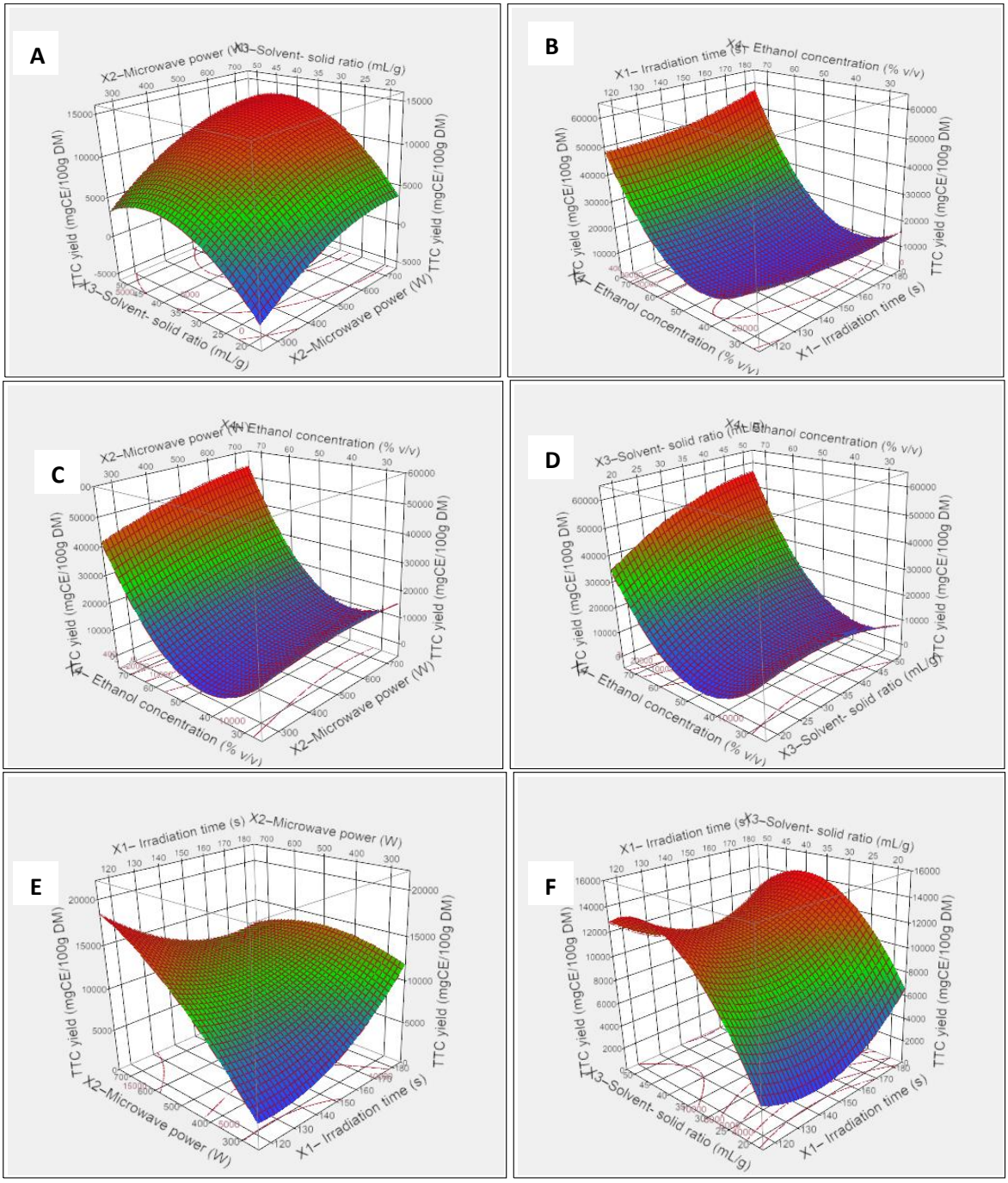


Figure 27: Response surface analysis for the total condensed tanins from *Z. lotus* pulp and peel with microwave assisted extraction with respect to ethanol concentration and solvent-to-solid ratio (A); ethanol concentration and extraction/irradiation time (B); ethanol concentration and microwave power (C); extraction/irradiation time and microwave power (D); microwave power and solvent-to-solid ratio (E); extraction/irradiation time and solvent-to-solid ratio (F).

Validation and verification of predictive model

The MAE optimal conditions obtained for TPC, TFC, TTC recoveries were: 51% ethanol, 600 W microwave power, 180 s irradiation time and 47 mL/g solvent-to-solid ratio. To ensure the equation of the model and the adequacy of the chosen experimental model compared to the predicted model, the optimal response values were tested at the selected optimal values.

The experimental values of extraction yield for the TPC, TFC and TTC were very close to the predicted values. According to Zhang, Hu **[16]** the adequacy of the regression response of the model compared to that of the experimental model is necessarily due to a very strong correlation between the actual results and the expected results to express the desired optimization, so we can say that the model has been validated.

Comparison between MAE, UAE and CSE

The efficiency of TPC, TFC, TTC using MAE was compared with other methods such as UAE and CSE (Table 12). The results indicate that MAE showed a significantly higher TPC extraction capacity (7473.38 ± 740.55 mg GAE/100 g) ($p < 0.05$) compared to UAE (6841.41 ± 4.61 mg GAE/100 g) and CSE (4818.72 ± 7.02 mg GAE/100 g).

Table 12: Comparison of extraction yield of bioactive compounds from *Z. lotus* pulp and peel by microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), and conventional solvent extraction (CSE). Results are expressed as means standard deviation.

Extraction method/ Factors	MAE	UAE	CSE
Irradiation time (min)	3	25	120
Ethanol concentration (%)	51	50	50
Microwave power (w)	600	-	-
Temperature (°C)	-	63	60
Solvent / solid Ratio (mL/g)	47	67	50
Results			
Recovery of total phenolic TPC (mg GAE/100g DM)	7473 ± 740[a]	6841.41± 4.61[b]	4818.72 ± 7.02[c]
Recovery of total flavonoids TFC (mg QE/100g DM)	1019.96 ± 75.03 [a]	1047.46 ± 1.89 [a]	934.41 ± 6.05 [b]
Recovery of condensed tannins TTC (mg CE/100g DM)	14253.11 ± 2453.86 [a]	10339.50 ± 0.1[b]	4629.68 ± 0.08[c]
DPPH scavenging EC_{50} (mg GAE/mL)	0.42 ± 0.23[a]	0.84 ± 0.14 [b]	0.89 ± 0.02 [b]
ABTS scavenging (mg TE/100g DM)	14573.85 ± 7.03 [a]	12788.87 ± 4.68 [b]	10103.96 ± 28.13 [c]
FRAP activity (mg GAE/100g DM)	3305.18 ± 9.75 [a]	1284.53 ± 10.99 [b]	672.86 ± 4.87[b]
FIC activity (mg EED/100g DM)	137.41 ± 6.49 [a]	104.16 ± 18.16 [b]	80.55 ± 12.78 [c]

Results may be due to the long extraction time and/or the amplitude of the UAE which had a negative influence on the recovery of the phenols. In the work of Al-Saeedi, Al-Ghafri **[24]** who reported a TPC of 64.89 ± 0.44 mg GAE/100 g) from the fruit of Oman *Zizyphus jujuba (Zj)* which is significantly lower than our results using the methanol as extraction solvent. In addition, Spanish jujube fruit has a content of 1442 to 3432 mg GAE/100 g according to Wojdyło, Carbonell-Barrachina **[25].** Similarly, Cosmulescu, Trandafir **[26]** have shown that the TPC content was between 475.3 to 1634.4 mg GAE/100 g from the fruit of *Zj* by a conventional extraction method, these quantities are lower than our results found from pulp and peel of Algerian. However, the TFC extracts obtained by MAE, UAE (1019.96 ± 75.03; 1047.46 ± 1.89 mg QE/100 g respectively) were significantly higher ($p < 0.05$) than those obtained by CSE (934.41 ± 6.05 mg QE/100 g). We sugges there, that the recovery of flavonoids was influenced negatively by longer extraction time. Our results are superior to those obtained by Al-Saeedi, Al-Ghafri **[24]** of Oman variety fruit of *Zj* 27.43 ± 0.18 mg QE/100 g using chloroform as extraction solvent, likewise for those obtained by Koley, Kaur **[27]** who reported TFC content from 8.36 to 21.97 mg QE/100 g from *Zj*. The flavonoid extract of *Zj* showed a significantly lower content than ours, ranging from 19.9 to 48.5 mg QE/100 g **[26].** Moreover, the TTC extracts by MAE were very significantly higher ($p < 0.01$) (14253.11 ± 2453.86 mg CE/100 g) than those obtained by UAE and CSE respectively (10339.50 ± 0.1; 4629.68 ± 0.08 mg CE/100 g). Gao, Wu **[28]** compared proanthocyanidin extracts from several *Zizyphus* varieties, among those containing the highest content about 413.7 ± 23.1 mg/100 g DM from *Zj cv. Zaowangzao* which is significantly lower than our extracts obtained from *Z. lotus.*

It is more interesting to mention that all the factors studied have a significant influence on the jujube yield and therefore on the antioxidant activity which were improved by MAE with the evaluation of each parameter and the interaction effects that may exist. In which this work has combined the three responses studied to have similar optimal conditions which will allow future readers to deduce that the methodology used for MAE from *Z. lotus* pulp and peel extracts made it possible not only to improve the yield of total polyphenols but also while knowing that of flavonoids and tannins for their possible individual or collective use. In addition, that the polyphenols are varied because of their capacity to react with each other and may be with other substances. Thus, where there has been an hydrolysis or a polymerization of these molecules in smaller or larger having a strong antioxidant activity **[7].** According to several authors **[16, 29],** MAE gives a very good yield in a minimum of irradiation time,

herein its advantageous compared with other methods. Moreover, the increase of the interaction of the cells of the matrix leads to the transfer of the tissues so facilitates the interaction of electromagnetic fields. The energy transfer from the inside to the outside world will allow the promotion of the solubility of the solvent.

I.1.3. Identification of phenolic compounds from *Z. lotus* pulp and peel

In order to identify the bioactive compounds from our samples, LC–MS/MS was carried out, Figure 28. LC–MS in the negative and positive mode was perfomed and the compounds are indicated in Table 13. Compounds were tentatively identified under the analytical conditions by Data Analysis program from Bruker, and the chemical structures were suggested by pubchem, metlin and published reference on the same plant species or genus. In order to visualise the relationship between the compounds by knowing the intensities and antioxidant activities, the heatmap represented in Figure 28 was used. This visual representation allows to confirm which of compounds are more active in terms of antioxidant effect. Table 13 indicated the presence of 34 bioactive compounds based on their occurence in plants from literature and by taking into account the exact mass and availability of reference standards and to the best of our knowledge have not been previously reported in *Z. lotus* pulp and peel.

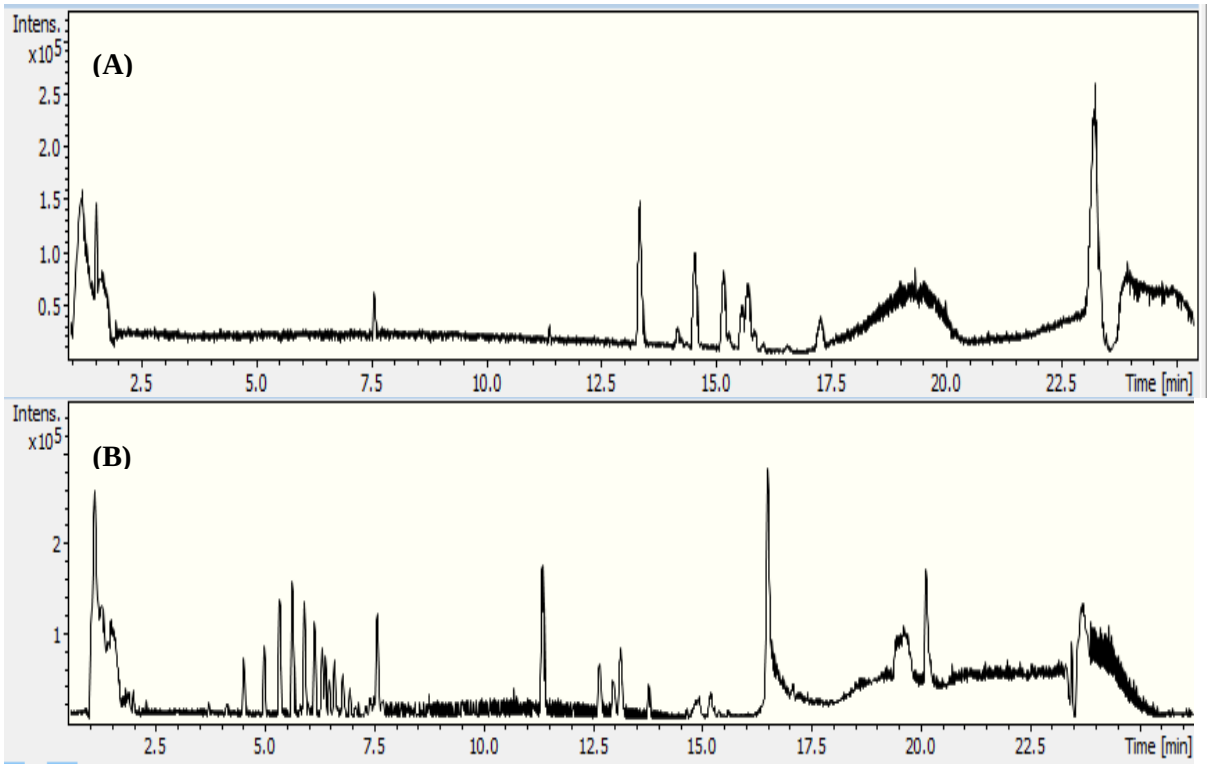


Figure 28 : Total ionic chromatogram and ionic chromatograms extracted from ions identified in *Z. lotus* pulp and peel : (a) LC-MS/MS (-) and (b) LC-MS/MS (+).

Table 13: Identification proposal of the phenolic compounds present in the *Z. lotus* pulp and peel by LC–MS/MS in ESI negative and positive mode. Peaks with a minus/plus superscript were analyzed in negative/positive mode. Compounds are indicated by retention time (R_t) order and numbered according to the appearance in each chromatogram, negative $[M-H]^-$ or positive mode $[M+H]^+$.

R_t (min)	Intensity	[M - H]-/ [M + H]+	Fragments ion (m/z) ; intensity(%)	Error (mg/kg)	Molecular formula	Tentative identification	References
1.05-	29390	387.1089	341.1061 (42.9) ; 119.0321(40.9) ; 101.0219(23.2) ; 161.0427 (13.5) ; 149.0425 (11.2) ; 83.01 (3.2)	2.5	$C_{21}H_{16}N_4O_4$	Indole derivative	125554521 (pubchem)
1.11-	17000	179.0535	59.0105(100) ; 71.0110(90.1) ; 96.9570(39.9)	14.6	$C_6H_{12}O_6$	Glucose	5793 (pubchem)
1.21+	52178	525.1609	354.1041(100) ; 273.0782(98) ; 245.0469(37.7) ; 145.0473(17.6) ;	-1.2	$C_{24}H_{28}O_{13}$	Barbatoflavan	47366 (metlin)
1.49-	7764	475.1235	133.0110(100) ; 115.0009(28.8) ; 71.0098(19.9) ; 56.9939(1.7)	2.3	$C_{23}H_{24}O_{11}$	Luteolin 5,3'-dimethyl ether 7-glucoside	49394 (metlin)
1.58-	14314	431.1347	89.0208(100) ; 119.0311(4.1) ; 341.1040(8.1)	0.1	$C_{22}H_{24}O_9$	Isosakuranetin-7-O-rhamnoside	52823 (metlin)
1.74-	9174	455.0950	112.9824(100) ; 68.9919(51.8)	7.4	$C_{23}H_{20}O_{10}$	Epigallocatechin 3-O-vanillate	47348 (metlin)
1.96-	6618	549.1590	179.0529(84.4) ; 383.1130(31.8) ; 323.0925(18.7) ; 161.0429 (19.4) ; 341.1037 (11.9)	19.8	$C_{26}H_{30}O_{13}$	Caffeic acid derivative glycosylated	985394 (metlin)
1.97+	11640	139.0486	121.0380(100) ; 93.0434(72.5)	19.4	$C_6H_6N_2O_2$	4-Methylpyrimidine-2-carboxylic acid	723920 (metlin) San and Yildirim [30]
1.98-	36208	191.0170	87.0054(100) ; 85.0257(47.9) ; 111.0066(37.2) ; 57.0306(17.7)	8.52	$C_6H_8O_7$	Citric acid	Abu-Reidah, Ali-Shtayeh [62]
2.01-	2632	117.0158	73.0260(100); 45.9963(96.0); 68.9922(94.7)	20.80	$C_4H_6O_4$	Succinic acid	Orčić, Francišković [63]
2.32-	290	169.0126	NF	3.25	$C_7H_6O_5$	Gallic acid	San and Yildirim [30], Zhao, Zhang [31], Benabderrahim, Elfalleh [32]
3.74+	4362	195.1203	45.0326(100) ; 109.9576(24.9) ; 135.0422(13.1) ; 107.0483(9.4)	19	$C_8H_{12}N_4O$	Pirimidine derivative	926880 (metlin)
5.67+	148518	393.2040	NF	5.17	$C_{25}H_{28}O_4$	5-Hydroxy-7-O-nerylflavanone	52674 (metlin)
6.11+	5386	465.1873	407.1842(16.4)	5.1	$C_{18}H_{24}N_8O_7$	Tetrapeptide (Asp-Gly-His-His)	121608 (metlin)
6.12+	9780	476.3004	45.0328(100) ; 133.0834(26.5)	3.8	$C_{21}H_{41}N_5O_5S$	Tetrapeptide (Leu-Cys-Lys-Leu)	176048 (metlin)
6.43+	60668	407.2194	254.9096(0.3) ; 365.1489(0.3) ; 372.3353(0.3)	6	$C_{26}H_{30}O_4$	5-Methoxy-7-prenyloxy-8-C-prenylflavanone	52663 (metlin)
6.50+	13542	611.1521	303.0459(100) ; 304.0494(20.4) ; 465.0962(11.8)	14.0	$C_{27}H_{30}O_{16}$	Quercetin-3-O-robinobioside	Wojdyło, Carbonell-Barrachina [25], Memon, Memon [64]
6.57+	11738	301.1146	106.0636(100) ; 132.0428(50.4) ; 225.0994(61.0) ; 197.1041(35.5)	-1.1	$C_{11}H_{16}N_4O_6$	5-Oxoprolylasparaginyl glycine	487255 (metlin)
6.59+	5296	467.2203	331.0835(1.2) ; 290.5074(0.9)	-4.6	$C_{20}H_{30}N_6O_7$	Tetrapeptide (Val-Pro-His-Asp)	244401 (metlin)
6.84+	6326	619.3095	103.0375(100) ; 191.0891(30.7) ; 235.1127(18.2)	16.6	$C_{28}H_{42}N_8O_8$	Pentapetide (Pro-Tyr-Pro-Arg-Ser)	264985 (metlin)
7.25+	7332	417.2332	45.0328(100) ; 89.0586(42.4) ; 133.0838(25.3) ; 177.1098(12.9) ; 144.0754(12.6)	5.4	$C_{17}H_{30}N_4O_7$	Tetrapeptide (Leu-Val-Asp-Ala)	182319 (metlin)
7.33+	11710	317.1460	106.0638(100) ; 132.0424(18.2)	-5.6	$C_{12}H_{20}N_4O_6$	Tripeptide (Ser-Pro-Asn)	19282 (metlin)
8.06+	3365	301.1136	106.0638(100)	-2.2	$C_{10}H_{20}O_{10}$	Dioside	70700334 (pubchem)

8.41+	13106	505.2559	419.2203(2.3) ; 113.0570(2.3) ; 124.0784(1.5)	-3	$C_{19}H_{36}N_8O_6S$	Tetrapeptide (Ala- Met-Gln-Arg)	107754 (metlin)
8.48+	4558	433.1185	61.0274(100) ;132.0427(50.3) ; 208.0571(18.6); 269.0881(17) ; 311.0968(13.1) ; 327.0893(12.2)	-12.87	$C_{21}H_{20}O_{10}$	Apigenin-7-O-glucoside	Khan, Haq [41], Orčić, Franciśković [63].
9.54+	25954	225.0990	132.0428(100) ; 104.0485(17.7)	-4.5	$C_8H_{16}O_7$	Glycoside	102183608 (pubchem)
10.15+	8300	255.1559	209.1506(100) ; 123.1152(47.8) ; 191.1413(44.7) ; 109.0998(28.9) ; 167.1052(22.8)	2	$C_{14}H_{22}O_4$	9-(3-Hydroxy-5-oxocyclopent-1-en-1-yl)nonanoic acid	689561 (metlin)
10.91+	4476	301.0728	182.0871(57.3)	-7.09	$C_{16}H_{12}O_6$	6-Methylluteolin	49158 (metlin)
10.99+	3366	255.0957	181.0607(26.5) ; 204.9606(23.3)	2.1	$C_{11}H_{14}N_2O_5$	Glycine derivative	500366 (metlin)
11.17+	7960	255.1568	209.1511(100) ; 123.1152(36.5) ; 191.1404(27.9) ; 109.1010(17.9) ; 167.1043(21.3)	-1.6	$C_{10}H_{18}N_6O_2$	Glycine derivative	347687 (metlin)
14.99+	6378	291.2494	45.0327(100) ; 161.0930(15.9) ; 151.0927(10)	31.7	$C_{13}H_{10}N_4O_3$	Guanidine derivative	104888008 (pubchem)
15.10-	11328	353.1957	96.9565(61.5) ; 122.9722(2.9)	2.0	$C_{19}H_{32}O_7$	Blumenol C-glucoside	95146 (metlin)
16.50+	49376	595.4105	309.1994(100) ; 310.2025(21.6) ; 221.1492(1.1)	-6.6	$C_{30}H_{58}O_{11}$	Stearyllisomaltse	9894835 (pubchem)
16.53+	98018	287.2188	69.0689(100) ; 111.1153(54.1) ; 199.1664(4.3)	13.9	$C_{16}H_{30}O_4$	3,6-Dihydroxy-4-ethylidene-6-ethyldecanoic acid ethyl ester	10803152 (pubchem)

The following three major peaks obtained at 1.96, 2.32 min detected with a deprotonated molecule at m/z 549.1590 and 169.0126 were assigned as caffeic derivative glycosylated and gallic acids. These compounds were analogous to those previously identified in *Zizyphus* species **[30-32].**

Flavan-3-ols and proanthocyanidin were characterised in this study. Compounds observed at R_t of 1.21 and 1.74 min with m/z 525.1609 ($[M+H]^+$), 455.0950 ($[M-H]^-$) with different ion fragments were proposed to be barbatoflavan and epigallocatechin 3-O-vanillate, respectively **[33, 34].** However, compounds with retention times of 5.67, 6.43 min showed a MS^2 fragmentation characteristic to flavanones with m/z of 393.2040, 407.2194 at positive mode were tentatively identified as 5-hydroxy-7-O-nerylflavanone and 5-methoxy-7-prenyloxy-8-C-prenylflavanone, respectively. In addition, a flavonoid with methoxy groups was at R_t = 10.91 with a [M+H]+ m/z of 301.0728 which was assigned as 6-methylluteolin **[35, 36].** The flavonoid aglycones were in accordance to those identified in previous reports from other plant materials that showed a significant antioxidant properties **[37].** To the best of author's knowledge, these compounds are reported from *Zizyphus* genus for the first time.

Four compounds were charachterized as flavanones glycosides in this stuy. Peaks observed at 1.49, 1.58 min, with $[M-H]^-$ ion at m/z of 475.1235 and 431.1347, were tentatively identified as luteolin 5,3'-dimethyl ether-7-glucoside and isosakuranetin-7-O-rhamnoside, respectively

[38, 39]. Our study is the first detecting the presence of these compounds from *Zizyphus* genus. However, two known flavones, including quercetin-3-O-robinobioside (R_t = 6.50 min), apigenin-7-O-glucoside (R_t = 8.48 min) were found with positive mode in the samples. These compounds were in accordance with privous funding from jujube samples **[25, 40, 41].** Figure 5 showed clearely that both glycoside and aglycone flavonoids are 44.51% of all phytochemicals and 80.75% of all seconderay metabolites detected in our samples. 5-hydroxy-7-O-nerylflavanone is found to be the most abundant flavonoid from our samples. We can say that the antioxidant activities of *Z. lotus* extracts will be mainly due to flavonoids group by knowing the intensities of each compound present in extracts as we mentioned them at different color in heatmap.

Alkaloid group was characterised in this sudy and observed at 1.05 min with m/z of 387.1089, this compound was assigned as indole derivative. This last is detected also from other plants and it is known as one of antimalarial, antioxidant and antiiflammatory agents **[42].** In addition, peak observed at R_t = 15.10 min with a $[M-H]^-$ ion at m/z 353.1957 was attributed to blumenol C-glucoside. This compound showed a good pharamcological effect in several plan material like major class of terpenes, thus it is demonstrated by Rameshwar, Kishor **[43]** from Indian *Bombax ceiba* that is a good antioxidant. Others suggested that it is one of inhibitors on proinflammatory cytokines **[44].**

Peaks observed at 1.11, 8.06, 9.54 and 16.50 min with a positive and negative modes at m/z of 179.0535, 301.1136, 225.0990 and 595.4105 were attributed to glucose, dioside glycoside and stearyllisomaltse, respectively. These compounds are not present with high intensities in our sample and some of them are in accordance to other data detecting sugars from *Zizyphus mauritana* fruits **[45].** Recently, several studies have given a considerable interest to the bioactive peptides derived from plant materials which have a large biological effects on human health. Otherwise, different parts of jujubes such as fruits, roots, seeds and leaves have been used against the treatment of several diseases **[46, 47].**

However, several small peptides are detected in *Z. lotus* extracts. Peaks observed at R_t of 3.74, 14.99 min with m/z of 195.1203, 291.2494 were tentatively assigned as pirimidine and guanidine derivative. In addition, three glycine derivatives were tentatively identified at 6.57, 10.99, 11.17 in positive mode with m/z of 301.1146, 255.0957 and 255.1568, respectively. Furthermore, five differents tertapeptides were identified tentatively in positive mode at differents retention times 6.11, 6.12, 6.59, 7.25 and 8.41 min as shown in Table 5. These

compounds were assigned with differents molecular formula $C_{18}H_{24}N_8O_7$, $C_{21}H_{41}N_5O_5S$, $C_{20}H_{30}N_6O_7$, $C_{17}H_{30}N_4O_7$ and $C_{19}H_{36}N_8O_6S$, respectively. However, one pentapeptide was detected at 6.84 min with m/z of 619.3095, and one other tripeptide was observed at 7.33 min with m/z of 317.1460. These compounds were in accordance to those identified in previous reports from *Zizyphus* species and others data isolated bioactive compounds from plants **[48-50].**

Figure 29 showed the presence of 14.04% peptides from total compounds prensent in our *Z. lotus* pulp and peel extracts and we mentioned 31.30% from total primary metabolites. Several studies have been characterised the antioxidant potential of peptides from several plants as demonstrated from *Zj* fruits **[51].** Thus, *Z. lotus* pulp and peel have a non negligeable amount of peptides which may participate in the studied antioxidant activities. The examination of chromatograms obtained at negative and positive modes indicated the presence of some organic acids at 1.97, 1.98, 2.01, 10.15 and 16.53 min with m/z of 139.0486, 191.0170, 117.0158, 255.1559 and 287.2188, respectively were assigned tentatively as 4-methylpyrimidine-2-carboxylic, citric, succinic, 9-(3-hydroxy-5-oxocyclopent-1-en-1-yl)-nonanoic and 3,6-dihydroxy-4-ethylidene-6-ethyldecanoic ethyl ester acids. In addition to other substances, these acids have found to excerse antioxidant and anti-Alzheimer effects from serval plants **[52-55].**

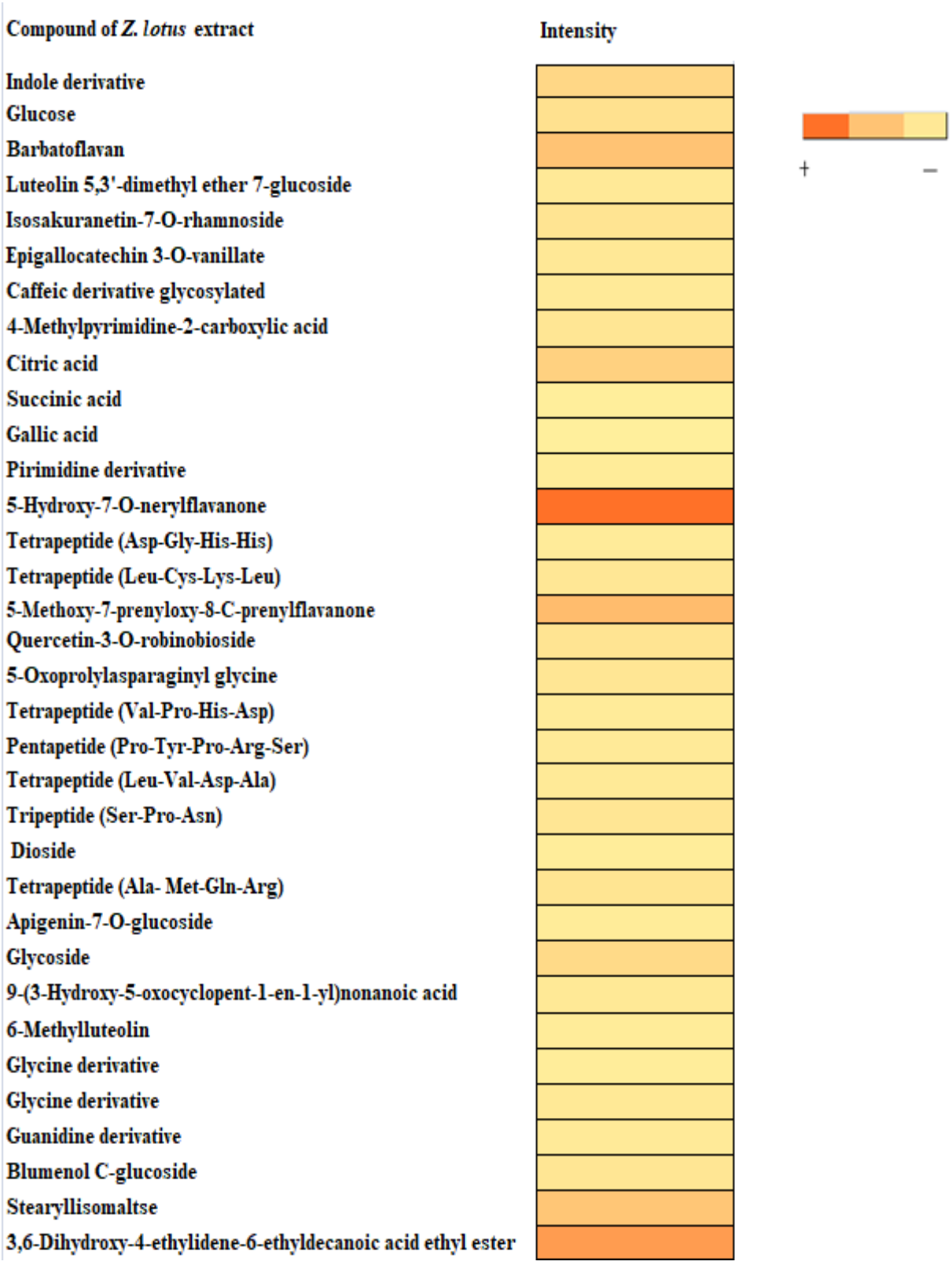


Figure 29 : Heatmap of the chemical profile and of *Z. lotus* pulp and peel extracts. Mean values refer to colors from minimum displayed in light orange to maximum represented with dark orange.

I.1.4. Antioxidant activities of jujube pulp and peel extracts

In order to evaluate the antioxidant activities of *Z. lotus* fruit extract obtained by the three methods studied, several tests were used such as $DPPH^{\cdot}$, $ABTS^{\cdot+}$, FRAP and FIC assays (Table 13). The greater antioxidant capacity of the phenolic extracts was attributed to a significantly lower IC_{50} ($p < 0.01$).

The MAE extract showed the higher antioxidant (0.42 ± 0.23 mg GAE/mL) than UAE extract (0.84 ± 0.14 mg GAE/mL) and CSE (0.89 ± 0.02 mg GAE/mL). Our results were in agreement with Ghazghazi, Aouadhi **[56]** and works who have used microwave extraction for *Zj* from Tunisia and Oman. Similarly our results obtained by UAE and CSE are greater than those of Hammi, Jdey **[57]** reported an IC_{50} of 0.2895 mg/ml for Tunisian variety extract but close to those of MAE extract. Moreover, MAE extract from *Z. lotus* showed also a higher ABTS activity (14573.85 ± 7.03 TE/100 g) compared to extracts from CSE and UAE. These results are almost in the same range as reported by Gao, Wu [**28]** for *Zj cv. Jiaxianmuzao.* In addition, the iron-reducing power of the *Z. lotus* extracts revealed a highly significant effect by the MAE extract (3305.18 ± 9.75 mg GAE/100 g), compared with the UAE and CSE extracts (1284.53 ± 10.99; 672.86 ± 4.87 mg GAE/100 g respectively). Our results are much higher than those of Wang, Cheng **[58]** (471.6 ± 30.8 mg CE/100 g DM) obtained from *Zj cv. Zaowangzao*. However, the ferrous iron chelation activity was well marked by the extracts obtained by MAE (137.41 ± 6.49 mg EED/100 g) than the other two methods (104.16 ± 18.16 and 80.55 ± 12.78 mg EED/100 g respectively). This is due to the ability of chelating agents (Fe^{2+} free ions) to form complexes with ferrozine **[59].**

The present study confirmed the results obtained by the other antioxidant activities that microwave extract presented a significant and high antioxidant and chelating activities than UAE and CSE extracts. The mechanism responsible may be due to the bioactive compounds present in jujube extract, such as phenolic and organic acids, flavonois aglycones and glycosides, peptides and some of sugars as we have seen by LC-MS/MS analysis. Figure 5 demonstrated that most of these phytochemicals with high intensities are polyphenols (55%) known which could provide hydrogen and/or electron to the radicals **[60].** Their activities could be attributed to the presence of a high number of hydroxyl groups which are present in their aromatic rings **[61].** As well as, the increasing of these activities due to the content of other compounds is associated to the strong reducing of the oxidized state which causes an increase in antioxidant capacity. Nevertheless, *Z. lotus* fruit extract showed another combination of phenolic compounds with other primary metabolites, mainly peptides and

derivative that are complexing together in plant extracts and increase the antioxidant effect **[51].** Furthermore, from a heatmap we clearly notice that combination between glycoside and aglycone flavonoids (80.75%) are the most determinant of the antioxidant activity in jujube extracts where 5-hydroxy-7-O-nerylflavanone was the most abundant. It should be emphasized that the proposed approach is limited for the identification of all the compounds contained in this extract mentionned for the first time from pulp and peel. This will open up to other more innovative studies requiring the purification of this extract and the isolation of this majority compound in order to benefit more from their use as a nutraceutical agent.

Optimization of microwave-assisted polysaccharide extraction from Algerian jujube (*Zizyphus lotus L.*)pulp and peel

Abstract

The active ingredient recoveries from the vegetable is a very attractive research field for the development of a Sustainable Economy, to revalue the jujube fruit (*Zizyphus lotus* L.) polysaccharide (*ZLPS*), an optimized green microwave assisted method was used for the recovery and enrichment of the antioxidants present in the distilled water extract. A series of 17 experiments including microwave power, irradiation time, and liquid-to-solid ratio independent parameters was designed by the response surface methodology to optimize the recovery of the polysaccharide extract.The optimal conditions were as follows: 600 W, 40 min, and 26.69 mL/g. Under these conditions, the experimental extraction yieldwas 13.98 ± 1.55%which is very close with the predicted value (14.08%), and this demonstrated the validation of the extraction model proposed. The polysaccharide extract exhibited a significant scavenging activity against ABTS.+ (70.45%), DPPH*.(66.02%), and FRAP (A = 0.63) with a very important anti-inflammatory activity using a protein denaturation method that showed a maximum inhibition of 95.33% at 200 μg/mL. Additionally, the membrane stabilization method showed a significant action and protection of human red blood cells (85.76%) in hypotonic-induced lysis solution and 86.45% in heatinduced lysis solution. This study demonstrated the possibility of exploiting the microwave process to obtain extracts remarkably enriched with invaluable antioxidants from the jujube matrix. The operation time is short, and the antioxidant and anti-inflammatory activities of the distilled water extract were preserved.

Keywords: *Zizyphus lotus* fruit . Polysaccharide . Response surface methodology . Microwave-assisted extraction . Anti-inflammatory and antioxidant activities

I.2.1. Preliminary study of plysaccharide extraction

The effects of various extraction parameters such as microwave power, irradiation time and liquid/solid ratio on the polysaccharide yield were studied using single-factor experiments**.**

Effects of microwave power

To study the effect of different microwave powers on polysaccharide extraction yields, the extraction process was carried out using the powers of 100, 200, 300, 400, 500, 600, 700 and 800 W. The extraction time and the liquid/solid ratio were fixed at 30 min, 50 :1 mL/g respectively. As shown in Fig. 30a, the maximum extraction yield of *ZLPS* (12.2%) was observed when the microwave power was set at 400 W and then slightly decreased for more than 600W. These results are in agreement with those obtained by Dahmoune, Nayak **[65]** who reported that an intermediate power of 400 W gives a better phenolic compounds recovery and activities. Rostami and Gharibzahedi **[66]** suggested that the use of maximum microwave power of 400 W for extraction of polysaccharides from jujube fruit enhances polysaccharide extract due to the heating effect and its solubility, which was observed in our results where the yield was decreased from 400 to 600 W. The higher power density may cause a degradation of extract which may conduce to polysaccharide caramelization. However, sometimes microwave treatment can contribute to the appearance of certain chemical reactions not appearing by conventional methods, but does not participate to the degradation of polysaccharide extract as demonstrated by Zhou, Yu **[67]** whostudied the effect of microwave treatment on degradation of polysaccharide from *Porphyray ezoensis* according to the Maillard reaction of d-glucose/glycine, UV and fluorescence reaction tests. Therefore, 200-600 W range was adopted for the RSM trials, while 400 W was kept for the next experiments.

Effects of extraction time

The extraction yield of *ZLPS* affected by irradiation time is another parameter that should be considered for the optimization step. Fig.30b showed that extraction time influenced significantly the extraction yield of *ZLPS*, when both factors (X_1, X_2) were fixed at 400 W and 50 :1 mL/g respectively. A significant increase of *ZLPS* extraction yield (10.9%) was observed from 5 to 30 min of microwave irradiation exposure, followed by a significant decrease until 30 min. This is in agreement with Adeli and Samavati **[68]** results about

polysaccharides recovery from *Zizyphus lotus* using UAE showing a maximum yield between 30 and 40 min of treatment.

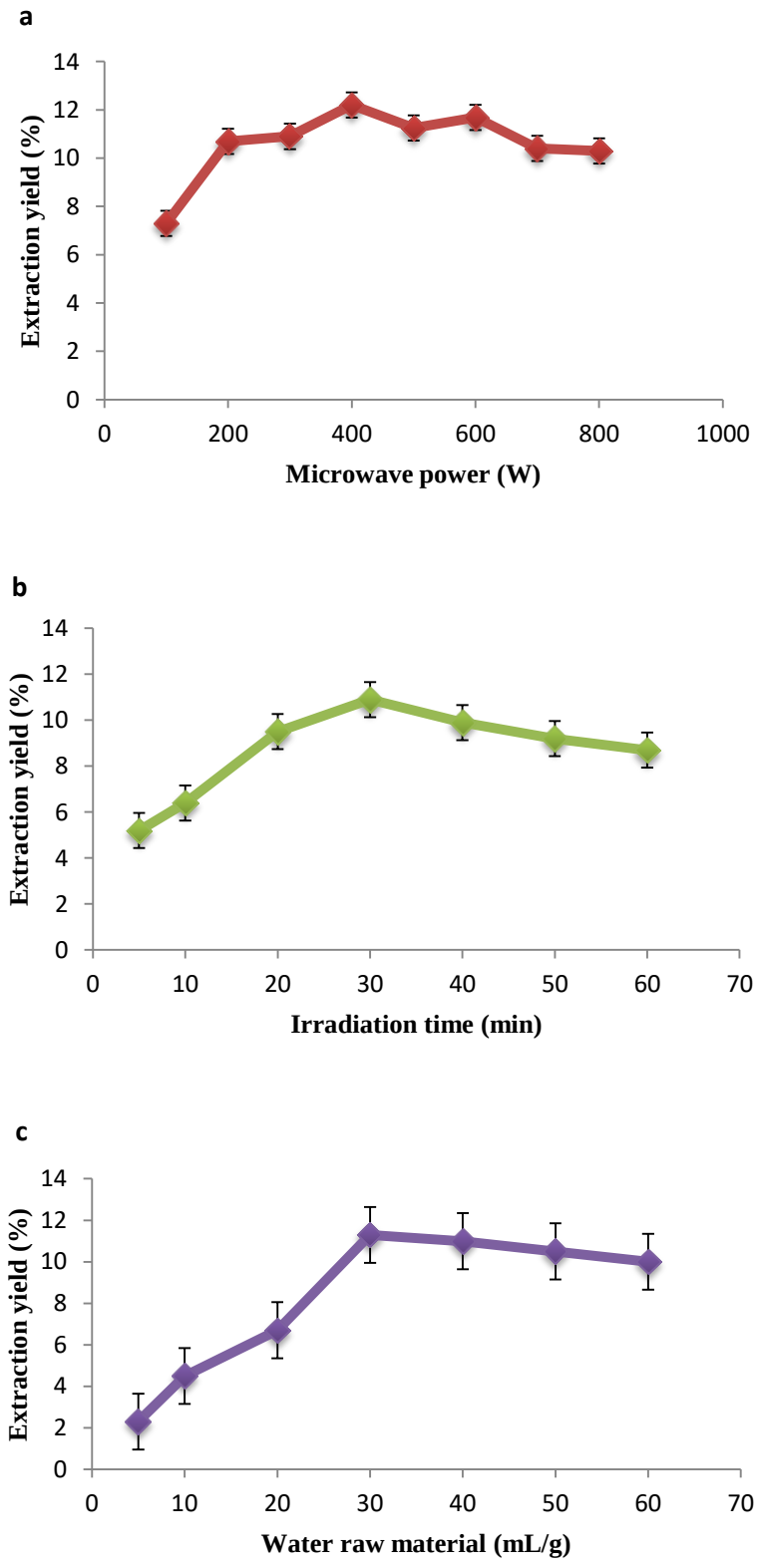


Figure 30 : Effect of microwave power (a), irradiation time (b) and water to the raw material ratio(c) on extraction yield of *ZLPS* (%).

Rostami and Gharibzahedi **[66]** showed also that polysaccharide extraction yield from *Z. jujube Mill* is higher when the irradiation time is between 30 and 60 min using MAE. These observations suppose the effect of the applied extraction procedure and the studied plant type. Other work suggested also that the extraction of polysaccharides from plants were favored by a longer extraction time which let the water penetrate the raw materials using both microwaves and ultrasound as an innovative extraction method **[69, 70].** The 20-40 min range was selected for the RSM trials, while 30 min was kept for the next single-factor trials.

Effect of liquid/solid ratio

In this present study, liquid/solid ratio was set at 5, 10, 20, 30, 40, 50 and 60 mL/g while the other two extraction factors were fixed at 400 W and 30 min for microwave power and extraction time respectively. Fig. 30c showed that the *ZLPS* yield increased significantly from 2.3% to 11.99% by increasing the liquid/solid ratio from 5 to 30 mL/g. Thereafter, it was observed that *ZLPS* was constant until the ratio of 60 mL/g. The increase of extraction ratio implies the diffusivity of the solvent inside the cells which allows the improvement of polysaccharides desorption from them as reported by Samavati and Manoochehrizade **[70].** Our results are in agreement with those obtained from jujube polysaccharide by Adeli and Samavati **[68]** using UAE (ratio of 24.44 mL/g) and as demonstrated by Rostami and Gharibzahedi **[66]** using MAE. For the liquid/solid ratio, the range of 20–40 mL/g was considered for the RSM optimization.

I.2.2.Response Surface Methodology Optimization of Polysaccharide Extraction

Referring to the results of the screening factor experiments approach, the major factors, namely microwave power (W), irradiation time (s) and liquid/solid ratio (mL/g) were varied according to CCD layout Table 14. Then, the optimization of polysaccharide yield extracted from jujube powder according to these three selected parameters is performed by the RSM.

Table 14 reports the applied second-order regression model using statistical analysis. The analysis of variance (ANOVA) was used to determine quadratic model adequacy for experimental data prediction of polysaccharide recovery using MAE. The ANOVA result showed that the higher model F-value (13.70) associated lower p-values ($p< 0.0012$) indicates that most variation in the response can be explained by the regression model. The lack of fit F-value of 2,50 and *p*-value of 0.30 showed that the lack of fit of the model was insignificant ($p> 0.05$).

Table 14 : Response surface design and results for extraction yield of *ZLPS.*

Run	X_1- Microwave power (W)	X_2 - Irradiation time (min)	X_3 – Liquid/solid ratio (mL/g)	*ZLPS* Extraction yield (%)	
				Actual	Predicted
1	200	20	20	9,40	8,86
2	600	20	20	10,20	10,79
3	400	30	20	9,90	10,03
4	200	40	20	8.00	8,46
5	600	40	20	13,70	13,04
6	400	20	30	9,70	9,19
7	200	30	30	10,70	10,45
8	400	30	30	10,80	10,76
9	400	30	30	9,80	10,76
10	400	30	30	10,70	10,76
11	600	30	30	13,10	12,83
12	400	40	30	11,10	11,09
13	200	20	40	4,80	5,58
14	600	20	40	6,10	5,76
15	400	30	40	7,50	6,85
16	200	40	40	7,60	7,13
17	600	40	40	9,32	9,97

Additionally, the determination coefficient (R^2 = 0.95) revealed that 95% of the total variations are well explained by the experimental data model. In order to determine whether is the good statistical quadratic model, the adjusted R^2 value should be close to 1 (0.87), this explained a high correlation degree as shown in Table 3. At the same time, coefficent of variation value (CV) was less than 10% that displayed also the perfect precision and better reliability of the experimental values and the predicted one **[65, 69].** Therefore, the model is adequate and can be used to optimize experimental variables.

As shown in Table 15, the independent variables have significant effects on *ZLPS* recovery using MAE at the level of $p < 0.01$. *ZLPS* extraction was affected more significantly by water to raw material ($p = 0.0004$), followed by microwave power ($p = 0.0021$) and by irradiation time at ($p = 0.0068$). Also, (quadratic liquid/solid ratio, X_3^2) and interaction (X_1X_2 : Irradiation time- liquid/solid ratio) terms affect significantly the response ($p < 0.0001$ and $p < 0.05$ respectively), while all the other interaction terms (X_1X_2, X_2X_3) were insignificant ($p > 0.05$). Consequently, the predictive mathematical equation (Eq. 12) is given below in terms of coded factors excluding non-significant terms ($p > 0.01$) :

$$Y(ZLPS) = 10.76 + 1.19X_1 + 0.95X_2 - 1.58X_3 - 2.32X_3^2 + 0.66X_1X_2 \quad (12)$$

Table 15 : Analysis of variance (ANOVA) for response surface quadratic model for extraction of *ZLPS.*

Parameter[a]	Estimated coefficients	Standard error	Degree of freedom	Sum of squares	F-value	Prob> F
Model	10,769859	0,339426	9	77,608047	13,7044	0.0012*
(*ZLPS*)B_0						
Linear						
X_1	1,192	0,250843	1	14,208640	22,5812	0,0021*
X_2	0,952	0,250843	1	9,063040	14,4035	0,0068*
X_3	-1,588	0,250843	1	25,217440	40,0771	0,0004*
Quadratic						
X_1^2	0,8777465	0,484614	1	2,064195	3,2805	0,1130
X_2^2	-0,622254	0,484614	1	1,037402	1,6487	0,2400
X_3^2	-2,322254	0,484614	1	14,448799	22,9629	0,0020*
Interaction						
X_1X_2	0,665	0,280451	1	3,537800	5,6225	0,0495*
X_1X_3	-0,435	0,280451	1	1,513800	2,4058	0,1648
X_2X_3	0,49	0,280451	1	1,920800	3,0527	0,1241
Lack Of Fit			5	3,7978978	2,5041	0,3096
Pure Error			2	0,6066667		
Total Error			7	4,4045645		
R^2					0,946294	
R^2Adjusted					0,877244	
CV%	7.34					
RMSE	0,793236					
Corr.Total			16	82,012612		

The effects of the operational parameters, the optimal levels of independent variables and their mutual interaction on the polysaccharides extraction yield according to Eq. (12) can be visualized on three dimensional response surface profiles (**Figure 31**). The plots were generated by sketching the response using the z-axis versus two independent variables (X_1 and X_2) while keeping the other independent variables (X_3) constant at their zero levels **[19].** Summarizing, only the interaction between microwave power and irradiation time (Fig.31a**)** with their corresponding linear parameters was significant, but water raw material did not influence the polysaccharide yield as an interactive factor with microwave power and irradiation timeas can be observed in Fig.31 b-c(non significant interaction effect), but it exhibited the best linear and quadratic effects.

In fact, through the 3-D response surface plot, Fig. 31a shows that the extraction yield of *ZLPS* increased rapidly with increase of microwave power from 400 to 600W and with the

increase of extraction time from 30 to 40 min. As reported by Thirugnanasambandham, Sivakumar **[71],** the increase of microwave power is not only for the increase of dipole rotations which lead to power degenerate inside the extract, but also the heat in the extract was reached rapidly which improve the solubility of the polysaccharides and extraction yield. This graph was consistent with results shown in Table 15, so it is evident that the interaction between microwave power and irradiation time had a very significant impact ($p < 0.05$) on the extraction efficiency of polysaccharide from *Z.lotus*.

From Fig. 31b, it can be observed that the *ZLPS* yield increased significantly from 5 to 10% with the increase of irradiation time (>40 min) and the decrease of liquid/solid ratio from 40 to 26 mL/g. The exposure time of 40 min provided a good penetration of water into the *Z. lotus* powder which dissolved its polysaccharides, as showed by Liu, Liu **[72]** for jujube powders during a large time of irradiation which enhances diffusivity of the water into cells and improves desorption of the polysaccharides from the cells when liquid/solid ratiowas <30 mL/g, Chouaibi, Mahfoudhi **[73]** suggested that the extraction yield decreased with the increase of liquid/solid ratio which is probably due to the breakage of cell membranes and its enhancing in the raw material. This confirmed and explained that the mutual interaction between these two variables was insignificant ($p = 0.12$).

As shown in Fig. 31c, it can be seen that the maximum extraction yield of the *ZLPS* could be reached when the liquid/solid ratio decreases from 40 to 30 mL/g to 600 W. The significant increase in liquid/solid ratio can affect certain rheological properties of the polysaccharide extract by affecting the extent of its gelatinization because of the increase in the amount of the solvent entering the mixture and thus the extraction efficiency can be decreased by affecting the transfer of the mass of the raw material **[66].**

From the model (Eq. (8)), the optimal conditions of polysaccharide yield using MAE were: microwave power 600 W, irradiation time 40 min, liquid/solid ratio 26.69 mL/g. Under the optimal conditions, the model predicted a maximum response of 14.08 ± 1.55%. To confirme the predictability of established model, three additional experiments under the optimum extraction conditions were carried out. The *ZLPS* yield was 13.98 ± 1.55% which is closed with the predicted value (14.08 ± 1.55%) , which was found to be not significantly different than the predicted one ($p > 0.05$) using a paired t-test, suggesting that the model had a high suitability of the optimizing extraction conditions for *ZLPS* recovery (Table 16).

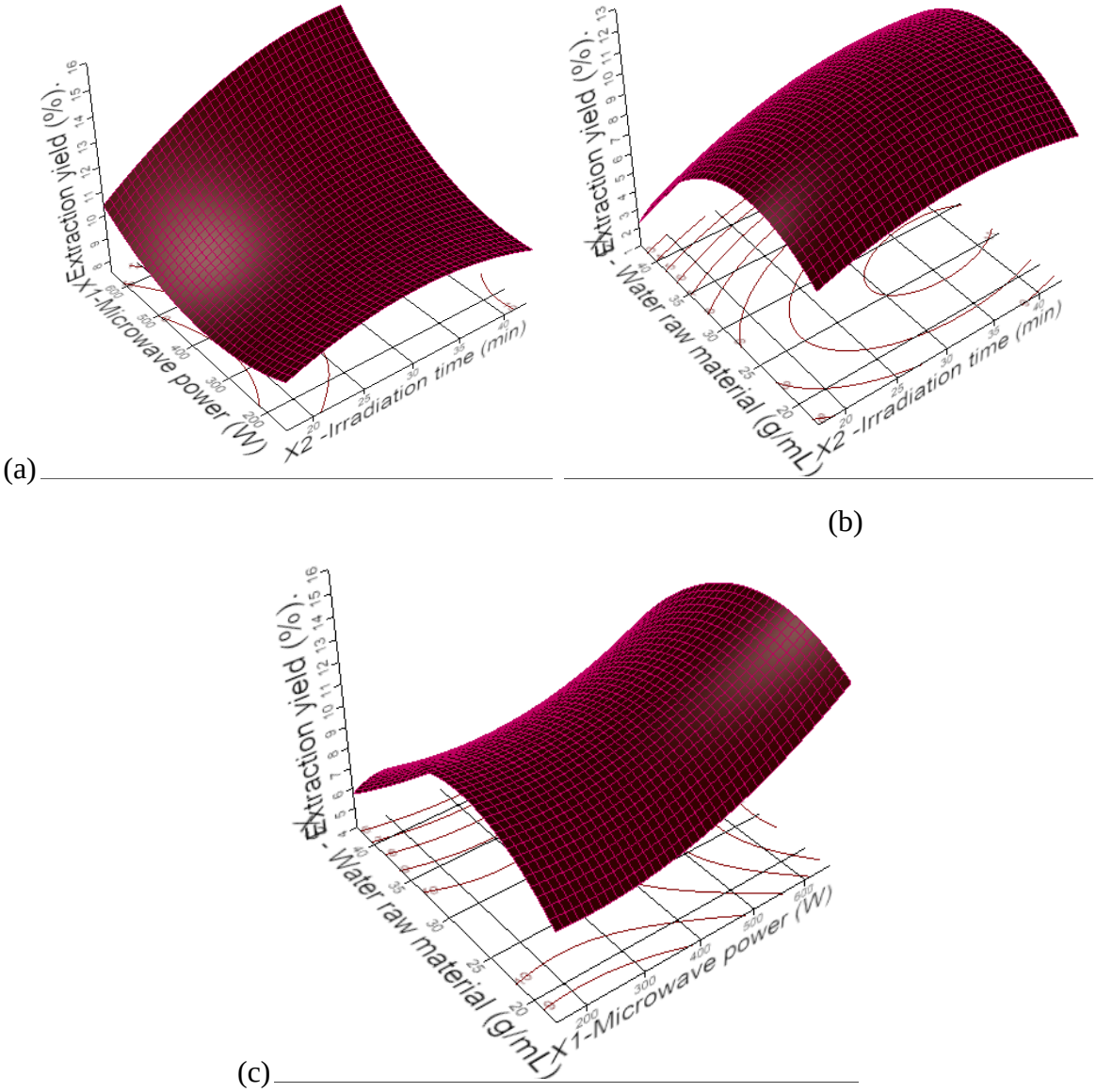


Figure 31 : The response surface plot (a, b and c) indicating the effects of factors (X_1, microwave power; X_2, irradiation time; X_3, liquid/solid ratio).

Table 16 : Predicted and experimental values of the responses at optimum conditions.

Optimum condition			Extraction yield of *ZLPS*(%)		
Microwave power (W)	Irradiation time (min)	Liquid/solid ratio (mL/g)	Experimental	Predicted	Desirabiliy
600	40	26.69	13.98 ± 1.55%	14.08%	0.987

I.2.3.Chemical analysis of crude polysaccharide

According to Table 17, the crude *ZLPS* extract produced under the optimal MAE conditions revealed a high carbohydrates content and low amount of protein. In fact, total sugars before

and after deproteinization were 98.92 ± 0.12 and 92.35 ± 0.52 % respectively, while, total proteins after deproteinization were 3.2 ± 0.23 %. This values are approximatively the same for *ZLPS* extract obtained by Chouaibi, Rezig **[74]** from Tunisia region, who found 93.15% of total sugars from purified polysaccharide, with a very small amount of protein (2.31%). In addition, comparable result was obtained from Tunisian *Zizyphus lotus* pulp and peel which showed 97.92% of total sugars and absence of protein contents **[69].**

Table 17 : Quantitative analysis of sugars and protein in *ZLPS.*

Polysaccharide extract	
Total sugars before deproteinization (%)	98.92 ± 0.12
Total sugars after deproteinization (%)	92.35 ± 0.52
Total proteins after deproteinisation (%)	3.2 ± 0.23

I.2.4.Infrared spectrum analysis

The Fourier transform-infrared spectroscopy (FTIR) is used to identify the characteristic organic groups in polysaccharides. The FTIR spectrum of *ZLPS* extract was depicted in Fig. **32,** and the results were analyzed in different characteristic regions **[75].** A large absorption band was detected at around 3349 cm-1 which is characteristic of hydroxyl group (O-H) due to inter and intra molecular interaction of the polysaccharides chains ; the band at 2933 cm-1 was referred to (C-H) absorption stretching vibrations. In addition, the peak at 10770 cm-1 was assigned to C=O stretching vibration of O-acetyl groups and the small band at 1605 cm-1 was attributed to the carboxylate ion stretching band (COO-) **[76-78].** Large absorption bands were detected around 1550, 1400 cm-1 and 1309 cm-1 for C-H band stretching and these were due to asymmetric and symmetric bending vibrations **[77],** which may confirm the presence of uronic acid **[78].** Furthermore, the presence of an absorption band at 1248 cm-1 may due to the stretching vibration of non-symmetrical C-O-C. The intense characteristic band at 1037 cm-1 in the infrared spectra indicated the stretching vibration of C-O-C of glycosidic structure **[78].** Finally, the two characteristics absorption bands at around 893 cm-1 and 820 cm-1 were observed for ZLP extract indicating the β and α-glycosidic C1-H deformation, respectively **[78].** Our results are in agreement with those obtained by Hammi, Hammami **[69]** for the *Zizyphus lotus* species obtained from Tunisia.

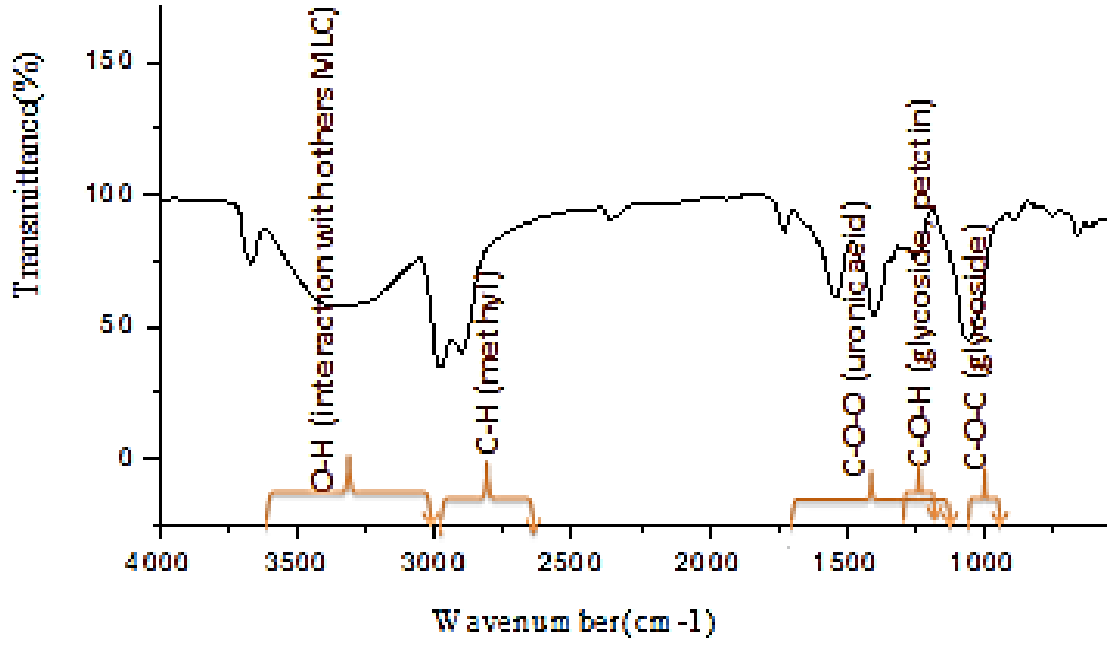


Figure 32 : FTIR spectrum of *ZLPS* extract.

I.2.5.Biological activities of *ZLPS*

I.2.5.1.Antioxidant activity

ZLPS extract scavenged strongly DPPH·radicals in a dose dependant manner (Fig. 33a) ; i.e, the percentage of inhibition increased significantly with the increase of sample concentration. The results indicated also that ascorbic acid used as standard presented higher scavenging activity than *ZLPS* extract. At the highest concentration of 200 µg/mL, the scavenging effect was 66.02 ± 0.89% and 86,49% for *ZLPS* and ascorbic acid respectively. Our results are in agreement to those obtained by Hammi, Hammami **[69]** for the Tunisian *Zizyphus lotus* pulp and peel using UAE. Wu, Li **[79]** reported that the hight value of antioxidant activity induced by polysaccharide extract could be explained by the presence of uronic acid. In addition, antioxidant activities against DPPH· of crude polysaccharide from *Zizyphus Jujuba cv. Jinsixiaozao* (CZSP) fruit was found to be higher (60%) at a concentration of 5 mg/mL when using both hot water (CZPH) and under ultrasonication (CZPU) **[80].**

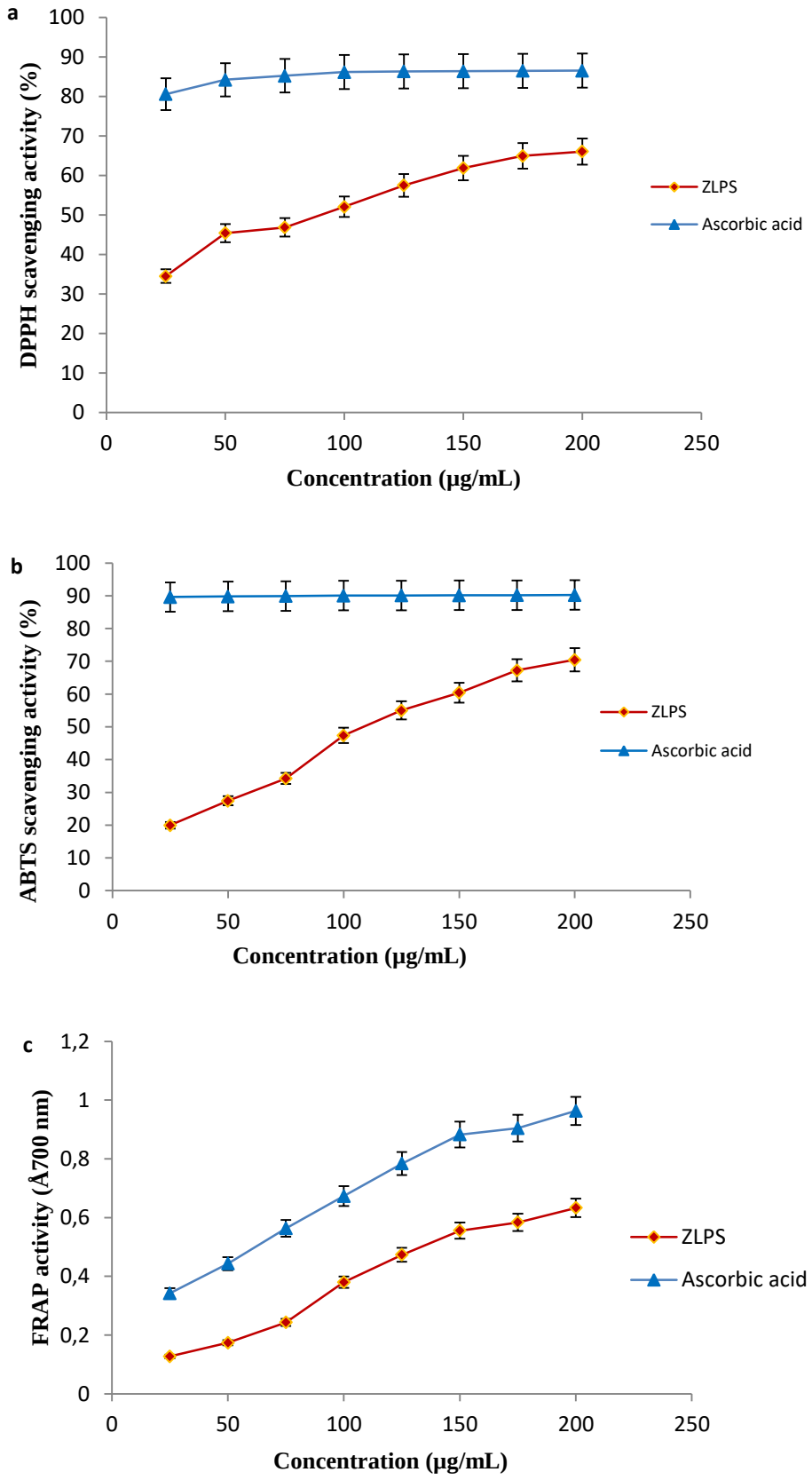


Figure 33 : Comparison of DPPH· radical scavenging activity (a) $ABTS^{\cdot+}$ scavenging activity (b) and FRAP scavenging activity of *ZLPS* and positive control (ascorbic acid) in the various concentrations.

More recently, Rostami and Gharibzahedi **[66]** showed that at a concentration of 200 µg/mL, purified polysaccharides extracted from *Ziziphus Mill* fruit using MAE exhibited obvious scavenging activity on $DPPH^{\cdot}$radical. Comparing to our results, Yuan, Liu **[81]** showed a lower antioxidant activity against $DPPH^{\cdot}$at 200 µg/mL (43.1%) of polysaccharide extract from *Ziziphus jujuba Mill. var. Spinosa.* Our results imply that *ZLPS* might act as electron or hydrogen donator to scavenge $DPPH^{\cdot}$(Fig. 4a**)** with an IC_{50} of 0.68 ± 0.10 mg. As shown in Fig. 4b, *ZLPS* extract exhibited higher antioxidant capacity to reduce $ABTS^{\cdot+}$ radical with dose dependant effect. At a highest tested concentration of 200 µg/mL, $ABTS^{\cdot+}$inhibition was 71% and 90 % for *ZLPS* and ascorbic acid respectively. Our results are higher than those obtained by Lin, Liu **[82]** for *Zizyphus jujuba* polysaccharide extracted from seeds which demonstrated a $ABTS^{\cdot+}$ scavenging activity of 33.41% at a concentration of 5 mg/mL. The ability of polysaccharide extracts to reduce the Fe^{3+}/ferricyanide complex was evaluated by following the formation of blue complex which absorbs at 700 nm **[83].** Higher absorbance at 700 nm corresponds to a higher reducing capacity. Figure 4c showed clearly that *ZLPS* extract and ascorbic acid reduce significantly the Ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) with a dose dependant effect. Our results were higher than those obtained by Hammi, Hammami **[69]** for the same species using UAE. Results obtained by Rostami and Gharibzahedi **[66]** showed that *Zizyphus* extracts exhibited less reducing power activity using MAE (0.65 at 700 nm) compared to our extacts obtained with optimal conditions using MAE as well.

These chemical tests used let suppose the potentiel capacity of *ZLPS* to donate atom and/or electron to the radical species. This implied the possible implication of these molecules to contreract oxidative damage which may occur in our organism and cause several pathogenesis.

I.2.5.2.Anti-iflammatory activity

Polysaccharides from natural sources have been reported to have multiple biological activities including antioxidant and antiinflammatory properties with no toxic effect **[84].** Several methods have been used in pharmacology for drug preparations especially chemicals. More recently, medicinal plant preparations with a high potential for anti-inflammatory drugs have been used. These techniques include inhibition of protein denaturation, stabilization of the erythrocyte membrane, lysosomal membrane stabilization, decoupling of oxidative phosphorylation and fibrinolytic and platelet assays aggregation **[85, 86].** In the present work,

inhibition of albumin denaturation, stabilization of erythrocyte membranes exposed to both heat and hypotonic that induced lyses were studied due to its simplicity and reproducibility.

Table 6a shows that the *ZLPS* extract prevent significantly and strongly albumin denaturation and the highest concentration tested of 200 µg/mL gives an inhibition of 95.33 ± 0.57% which is very close to the profenid as anti-inflammatory drug standard giving an inhibition of 95% with 100 µg/mL. Mizushima and Kobayashi **[87]** suggested that denaturation of proteins is well documented and is caused by inflammation and rheumatoid arthritis. However, the main mechanism action to protect against protein denaturation was NSAIDs (Non-Steroidal Anti-Inflammatory Drugs). The ability of *ZLPS* extract to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity. Additionally, the anti-inflammatory activity of *Zizyphus* extract may be due to the presence of therapeutically active polysaccharide, as demonstrated by Mzoughi, Abdelhamid **[88],** who have shown that many polysaccharides exhibited anti-inflammatory activity and it may be a potential therapeutic agent for inflammatory disorder **[89].**

Table 18 : Effect of *ZLPS* on heat induced protein denaturation (a), hypotonicity induced haemolysis (b) and heat induced haemolysis (c).

Concentration (µg/mL)	% Inhibition					
	(a)		(b)		(c)	
	ZLPS	Profenid	*ZLPS*	Profenid	*ZLPS*	Profenid
25	12.22 ± 1.53		10.63 ± 0.14		10.09 ± 1.92	
50	37,33 ± 0.57		16,38 ± 0.00		21,22 ± 0.30	
75	53 ± 0.70		22,97 ± 0.01		25,26 ± 0.06	
100	63,66 ± 0.57	95 ± 0.00	36,81 ± 0.07	85 ± 2.32	37,44 ± 0.01	88 ± 1.09
125	73 ± 1.41		54,97± 0.01		43,13 ± 0.07	
150	80,66 ± 0.57		56,06 ± 0.01		54,05 ± 0.10	
175	88 ± 1.37		60,80 ± 0.07		61,72 ± 0.01	
200	95.33 ± 0.57		85.76 ± 0.56		86.45 ± 0.12	

To confirm the membrane stabilizing action of *ZLPS* extracts, experiments were performed on the erythrocyte's membrane. Table 18 (b-c) demonstrates that *ZLPS* extracts inhibited the haemolysis of erythrocytes induced by both hypotonic solution and heat.

The whole plant extract exhibited a minimum membrane stability of 10.63 ± 0.14% and a maximum activity of 85.76 ± 0.56% (Table 18b). The standard anti-inflammatory drug (profenid) at 100 µg/mL exerted maximum membrane stability of 85 ± 2.32%.

Morever, Table 18c shows the membrane stability of 10.09 ± 1.92% and 86.45 ± 0.12% as minimum and maximum percentage activity using diffferent concentrations respectively. The response of the red blood cells was also monophasic and biphasic to the fractions. The standard anti-inflammatory drug (profenid) at 100 µg/mL exerted maximum membrane stability of 88 ± 1.09%. The activities of the extracts/fractions were approximatively higher than that of the standard drugs even at lower concentration ranges.

Chapter II. Optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from *Zizyphus lotus* L seed using Response Surface Methodology and its biological effects

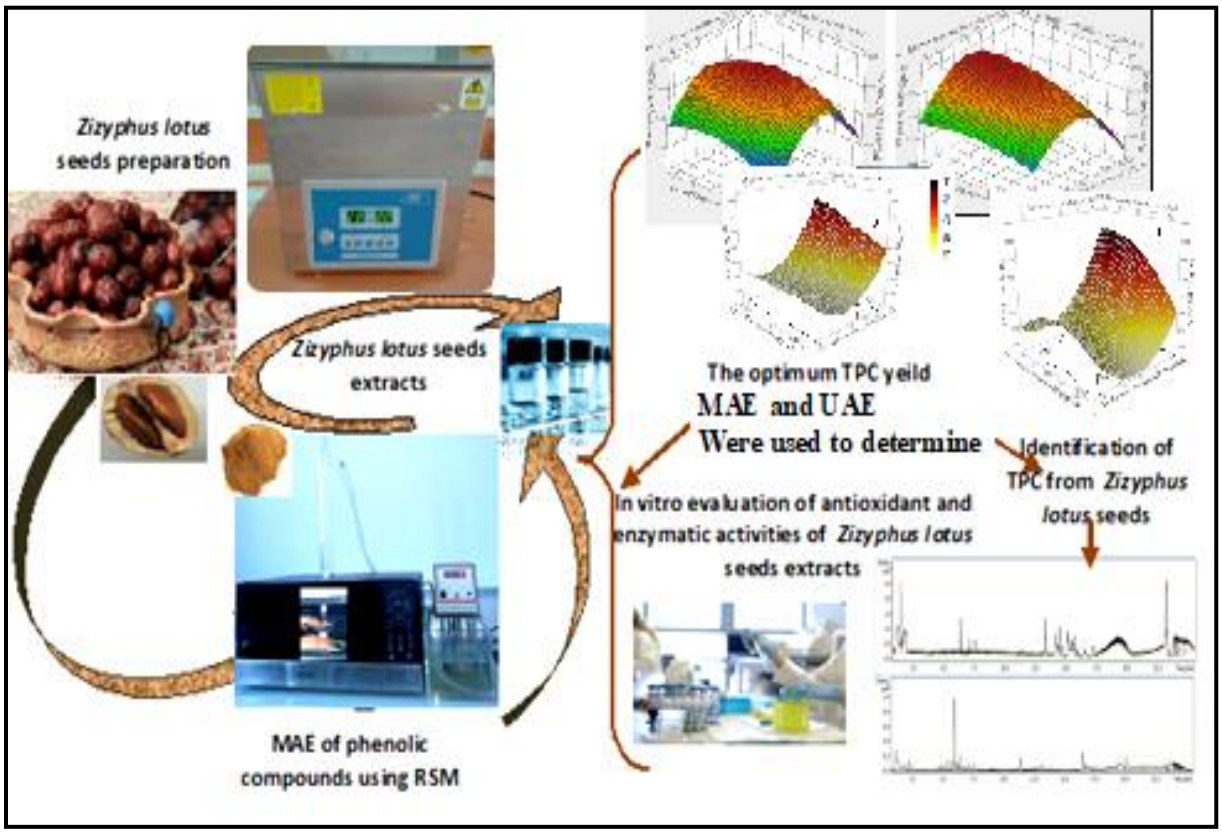


Figure 34: Graphical abstract of microwave and ultrasound assisted extraction of polyphenols from jujube seeds

Optimization of microwave-assisted phenolic compounds extraction from Algerian jujube (*Zizyphus lotus L.*)seeds

Abstract

The present study was aimed to optimize the extraction conditions of total phenolic contents (TPC) from *Zizyphus lotus* seeds (*Zls*) samples by microwave procedure using response surface methodology (RSM) in order to obtain maximum extraction yields. Central Composite Design (CCD) was employed with three factors defined as solvent concentration (X_1), irradiation power (X_2) and microwave power (X_3) at three coded levels on the TPC recovery as response. The optimal conditions for X_1, X_2 and X_3 were : ethanol 60%, 210 s and 600 W, respectively. The experimental value of TPC yields was for 6709.01 ± 2.20 mg GAE/100 g which is in close agreement with the predicted value indicating the success of RSM model. Results showed that optimized *Zls* extract exhibited a high inhibitory effects on some biological activities including 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, ferric iron reducing power (FRAP), Acetylcholinesterase (AChE) and 3-hydroxy-3-methylglutaryl reductase (HMGR) inhibitory. The liquid chromatography-high resolution tandem mass spectrometry (LC/MS-MS) profile revealed 47 active compounds where 21 were never been detected in *Zizyphus* genus. The *Zls* extract was found to contain significant major compounds comprised 6-methyl-2-O-glucoside xanthone, jasminoside isomer, citric acid, gallocatechin, imidazole carboxylate derivative, kaempferol-3-O-robinobioside, 6-gingerol, 2-hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy)propyl]phenyl]propan-1-one, 3-(decyloxy)-2-hydroxypropyl prop-2-enoate, tenasogenin and some small peptides. The findings demonstrated the beneficial application of microwave method for an increase extraction of TPC amounts from *Zls* extracts that could be valorized in food and pharmaceutical industries.

Keywords : *Zizyphus lotus*, mircowaves, response surface methodology, phenolic compounds, LC/MS-MS, biological activities.

II.1.1. Preliminary study of phenolic compounds extracted by MAE

The phenolic recovery of jujube seeds sample was optimized in two experimental sessions. Table 19 shows the TPC yield obtained from a single factor experiment of seeds samples with different solvents types, a best selected solvent concentration (ethanol), extraction time and MW power by MAE. It is clear that the yield obtained by ethanol was statistically highly significantly different compared to those obtained by methanol, acetone and water ($p < 0.01$). On the other hand, mass transfer within the sample powder in ethanol was more appropriate with 9, 3, and 2 fold heighted to the recovery reached by water, acetone and methanol respectively. Similar findings have been observed and reported in our previous work **[10].** Therefore, ethanol was used for extracting TPC in next optimization study.

As shown in Table 19, the best result was obtained when ethanol set at 40% (v/v) with a recovery enhanced from 2606.41 ± 1.35 to 4085.13 ± 1.08 mg GAE/100 g. The percentage of ethanol in water was very important in extraction assisted by microwave which resulted in a higher absorption of microwave irradiation power and higher recovery due to the higher of solubility and diffusion coefficient of TPC compounds. The obtained results were in agreement to those demonstrated by several authors using MAE for extraction of phenolic from vegetable matrix **[10, 65, 90, 91].** For this, 40% was fixed for the next single-factor experiments and 20–60% was selected for the RSM trials. For the effect of irradiation time on the extraction process, as indicates in Table 19, a TPC recovery has two stage. The recovery increase rapidly and then decreases slowly as extraction progresses. In general, extraction time in MW process is considered a very important parameter that must be taken in consideration because the energy absorbed by matrix causes rapid microwave heat. Our results are in agreement with those of other literature, mentioning that extracts exposed to a longer irradiation times may causes a degradation of TPC following the thermal microwave effect **[92].**

Moreover, as explained in the previous paragraph, the irradiation time is also influenced by the dielectric properties solvent, like ethanol that can undergo tremendous heating during prolonged exposure to microwave irradiation and so risk losing the desired bioactive components. In the statistical analysis reported in Table 19, 150 s was selected for the next single-factor trials, while the range 90–210 s was selected for the RSM trials.

Table 19: Optimized MAE procedure of phenolic constituents from jujube seeds.

Step 1 : Vary one factor at a time approach				
Solvent type (Type) [a]TPC yield (mg [b]GAE/100 g)	Ethanol concentration (% v/v) TPC yield (mg GAE/100 g)	Irradiation time (s) TPC yield (mg GAE/100 g)	Microwave power (W) TPC yield (mg GAE/100 g)	Solvent-to-solid ratio (mL/g) TPC yield (mg GAE/100 g)
Water : 1996.08 ± 1.35[d]	20 : 2606.41 ± 1.35[f]	60 : 2465.72 ± 1.62[e]	300 : 1914.71 ± 1.35[d]	20/1 : 2254.30 ± 1.35[c]
50% MeOH : 2254.30 ± 1.35[b]	30 : 3006.25 ± 2.71[c]	90 : 2324.10 ± 0.13[g]	400 : 2255.08 ± 2.71[c]	25/1 : 2347.49 ± 0.13[b]
50% EtOH : 2819.24 ± 2.34[a]	40 : 4085.13 ± 1.08[a]	120 : 2349.13 ± 1.08[f]	500 : 3051.72 ± 0.13[a]	30/1 : 3053.20 ± 2.71[a]
50% Acetone : 2159.70 ± 0.13[c]	50 : 2819.32 ± 2.23[d]	150 : 3145.61 ± 0.14[a]	600 : 2582.47 ± 0.54[b]	35/1 : 1809.15 ± 2.84[d]
	60 : 3875.58 ± 4.06[b]	180 : 2930.35 ± 1.35[b]		
	70 : 2676.60 ± 0.94[e]	210 : 2841.94 ± 2.71[c]		
	80 : 2176.68 ± 1.08[g]	240 : 2488.49 ± 0.40[d]		

Step 2 : Central Composite Design on Face					
Run	X_1- Ethanol concentration (% v/v)	X_2- Irradiation time (s)	*X3*- MW power (W)	TPC yield (mg GAE/100 g)	
				Experimental	Predicted
1	-1(20)	-1(90)	-1(400)	2198.75	2381.91
2	1(60)	-1(90)	-1(400)	2363.07	2039.97
3	0(40)	0(150)	-1(400)	1838.81	1236.04
4	-1(20)	1(210)	-1(400)	2370.89	2336.14
5	1(60)	1(210)	-1(400)	3192.49	3969.94
6	0(40)	-1(90)	0(500)	1987.48	2140.64
7	-1(20)	0(150)	0(500)	2887.32	2990.34
8	0(40)	0(150)	0(500)	2871.67	2611.69
9	0(40)	0(150)	0(500)	2425.66	2611.69
10	0(40)	0(150)	0(500)	2668.23	2611.69
11	1(60)	0(150)	0(500)	5633.80	5124.93
12	0(40)	1(210)	0(500)	2699.53	3082.73
13	-1(20)	-1(90)	1(600)	2261.35	2084.18
14	1(60)	-1(90)	1(600)	4084.51	4719.55
15	0(40)	0(150)	1(600)	2230.05	2426.96
16	-1(20)	1(210)	1(600)	2112.68	2038.41
17	1(60)	1(210)	1(600)	7230.05	6649.52

Moreover, microwave power significantly influenced the TPC yield as shown in Table 19, the yield increased with increasing microwave power from 200 to 500 W and then decreased for higher powers, the best yield was at 500 W (3051.72 ± 0.13 mg GAE/100 g) which was selected for the last single-factor trials, while the range of 400–600 W was selected for the RSM study. Several reports have demonstrated the main effect of microwaves, and in many cases the only, is the heating effect **[65, 93].**

Finally, the TPC yield increased significantly from 2254.30 ± 1.35 to 3053.20 ± 2.71 mg GAE/100 g (Table 19) with the increase of solvent-to-solid ratio up to 30 mL/g. This result

could be related to the quantity of solvent in which the vegetable matrix powder is immersed must be sufficient in order to have a good amount of extraction of the TPC, this is generally due to the effect of the microwave heat which accelerates the diffusion of the solvent **[7].** The TPC yield in excess to 30 mL/g was non significant. Thus, this we are referred to the screning study and other works focused on the extraction of herbs by microwaves and 30 mL/g was choosed for the RSM trials. A quadratic model fitted to the TPC recovery from a jujube seeds data involving X_1- ethanol concentration (%), X_2- irradiation time and X_3- MW power is represented in Table 20.

Table 20 : Regression coefficients estimation and their *p*-values for the reduced fitted quadratic model for microwave assisted ethanol extraction processing of phenolic compounds from jujube seeds.

Parameter[a]	Estimated coefficients	Standard error	DF[b]	Sum of squares	F-value	Prob > F
Model B_0	2611.69	207,0626	9	30706273	14,5702	<0,0001*
Linear						
X_1- Ethanol	1067,2926	153,0239	1	11391136	48,6462	0,0001*
X_2- Time	471,04851	153,0239	1	2218867	9,4757	0,0193*
X_3- Power	595,46166	153,0239	1	3545746	15,1422	0,0058*
Quadratic						
X_1^2	1565,3861	295,633	1	6565312	28,0374	0,0010*
X_2^2	-351,6718	295,633	1	331351	1,4150	0,2730
X_3^2	-660,7485	295,633	1	1169728	4,9954	0,0291*
Interaction						
X_1X_2	493,93584	171,0859	1	1951781	8,3351	0,0257*
X_1X_3	744,32707	171,0859	1	4432182	18,9278	0,0030*
X_2X_3	249,41315	171,0859	1	497655	2,1253	0,1882
Lack Of Fit			5	1539423,7	6,1752	0,1452
Pure Error			2	99717,3		
Total Error			7	1639141,0		
R^2					0,949324	
R^2 Adjusted					0,884169	
CV%	6.21					
RMSE	483,9039					
Corr.Total			16	32345414		

The model is statistically highly significant ($p < 0.01$) with a *p*-value of 0.0002, while a lack of fit *p*-value of 0.1344 is non-significant, a strong correlation between the observed and predicted values are observed by the coefficient of determination ($R^2 = 0.92$) and adjusted

determination coefficient (R^2_{Adj} = 0.86) which were reasonably close to 1, the residuals were distributed randomly and non-patterned therefore the overall model indicating a good deal of reliability of the input response and output factor.

Table 20 provides the ANOVA analysis of all significant coefficients for the reduced model given in Eq. (13).

$$Y = 2611.69 + 1067.29X_1 + 471.05X_2 + 595.46X_3 + 493.93X_1X_2 + 744.33X_1X_3 + 1445.95X_1^2 - 780.19X_3^2 \quad (13)$$

A quadratic model fitted to $Y_{TPCyield}$ data showed that linear terms for all independent parameters were statistically significant ($p < 0.05$) and only ethanol concentration and irradiation time ($p = 0.0257$) as well as ethanol concentration and MW power ($p = 0.0030$) showed significant interaction. At same time, the quadratic terms of ethanol concentration and MW power had a significant effect on TPC yield (Table 20). The desirability function was used in the prediction profiler to find optimal factor settings and indicated an extraction efficiency of 6649.52 mg GAE/100 g.

The influence of the process parameters and the mutual interactions on the TPC yield is depicted by the three dimensional (3D) response surface graphs as shown in Figure 35. The recovery of TPC extracted from *Zls* is increased with increasing of ethanol concentration gradually from 40% to 60% at longer irradiation time (210 s) to reach a value of 8000 mg GAE/100 g (Fig. 35A). Thus, increasing of exposition to irradiation time affect positively the efficiency of extraction yield as its linear effect that was highly significant. The extraction of TPC from jujubes using microwaves could be enhanced using ethanol in water over a limited range that facilitate the increase of the contact surface between the solvent and vegetable material which results an increase in extraction yields **[10, 93].**

Additionally, increasing ethanol/water favorite the extraction of more phenolic groups than non-phenolic contents mainly carbohydrates and terpenes. While, from structure plan, some phenolic compounds extracted from plant materials were found to be in complexing forms when are only extracted with organic solvents, for instance, ethanol and methanol as shown by Kamarudin, Esa **[94].** Furthermore, Fig. 35B showed clearly that increase in ethanol concentration from 20% to 60% causes the increase of TPC in few minutes at a slightly increase of microwave power to 600 W from 1500 to around 3000 mg GAE/100 g. After that, additional microwave power causes negative effect and decreased the yield to 2000 mg GAE/100 g after 600 W, as its significant quadratic negative effect illustrated in Table 3.

These results confirm those of single factors experiments illustrating the remarkably significant positive interaction effect between ethanol X_1 and X_3 (**Table 20**).

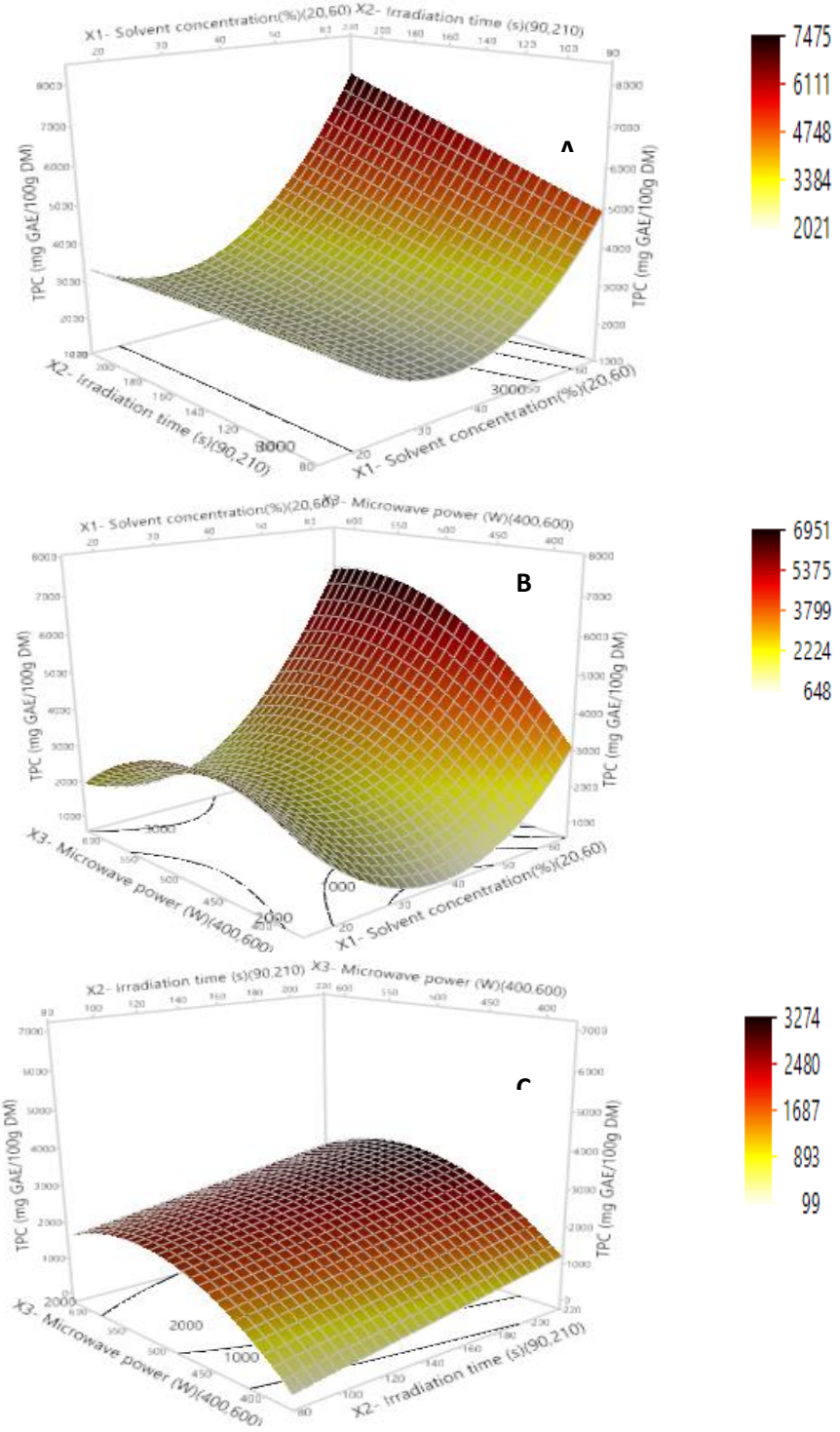


Figure 35 : Response surface plots for the total phenolic recovery from jujube seeds extracted by microwave extraction method with respect to ethanol concentration and irradiation time (A); ethanol concentration and microwave power (B); microwave power and irradiation time (C).

Higher microwave power decrease the viscosity of extraction solvent and enhances the diffusion rate of soluble phenolic compounds due to higher heat activation in the extraction process. However, increasing in mass transfer phenomena up to a certain power value (in our case > 600 W) can lead to thermal degradation of some bioactive compounds **[9, 94].** Moreover, in present work, no significant interaction between irradiation time and MW power was found ($p > 0.05$),but its significant contribution to the extraction efficiency was observed only in their linear terms (Fig. 35C and Table 20). This study suggested clearly that phenolic compounds extracted from *Zls* using MAE are mainly depended on the ethanol concentration as its linear, quadratic and interaction effects with MW power and time confirming the preliminary experiment results. In meantime, the obtained results are satisfactory by founding ethanol as best solvent giving higher extraction TPC yield which is in agreement with other works demonstrating the use the ethanol as green solvent to enhances the bioactive compounds extracted from plants which is considered to have low toxicity than other organic solvents and permitted to be used in food additives **[94].**

Optimal conditions for yield optimization were 60% ethanol, 210 s and 600 W with a predicted TPC yield of 6649.52 mg GAE/100 g. MAE was carried out at these optimal conditions obtaining a TPC yield of 6709.01 ± 2.20 mg GAE/100 g, close to the value predicted by the model.

The recovery of TPC extracted with microwaves through RSM optimization study showed a significantly higher value than those obtained from conventional extraction method (CE) of jujube seeds using ethanol 70% (5100.21 ± 0.20 mg/100 g) **[95].** Additionally, TPC value extracted from Spanish jujube fruits using methanol ranged between 1442 and 3432 GAE mg/100 g is significantly lower than obtained results **[25].** Simillarly, our samples revealed a higher TPC values than those obtained from Chinese jujubes ranged from 454.3 to 1298.9 GAE mg/100 g Zhao, Zhang **[31].** Furthermore, the TFC of *Zls* extracts using MAE (Table 21) was significantly higher than those obtained from other jujubes species (499.21 ± 0.59 mg QE/100 g), in comparison to TFC reported from *Zizyphus jujuba* seeds using ethanol 70% by CE (200.0.1 ± 0.15 mg/100 g) **[95].** Similarly, from two different Chinese jujubes that were around 122.1 to 319.5 and 65.1 to 158.6 mg/100 g, respectively **[31, 96].** More recently, TFC of *Zj* fruits showed a significantly lower content than ours at different stage of ripening, ranging from 26.7 to 48.5, 19.9 to 34.6 mg QE/100 g in 'Ya Tsao', 'Ta-Jan Tsao' cultivars, respectively **[26].** While, TTC of *Zls* was for 25000.74 ± 1.28 mg CE/100 g which was very higher in comparison to polymeric proanthocyanidins extracted from *Zizyphus jujuba* fruits

obtained by Wojdyło, Carbonell-Barrachina **[25]** that showed a value between 939 and 2548 mg/100 g in depends on cultivars. In addition, Gao, Wu **[97]** compared proanthocyanidin extracts from several *Zizyphus* varieties, among those containing the highest content about 413.7 ± 23.1 mg/100 g from *Zizyphus jujuba cv. Zaowangzao* which is significantly lower than our extracts.

Table 21 : Phenolic yields of jujube seeds samples and some biological activities under optimum extraction conditions.

Factors	Microwave extraction
Irradiation time (s)	210
Ethanol concentration (%)	60
Microwave power (W)	600
Solvent solid/ratio (mL/ g)	30
Results	
Recovery of total phenolic TPC[a] (mg GAE[b]/100 g)	6709.01 ± 2.20
Recovery of total flavonoids TFC[c](mg QE[d]/100 g)	499.21 ± 0.59
Recovery of condensed tannins TTC[e] (mg CE[f]/100 g)	25000.74 ± 1.28
DPPH[g] scavenging EC_{50} (mg/mL)	0.00006 7 ± 0.00
FRAP[h] (mg GAE/100 g)	2039.60 ± 8.43
AChE[i] assay IC_{50} (mg/mL)	0.88 ± 0.02
HMGR[j] assay (%) (for 100 µg/mL)	28.71

The variation results in TPC, TFC and TTC from our study using MAE and others compared in literature is related to many factors such as family, geographical conditions, varieties of plants, extraction method and environemental effects **[31, 65, 98, 99].** This study provides a successful RSM optimization study of polyphenols extraction conditions from *Zls* with combination to microwave process instead of traditional extraction methods which is a crucial step for an industrial giving a greater recovery of phenolic compounds in less time and solvent consumption due to its heating microwave energy. In addition, it is more important that it contribute to valorization of natural recoursses that is more economic for them and will be more healthy for us due to its protection to the environment and its uses to green method without toxicity effect. Results are in accordance with other research data demonstrating the advantageous of microwaves on the extraction of phytochemicals from plant matrix **[7, 100-102].** Howerver, it will be more interesting to know the different phytochemicals present in the *Zls* extracts and evaluation each of them are responsible for some biological activities studied in this work.

II.1.2. Identification of bioactive compounds from *Zls* extracts

The content of bioactive compounds extracted from *Zls* pleonasm obtained by LC-MS/MS analysis in the negative and positive mode (Figure 36) were demonstrated in Table 22. The identification of phytochemicals was carried out using Data Analsis program from Bruker, and pubchem, metlin and others references on the *Zizyphus* genus were used to find the chemical structures. Results indicated the presence of 47 biomolecules based on the exact mass and on the fragmentation patterns. The heatmap was made to compare the intensities of identified compounds and to visualize the most higher amount as shown in Figure 36.

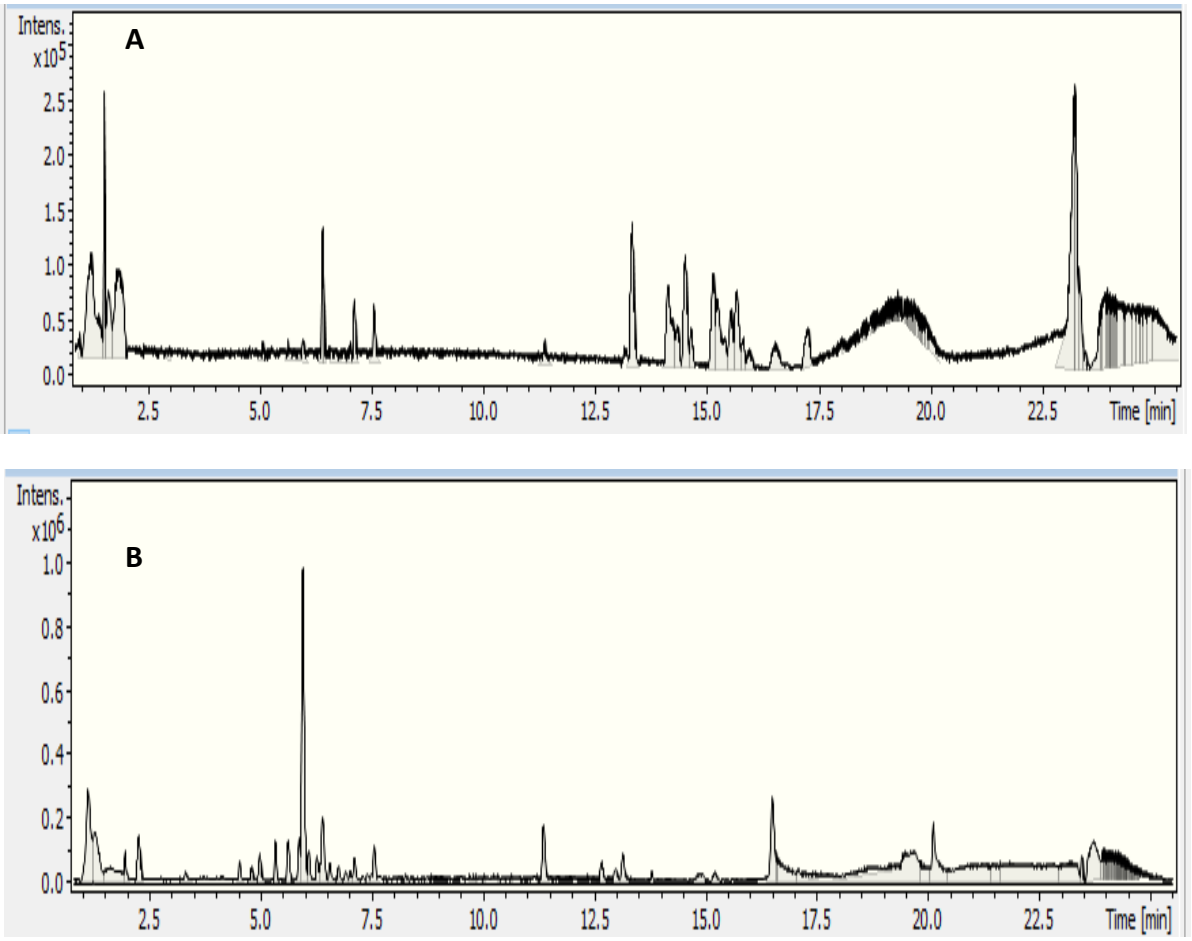


Figure 36 : Total ionic chromatogram and ionic chromatograms extracted from ions identified in *Z. lotus* seeds samples : (A) LC-MS/MS (-) and (B) LC-MS/MS (+).

a. Secondary metabolite

Like all seeds found in vegetable plants, *Zls* extracts have a high content of primary metabolites by comparing the secondary one, but these last are not negligeable given their beneficial effect on human health. The results obtained during the screening phytochemical by

khnowing their intensities (Figure 36) showed that the secondary metabolite are 39.62% of all compounds detected in *Zls* samples. It seems that 6-gingerol being the most abundant compound as demonstrated in dark color and found to be around 24.71% of all secondary metabolites. This compound is originated from *Zingiber* and found to exercise a significant biological potential such as anticancer, antioxidant and anti-Alzheimer effects **[103, 104].** This compound was previousely detected from jujubes **[105-108].**

Compound with a $[M-H]^-$ ions at m/z of 701.1780 was detected at 1.44 min and tentatively assigned as paenoside A **[109].** This compound was detected from *Delphinium staphisagria* that showed a significant antiproliferative activity against Trypanosoma cruzi (epimastigote, amastigote, and trypomastigote forms) **[110].** However, compound detected at 1.04 min ($[M+H]^+$) was assigned as biflavonoid at m/z of 543.1253. This compound was assigned as chamaejasmin. This compound was detected from other plants mainly *Stellera chamaejasme* L which showed a very good antioxidant and anticancer effects **[111, 112].** This is the first time detecting the presence of this flavonoid in *Zizyphus* genus. Some flavanones were detected at different retention times 1.03 and 7.55 min with negative mode at m/z of 711.2074 and 433.1546, respectively. These compounds were tentatively identified as glucoliquiritin apioside and 5,6,7,8,3',4',5'-heptamethoxy flavanone, respectively. To the best of our knowlege, this is the first report identifing these compounds in *Zizyphus* genus. Compound at R_t = 1.49 min was observed as isoflavanone at a positive mode with m/z of 435.1288 and was tentatively identified as robustaside D. This compound was detected previously from other plants and was found to excercise antimalarial, antioxidant and antitumor activities against human breast cancer cells from *Peganum harmala L* seeds **[113].** However, peaks at 1.60, 1.75 min showed a deprotonated molecule with m/z of 431.1348 and 601.1195 with different ion fragments was tentatively identified as medicarpin 3-*O*-glucoside, Cicerin-7-(6-malonylglucoside), respectively. These compounds were deduced for the first time in *Zizyphus* genus **[114, 115].** Peaks at R_t of 1.46, 7.03 and 7.58 min with $[M+H]^+$ and $[M-H]^-$ ions at m/z of 345.0988, 813.2084, 531.1201 were tentatively assigned as 5,7-dihydroxy-8,3',5'trimethoxy-flavone, luteolin 7-(6""-acetylallosyl-(1->3)-glucosyl-(1->2)-glucoside and apigenin-7-methyl ether 5-(6"-malonylglucoside), respectively. These compounds were reported for first time in *Zizyphus* genus. However, two known flavones were characterized in this study. Peak at 5.62 and 7.10 min with m/z of 595.1579($[M+H]^+$), 753.1895 ($[M-H]^-$) were assigned as kaempferol-3-O-robinobioside, Kaempferol 3-[6"-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside. Only kaempferol-3-O-robinobioside was

previousely detected in jujube plant **[25].** Furthermore, gallocatechin was the only flavan-3-ols detected at R_t = 1.99 min with m/z 307.0791 at postive mode as privously found in Algerian *Z. lotus* and other *Zizyphus* species from leaves and fruits **[40].**

Figure 37 showed clearly that our samples constitue a significant flavonoids part which are 18% of all metabolites and 50% of all secondary metabolite. *Zls* is one of the used part of jujubes for the treatment of several diseases due to their high content of phenolic compounds especially flavonoids. These were found to be one of the most known substances for their antibacterial, antioxidant and antifungal effect agents **[116, 117].** Peak at 6.49 min showed a deprotonated molecule with m/z of 529.242 and was tentatively assigned as scuterivulactone A. This diterpenoid was never been detected from jujubes and it is known to excerse a good antiinflammatory and anticancer effects from other plant material **[118, 119].** Morever, one iridoid glucoside was detected at 7.57 min with m/z of 469.1315 and tentatively identified as plumieride **[120].** Serveral plumieride derivative were detcted from other *Zizyphus* species and found as an efficient antioxidant agent **[121, 122].** In addition, two sesquiterpenes were observed at 15.45, 18.19 with m/z of 397.2217, 253.2133 in negative mode. These compounds were assigned as stephanol and geranyl ethyl butirate, respectivly **[123].** Compound at R_t = 16.53 min, which showed an m/z of 449.2965 in postive mode was tentatively identified as tenasogenin. From Figure 37 we observed clearly that terpenoids are present with high intensities in *Zls* extracts and arround 19% of all secondary metabolites in which tensagenin are 68% of all terpenoids, this is never been detected from other *Zizyphus* species. This compound was very used due to its antidiabetic effect and identified as a pregnane ester from *Marsdenia tenacissima* **[124, 125].**

The analysis showed the detection of two secoiridoids with a deprotonated molecular having the same formula ($C_{26}H_{30}O_{13}$) at 1.01 and 1.71 min with a m/z of 549.1574, these compouns were isotopes and identified tentatively as jasminoside. This compound is detected for a long time from *Jasminum primulinum Hems* **[126].** However, compound with $[M-H]^-$ molecualr ions with m/z 387.1092 at R_t = 1.09 min was assigned tentitatively as 6-methyl-2-O-glucoside xanthone **[127].** For instance, no data detected the presence of these compounds from *Z. lotus*. Morever, peak observed at 17.11 with m/z of 319.2797 $(M+H)^+$ was attributed to quinoline derivative. Several works were previously detected quinoline alkaloids in some *Zizyphus* species which is one of most known as antioxidant and anti-aging agent **[128, 129].**

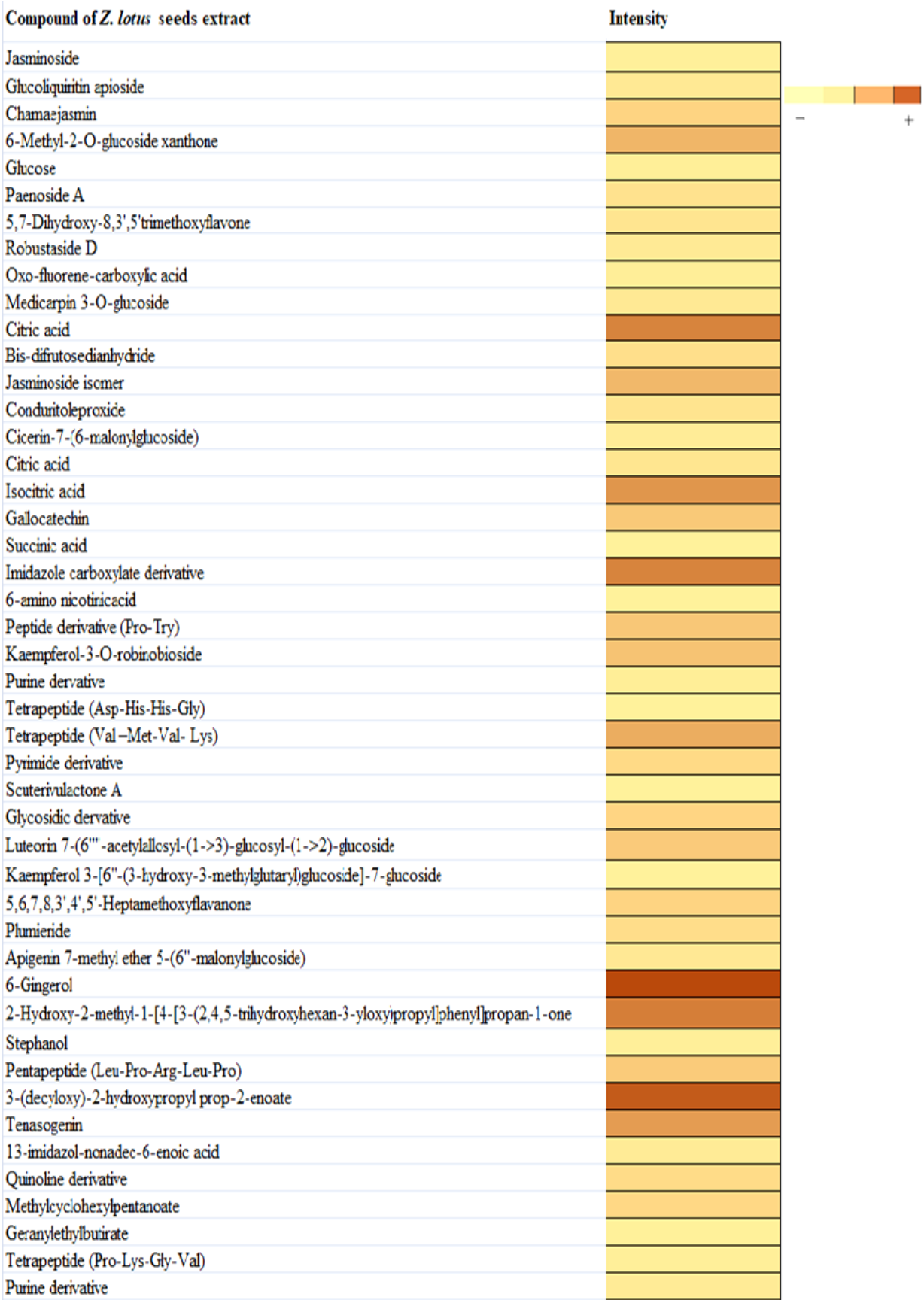


Figure 37 : Heatmap of the chemical profile and of *Z. lotus* seeds samples. Mean values refer to colors from minimum displayed in beige to maximum represented with dark brown.

b. Primary metabolite

Figure 37 showed that the primary metabolite contain arround 56% of all compounds found in our samples. It has been noticed that 3-(decyloxy)-2-hydroxypropyl prop-2-enoate was found as the major abundant compound with 16% of all primary metabolites. This compound was never been reported from *Z. lotus*. Thus, it is used under another structure of 2-propenoic acid from jujubes and showed a significant effect as antioxidant, antiinflammatory and anticancer against breast cell lines **[130, 131].** In addition, peptides are present with high intensities in comparison to other primary substances with 13% of *Zls* extracts and 23% of all primary metabolites. *Zls* exctrats have been previously found with high protein content (19.11%) which is known by its functional properties (emulsifying activity, foaming capacity, emulsifying stability, water retention and solubility) and nutritional value **[132].** Therefore, small peptides are found from *Z. jujuba* seeds as most potentially valuable compound which play an important antioxidant, anticholinesterase roles that can be used as functionnal food **[133, 134].** The examination of chromatograms obtained at negative mode indicated the presence of some sugars at 1.12, 6.92 min with m/z of 179.0529, 539.2965 were clearly identified as glucose and glycosidic derivative **[135].** Furthermore, peak observed at 1.67 min with m/z of 325.1090 $(M+H)^+$ was tentatively attributed to bis-D-frutose dianhydride, this compound is considered one of rare class of spirodisaccharides and found often in plants and isolated from microorganisms **[136].** The mass spectrometric characterization of compounds observed at 4.98, 6.29 and 16.49 with m/z of 305.1524, 271.1265, 595.4093 $(M+H)^+$ were identified tentatively as dipeptide, pyrimide derivative and pentapeptide. Others small peptides were identified tentatively at retention times of 6.05, 6.09, 6.66 and 18.60 min with m/z of 465.1878, 476.3003, 573.2665 and 469.2778 at positive and negative modes were as tetrapeptides with different molecular formula (Table 22).

The mass spectrometric characterization of compound at 1.16, 1.95 and 1.98 min with m/z of 191.0168, 191.0169 and 191.0167 were isomers and identified as citric acid and isomers, respectively. In addition, peaks observed at 1.52, 2.03 min displayed a deprotonated molecular ions at m/z of 223.0426 and 117.0163 were identified as oxo-fluorene-carboxylic and succinic acids, respectively. These organic acids were detected previously from jujube plant and other herbs and showed a good biological activities **[25, 137].** Additionaly, two peaks were observed at 5.98 and 19.36 min with m/z of 376.1267, 355.1531 with $C_{16}H_{19}N_5O_6$ and $C_{17}H_{20}N_6O_3$, respectively at negative mode were assigned as purine derivative.

Table 22 : Phytochemical profile of *Z. lotus* seeds extracts under optimal conditions. Peaks with a minus/plus superscript were analyzed in negative/positive mode.

R_t (min)	[M - H]- / [M + H]+[c]	Fragments ion (m/z) ; intensity(%)	Error (mg/kg)	Molecular formula	Tentative identification	References
1.01-	549.1574	179.0541(75.4) ; 221.0647(12.4) ; 504.1554(21.1) ; 323.098 (16.8) ; 324.0945(17.8)	7.2	$C_{26}H_{30}O_{13}$	Jasminoside	985381(metlin)
1.03-	711.2074	665.2041(100) ; 101.0210(17.9) ; 221.0640(14.2) ; 383.1144(19.2)	9.5	$C_{32}H_{40}O_{18}$	Glucoliquiritin apioside	95577(metlin)
1.04+	543.1253	365.1016(4.2)	5.9	$C_{30}H_{22}O_{10}$	Chamaejasmin	985030 (metlin)
1.09-	387.1092	89.0206(100) ;179.0536(48.3) ; 161.0434(22) ; 101.0211(20.06)	-2	$C_{20}H_{20}O_8$	6-Methyl-2-O-glucoside xanthone	Kulula-Koch et al., 2015 ; Pinto et al (2005)
1.12-	179.0529	44.9952(100) ; 96.9567(90.8)	3.2	$C_6H_{12}O_6$	Glucose	5793 (pubchem)
1.44-	701.1780	665.2015(100) ; 383.1141(20.1)	-8.2	$C_{33}H_{34}O_{17}$	Paenoside A	Pubchem
1.46+	345.0988	85.0270(100) ; 145.0478(59.6) ; 183.0466(50.0) ; 127.0376(50.8) ; 264.0729(44.3)	-5.6	$C_{18}H_{18}O_7$	5,7-Dihydroxy- 8,3',5' trimethoxy-flavone	49760 (metlin)
1.49+	435.1288	353.0869(100) ; 264.0739(74.6) ; 145.0463(34.3) ; 191.0373(13.5) ; 305.0674(23.9)	-0.5	$C_{21}H_{22}O_{10}$	Robustaside D	38358972 (pubchem)
1.52-	223.0426	72.9890(100) ; 129.0160(91.2) ; 180.9930(13.7)	11	$C_{14}H_8O_3$	Oxo-fluorene-carboxylic acid	33295 (metlin)
1.60-	431.1348	179.0524(6.4) ; 119.0314(3.5); 90.0321 (2.6); 89.0207(100)	-0.1	$C_{22}H_{24}O_{09}$	Medicarpin 3-O-glucoside	48094 (metlin)
1.61-	191.0168	87.0050(100)	9.7	$C_6H_8O_7$	Isocitric acid	572279 (metlin)
1.67+	325.1090	85.0272(100) ; 163.0578(19.9) ; 115.0380(6.1)	12	$C_{12}H_{20}O_{10}$	Bis-difructose dianhydride	66164 (metlin)
1.71-	549.1574	503.1534(100) ; 179.0530(64.7) ; 221.0632(25.6)	-3.5	$C_{26}H_{30}O_{13}$	Jasminosideisomer	985381(metlin)
1.72+	163.0578	85.0271(100) ; 55.0158(34.7)	14.0	$C_6N_{10}O_5$	Conduritoleproxide	2859 (pubchem)
1.75-	601.1195	96.9564 (100) ; 171.9425 (0.6)	0.7	$C_{26}H_{26}O_{15}$	Cicerin-7-(6-malonylglucoside)	74413691 (pubchem)
1.95-	191.0169	87.0051(100) ; 111.0047(54) ; 121.0257(8.3)	7.2	$C_6H_8O_7$	Citricacid	643187 (pubchem)
1.98-	191.0167	87.0051(100) ; 111.0047(54)	10.09	$C_6H_8O_7$	Isocitric acid	98259 (pubchem)
1.99+	307.0791	84.0430(100) ; 130.0482(30.4) ; 177.0300(20.9) ; 231.0394(18.0) ; 235.0185(9.5)	6.9	$C_{15}H_{14}O_7$	Gallocatechin	47219 (metlin)
2.03-	117.0163	44.9945(100)	16.5	$C_4H_6O_4$	Succinic acid	1110 (pubchem)
2.23+	254.1582	195.1102(100) ; 125.0692(13.2)	11	$C_{11}H_{19}N_5O_2$	Imidazole carboxylate derivative	575448 (metlin)
4.52+	139.0492	56.9632(100) ; 121.0383(20.8) ; 113.0359(13.9)	5.7	$C_6H_6N_2O_2$	6-amino nicotinicacid	18496(pubchem)
4.98+	305.1524	242.0537(1.6) ;127.9730(1.1) ; 292.4428(1.2)	-21.6	$C_{16}H_{20}N_2O_4$	Peptide derivative (Pro-Try)	938763 (pubchem)
5.62+	595.1579	325.0670(100) ; 457.1064(88.3) ; 337.0664(72.1) ; 459.1094(5.4)	13.0	$C_{27}H_{30}O_{15}$	Kaempferol-3-O-robinobioside	Wojdyło, Carbonell-Barrachina et al. (2016)
5.98-	376.1267	224.0446(34.1) ; 325.1276(100) ;	-1.2	$C_{16}H_{19}N_5O_6$	Purine derivative	62883 (metlin)
6.05+	465.1878	407.1842(16.4) ; 349.1772(12.4) ; 293.8551(7.4) ; 232.0459(7.7)	7.9	$C_{18}H_{24}N_8O_7$	Tetrapeptide (Asp-His-His-Gly)	47776 (metlin)
6.09+	476.3003	45.0328(100) ; 133.0839(29.5) ; 177.1091(22.2)	2.2	$C_{21}H_{41}N_5O_5S$	Tetrapeptide (Val –Met-Val-Lys)	144183810 (pubchem)
6.29+	271.1265	45.0327(47.4) ; 56.9641(21.7) ; 100.99(10.2) ; 249.1139(43)	12.7	$C_{12}H_{18}N_2O_5$	Pyrimide derivative	13845019 (pubchem)

6.49-	529.2421	471.2401(26.1) ;485.2521(8.3)	4.2	$C_{29}H_{38}O_9$	Scuterivulactone A	101661883 (pubchem)
6.66-	573.2665	515.2652(19.7) ; 101.0213(8) ; 471.2431(6.8)	-2	$C_{27}H_{38}N_6O_8$	Tetrapeptide (Trp-Gln-Glu-Ile)	252746 (metlin)
6.92+	539.2965	137.0617(0.7) ; 303.5529(0.6) ; 379.4403(0.6) ; 189.1038(0.6)	-0.37	$C_{27}H_{42}N_2O_9$	Glycosidic derivative	67816437 (pubchem)
7.03-	813.2084	292.0336(24.8) ; 307.0574(22)	2.1	$C_{35}H_{42}O_{22}$	Luteorin 7-(6""-acetylallosyl-(1->3)-glucosyl-(1->2)-glucoside	49148 (metlin)
7.10-	753.1895	145.0269(33.8) ; 292.0335(26.2) ; 161.0572(20.3) ; 307.0588(18.7) ; 205.0479(11.7)	-2.8	$C_{33}H_{38}O_{20}$	Kaempferol 3-[6"-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside	50223 (metlin)
7.55-	433.1546	91.0364(100) ; 249.0628(84.3) ;	-9.7	$C_{22}H_{26}O_9$	5,6,7,8,3',4',5'-Heptamethoxy flavanone	53165 (metlin)
7.57-	469.1315	249.0634(100)	6.8	$C_{21}H_{26}O_{12}$	Plumieride	67941 (metlin)
7.58-	531.1201	96.9564(100) ; 79.9527(2.5) ; 98.9529(4.5)	-10.7	$C_{25}H_{24}O_{13}$	Apigenin-7-methyl ether 5-(6"-malonylglucoside)	49440 (metlin)
13.32-	265.1446	96.9564(100)	2.9	$C_{15}H_{22}O_4$	6-Gingerol	263539 (metlin)
15.13-	353.1956	94.9770(1.1), 83.9551(0.2) ; 95.9490(16.9)	3.8	$C_{19}H_{30}O_6$	2-Hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy)propyl] phenyl]propan-1-one	138525818(pubchem)
15.45-	397.2217	96.9566(30.2) ; 325.1814(13)	-2.2	$C_{21}H_{34}O_7$	Stephanol	101289689 (pubchem)
16.49+	595.4093	309.1993(100) ; 310.2031(19.2)	-28	$C_{28}H_{50}N_8O_6$	Pentapeptide (Leu-Pro-Arg-Leu-Pro)	264807(metlin)
16.52+	287.2178	69.0689(100) ; 111.1152(47.2) 71.0482(9) ; 55.0533 (22.8)	5.0	$C_{16}H_{30}O_4$	3-(decyloxy)-2-hydroxypropyl prop-2-enoate	Benabderrahim, Elfalleh [32] 920161 (metlin)
16.53+	449.2965	69.0690(100) ; 111.1154(49.3) ; 271.1710(19.9) ; 204.063 (3.9) ; 145.050(18.9) ; 163.061 (18.7)	-15	$C_{26}H_{40}O_6$	Tenasogenin	101277354 (pubhem)
17.06+	363.3061	45.0328(100) ; 133.0838(14.6) ; 251.0399(6.0) ; 71.050 (26.3) ; 85.029 (8.1) ; 94.042(6) ; 177.092(5.3)	-12.1	$C_{22}H_{38}N_2O_2$	13-Imidazol-nonadec-6-enoic acid	123673046 (pubchem)
17.11+	319.2797	45.0325(100) ; 89.0586(41.1) ; 151.0924(15.3) ; 71.013 (31) ; 55.018(14.8)	-16.9	$C_{20}H_{34}N_2O$	Quinoline derivative	100310492 (pubchem)
17.23+	199.1663	69.0691(100) ; 55.018 (29.9) ; 56.026 (3.1); 68.026 (2.8)	14.5	$C_{12}H_{22}O_2$	Methylcyclohexyl pentanoate	694618 (metlin)
18.13-	253.2133	99.9219(100) ; 116.9321(50.6) ; 112.9846(33.1)	11.4	$C_{16}H_{28}O_2$	Geranylethyl butirate	6437648(pubchem)
18.60-	469.2778	96.9566(46.6) ; 95.9489(12.7) ; 79.9730(3.7) ; 116.9267(3.1)	0	$C_{21}H_{38}N_6O_6$	Tetrapeptide (Pro-Lys-Gly-Val)	202956 (metlin)
19.36-	355.1531	99.9220(100) ; 116.9251(98.2) ; 115.9164(19.5) ; 343.1484(1.9)	1	$C_{17}H_{20}N_6O_3$	Purine derivative	421671 (metlin)

Compounds observed at 1.72, 15.13, 16.52 and 17.23 min in positive mode with m/z of 163.0578, 353.1956, 287.2178, 199.1663 were assigned as conduritol peroxide, 2-hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy) propyl] phenyl]-propan-1-one, 3-(decyloxy)-2-hydroxypropyl acryate and methylcyclohexyl pentanoate. However, other peaks obtained with $(M+H)^+$ showed the presence of some compounds with retention times of 2.23, 4.52 and 17.06 min with m/z of 254.1582, 139.0492, 363.3061, respectively were identified as imidazole carboxylate derivative, 6-amino nicotinic and 13-imidazol-nonadec-6-enoic acids,

respectively **[138].** All these compounds were reported for the first time from jujubes and can participate in the studied activities what we have noticed in the Figure 37.

II.1.3. Effect of MAE on biological ativities

Due to the presence of this type of compounds as they are reported to have biological actvity, the antiioxdant activity and the inhibiiton of AChE and HMGR were looked for.

II.1.3.1. Determination of DPPH· and FRAP activities

The antioxidant activity was evaluated with DPPH· radical scavenger as shown in Table 21. The *Zls* extracts that exhibited high content of phenolic compounds with a remarkably significant antioxidant effet with EC_{50} value of 0.67 ± 0.00 µg/mL. In comparison with other *Zizyphus* species, Choi, Ahn **[139]** obtained from Korian *Z. jujuba* seeds (mechu and sanzoin) a lowest DPPH· activity than obtained results (0.3 and 0.1 mg/mL respectively). Simillarly, Ghazghazi, Aouadhi **[56]** demonstated DPPH· Activity of 0.31 ± 0.005 mg/mL from *Z. lotus* fruits extracts which is lower than ours from seeds. Our results gave higher antioxidant effects than results obtained by Hammi, Jdey **[57]** that reported an EC_{50} of 0.28 mg/mL for Tunisian *Z. lotus* pulp and peel extracts using UAE. Regarding the iron-reducing power FRAP assay for the *Zls* extracts which is used as one direct method for determination of antioxidant effect from jujubes. The results showed a significant value of 2039.60 ± 8.43 mg GAE/100 g. Our finding are significantly higher than those of Wang, Cheng **[58]** (471.6 ± 30.8 mg CE/100 g) obtained from *Z. jujuba cv. Zaowangzao*. The antioxidant capacity of *Z. lotus* extracts have also been evaluated in previous works **[57, 99, 140, 141].**

Morever, we can suggest that the solvent found in this sutdy have strong effect on antioxidant activity wich is in agreement to Kim and Son **[95].** This activity value is depending on the phenolic compounds contain in the extracts, as well as the extraction method. As demonstarted by Wojdyło, Carbonell-Barrachina **[25],** Dzoyem and Eloff **[142],** the presence of flavonoids, such as flavan-3-ols and flavonols in jujube extracts and particularly hydroxyl group position can act as proton donating and contribute to increasing radical scavenging activity. In addition to the presence of others phytochemicals mainly 6-gingerol and other small peptides which probably have a good antioxidant potential. Furthermore, it has been reported that the high antioxidant power should be attributed to the effect of microwaves on the structure of the cell of extracts due to the increase of internal pressure and temperature **[7, 65, 93].**

II.1.3.2. Determination of AChE inhibitory activity

The inhibition of acetylcholinesterase (AChE) is very used against several neurological disorders such as Alzheimer's disease, senile dementia, ataxia, myasthenia gravis **[143]** and severe constipaption **[144].** Several plants have been reported as having AChE inhibitor activity which was attributed to the presence of mainely phenolics compounds and alkaloids and some primary metabolites **[142].** The *Zls* extracts were analysed to determine their ability as acetylcholinesterase inhibitors (Table 21). The study shows that our samples obtained at different concentrations diluted by a factor of 10 exhibited a significant inhibitory activity (0.88 ± 0.02 mg/mL). These results are in accordance to those found by Hernandez, Falé **[145]** from *Hypericum* (*Hp_2*) extract that showed an IC_{50} value of 0.88 ± 0.08 mg/mL using water for decoction preparation. In the meantime, the same autors demonstrated that other *Hypericum* species (*$Hp_,$ $Hp_3,$ Hp_4* and *Hp_5*) obtained in a local supermarket from Portugal showed a lowest AChE activity than *Zls* extracts varying from 0.99 ± 0.12 to 1.79 ± 0.34 mg/mL in which some flavonoids were found to be responsible for the AChE activity. In addition, *Globularia alypum* extracted from leaves which show an IC_{50} value of 0.82 ± 0.05 mg/mL **[146].** which is in the same range as *Zls* extracts. Morever, the *Zls* extract had a highest significant AChE inhibitory potential than that reported from *Zizyphus oxyphylla* extracts with an IC_{50} value of 9.58 ± 0.08 mg/mL using *n*- butanol which exhibited a maximum inhibitory effect than other soluble fractions mainly *n*- hexane from the same sample (165.15 ± 0.94 mg/mL) **[147].** This indicates that *Zls* extracts obtained using ethanol as extraction solvent exhibited a best inhibition of this enzyme which could be used against Alzheimer's disease or alleviation of severe constipation.

The obtained results showed that MAE extracts contained some level of inhibitory activity against AChE, this may suggest that extraction method were able to extract more active compounds than other extraction methods as seen in previous part with possible AChE inhibitory activity. It could be postulated that *Zls* extracts inhibited AChE activity might be due to phenolic compounds. LC-MS/MS analysis revealed that *Zls* represents a rich sources of phenolic compounds beside of other secondary metabolites and small peptides found with high intensities Figure 37 which may contribute also to AChE inhibitory activity, and this is precisely where the interest of this study arises in order to have a global idea of the various compounds considered be responsible for this activity, where other future studies requiring to focus on the isolation of certain compounds and to get more from the virtues of this plant by doing an in-depth study on the effectiveness of *Zls* to be exploited as an anti-alzheimer agent.

Several authors described the role of the presence of some bioactive compounds to their AChE inhibitory effects **[148-151].** Morever, the obtained inhibitory effects could be related to the content in different phytochemicals found in the extracts. According to our knowledge, it is the first time that the in vitro inhibition of the acetylcholinesterase enzyme by *Zls* extracts is reported.

II.1.3.3. Determination of HMGR Inhibitory activity

In this study, HMG-CoA reductase activity was measured from *Zls* extracts at optimum extraction conditions that contain highest amount phenolic compounds. The results are demonstrated in Table 21, *Zls* extracts showed an activiy of 28.71% as a HMGR inhibitors using 100 µg/mL that is as satisfactory as those obtained from *C. olitorius* leaves methanol extracts. Our results are probably due to the components present in *Zls* in high level. The high activity is related to the type of bioactive compounds present in the extracts that can fit the enzyme active site. The modest activity obtained with *Z. lotus* can be explained by the fact that only some bioactivie compounds present in *Zls* are responsible for enzyme inhibition and it can only be said that seeds extracts are modest HMGR inhibitors with IC_{50} value similar to those found in literature describing results from polar plant extracts. The results found in this study is more satisfatory than those obtained from seeds extracts of some plants, mainly *Cannabis sativa* (7.4%), *Cuminum cyminum* (26%), *Nigella sativa* (0%), *Ocimum basilicum* (0%), *Pimpinella anisum* (10.5%) and *Trigonella foenumgraecum* (0%) **[152].** While, it is in the same range than HMGR inhibitors obtained from aerial parts extracts of *Peganum arma* (28.5%) and *Tencrium polium* (28.8%) **[152].**

However, several *Ziziphus* species that have a high level of secondary substances such as polyphenols, saponin and triterpene that are complexing with cholesterol as binding plasma lipids is due to the sugar chains contained in saponins that attach themselves to triterpene or sterol **[153].** These compounds were found to contribute on the inhibition of HMGR and exerce a a good hypolipidemic activity **[154, 155].** Morever, *Ziziphus mauritiana* leaves extracts are found to reduce levels of cholesterol, triglyceride and can be used for the treatment of fatty liver and atherosclerosis **[156].** More recently, alkaloids from Magnoflorine-containing extracts showed a significant HMGR inhibition **[157].** From these reports it seems that especially flavonoids, terpenes and alkaloids are mainly compounds responsible for HMGR inhibitors in our samples as we showed in previous paragraphs.

Several studies found that phenolic compounds are responsible for good HMGR inhibitory which is controled by the decrease of LDL/HDL ratio as found by Bahramikia and Yazdanparast **[158]** from hydroalcoholic extracts of *Nasturtium officinale* leaves. Others have reported from that the increase in the biosynthesis and decrease in catabolism of both fatty acids and cholesterol are affected by alcohol extracts which is implicated in the development hyperlipidemia and fatty liver in rats **[159].** The good HMGR inhibitory activity is affected also by all factors studied due to the different extaction of bioactive compounds using MAE in our samples especially solvent type and to the best of our knowlege our study is the first improuving the HMGR activity from *Z. lotus* species.

Optimization of ultrasound-assisted phenolic compounds extraction from Algerian jujube (*Zizyphus lotus L.*)seeds

Abstract

The purpose of this study was to investigate the application of ultrasound technique for extracting phenolic compounds (TPC) from seeds of *Zizyphus lotus* under optimization conditions based on response surface methodology (RSM). A maximum TPC of 2406.0835 mg GAE/100g obtained under ethanol concentration 50.16%, sonication temperature 29.01 °C, sonication time 15.94 min and solvent-to-solid ratio 34.10:1 mL/g. The optimized extract was then evaluated for its antioxidant, antiacethylcholinesterase, anticholesterolemia and antiproliferative activities. The results showed that ultrasound method is a green and safe method that can be used to effectively extract TPC from jujube seeds. The biological activity of *Zizyphus* extract exhibited a very good antioxidant against DPPH (EC_{50} = 0.39 µg/mL) and FRAP (1670.42 ± 6.5 mg/100g). Additionnaly, it possess acetylcholinesterase inhibitory effect (IC_{50} =0.93 ± 0.01 mg/mL) and HMGR inhibition (45.41 %) using 100µg/mL. The extract significantly inhibits cell proliferation on the MCF-7 and $HepG_2$ tumour cell lines with an IC_{50} values of <0.05 and 3 ± 0.55 mg/mL, respectively. Therefore, the ultrasound method can be considered a method for obtaining a significant anticancer activity with respect to the lines and therefore makes it possible to recover a maximum of phenolic compounds in less time with an antiacetylcholinesterase and HMGR inhitiory activity. Thus, it can be suggested that *Zls* extract is a promising nutraceutical and pharmaceutical agent that can be used in industries.

Keywords : Jujube, ultrasound, response surface methodology, polyphenols, biological activities.

II.2.1. Phenolic recoveries and UAE optimized process

The factorial experimental design based on a BBD using UAE method and corresponding responses for the obtaining of TPC from *Zls* extracts was investigated and presented at various conditions with a model of the total of 27 experiments in Table 23. In order to estimate experimental error measurement, three replications at the central points were made **[160].**

Table 23 : Box–Behnken design with the observed responses and predicted values of TPC from seeds using UAE.

Run	X_1 - Ethanol concentration (% v/v)	X_2 - Sonication temperature	X_3 - Sonication time (min)	X_4 -Solvent-solid ratio (mL/g)	Experimental	Predicted
1	50	30	20	25	1643.19249	1683.34855
2	50	20	30	25	1877.93427	1895.48557
3	20	30	30	25	1525.8216	1497.67432
4	80	30	30	25	1154.14711	1117.74039
5	50	40	30	25	1760.56338	1734.20927
6	50	30	40	25	1741.00157	1774.20231
7	50	20	20	30	1956.18153	1889.99739
8	20	30	20	30	1314.55399	1308.63111
9	80	30	20	30	1377.1518	1353.84064
10	50	40	20	30	1791.86229	1786.75448
11	20	20	30	30	1658.84194	1701.38889
12	80	20	30	30	1314.55399	1382.74865
13	50	30	30	30	2230.04695	2310.90245
14	50	30	30	30	2425.6651	2310.90245
15	50	30	30	30	2276.99531	2310.90245
16	20	40	30	30	1384.97653	1410.35255
17	80	40	30	30	1408.4507	1459.47444
18	50	20	40	30	2057.90297	2036.2763
19	20	30	40	30	1674.49139	1630.96635
20	80	30	40	30	1377.1518	1316.23848
21	50	40	40	30	1885.759	1925.20866
22	50	30	20	35	1990.08868	2050.45862
23	50	20	30	35	2300.46948	2259.98739
24	20	30	30	35	1661.45018	1671.12241
25	80	30	30	35	1780.1252	1781.53799
26	50	40	30	35	2291.34064	2206.95314
27	50	30	40	35	2190.92332	2244.33794

The factors studied were ethnaol concentration, sonication temperature , sonication time and solvet/solid ratio, that is ethanol/seed quantity. The parameters were estimated from the experimental TPC results and ANOVA analysis was carried out to determine the applicability of the model, Table 23. The second order polynomial equation was generated to describe the empirical relationship between the *Zls* extract and operational conditions in terms of coded values. The mathematical models were simplified by neglecting statistically the insignificant terms (p>0.01) following predictive equation :

$$Y = 2310,90 - 60.37\,X_1 - 53.57\,X_2 + 71.18\,X_3 + 209.31\,X_4 - 664.77X_1^2 - 157.63X_2^2 - 243.70X_3^2 - 129.10X_4^2 + 91.94X_1X_2 - 89.98X_1X_3 + 122.58X_1X_4 \quad (14)$$

Table 24: Analysis of variance (ANOVA) for the experimental results obtained by using UAE.

Parameter[a]	Estimated coefficients	Standard error	DF[b]	Sum of squares	F-value	Prob > F
Model B_0	2310.9025	42.28475	14	3245323.8	43.2157	<0.0001*
Linear						
X_1-Ethanol	-67.37959	21.14238	1	54480.1	10.1566	0.0078*
X_2-Temperature	-53.57764	21.14238	1	34446.8	6.4218	0.0262*
X_3- Time	71.183272	21.14238	1	60804.7	11.3357	0.0056*
X_4- Ratio	209.31142	21.14238		525735.3	98.0118	<0.0001*
Quadratic						
X_1^2	-664.7757	31.71357	1	2356942.5	439.4001	<0.0001*
X_2^2	-157.6356	31.71357	1	132528.0	24.7069	0.0003*
X_3^2	-243.7076	31.71357	1	316764.8	59.0538	<0.0001*
X_4^2	-129.108	31.71357		88900.6	16.5736	0.0016*
Interaction						
X_1X_2	91.940532	36.61967	1	33812.2	6.3035	0.0274*
X_1X_3	-89.98435	36.61967	1	32388.7	6382	0.0302*
X_1X_4	122.58738	36.61967		60110.7	11.2063	0.0058*
X_2X_3	-1.956182	36.61967	1	15.3	0.0029	0.9583
X_2X_4	27.060511	36.61967		2929.1	0.5461	0.4741
X_3X_4	25.75639	36.61967		2653.6	0.4947	0.4953
Lack Of Fit			10	43510.243	0.4172	0.8589
Pure Error			2	20857.773		
Total Error			12	64368.016		
R^2					0.980552	
R^2 Adjusted					0.957862	
CV%	4.11					
RMSE	73.23934					
Corr.Total			26	3309691.8		

It can be seen that all factors influence the extraction yield as lineaire and quadratic effects, but the interaction effects were significant for all the four parameters, positive for ethanol concentration and sonication temperature, negative for ethanol concentration and sonication time. While, positive also for ethanol concentration and solvent-solid ratio which was highly significant then the others. On the other side, it can be seen in Eq. (14) that X_2X_3, X_2X_4, X_3X_4 didn't exhibit any significant effet on extraction yield.

However, if there is any significance of each factor, it was demontrated by a presence of $p<0.05$ and the contrary, it was also demontrated p-values ($p<0.001$) which indicates high significance. Very low p-values ($p<0.0001$) indicated that each generated model was statistically significant and suggests that the UAE of *Zls* could be well described with those appropriate models.

The values of R-squared are close to 1 for the model (0.98 and 0.95 for R^2 and R^2 Adjusted respectively), which are very high and indicates a good correlation between the experimental and the predicted values, also indicated that 98% could be explain by the model of the variation in the TPC extracts using UAE method. In addition, other parameters were insignificants as F-value for the lack of fit ($p>0.05$) and values of coefficient of variation ($CV = 4.11$) which provide also the validity of the deduced model.

II.2.2. Effect of experimental coditions on TPC extraction yield

The influence of the four parameters on TPC yields is shown in Figure 38. Confirming the results of the single-factor trials, Fig. 38(a-c) shows that the TPC yield reached a maximum level when ethanol concentration was set at medium levels (0 coded value) as it mentioned in Table 24, we can notice that the yield of TPC using UAE mainly depends on the ethanol concentration as its quadratic, interaction and linear effects were highly significant ($p<0.01$), which showed the increase on TPC for all other parameters.

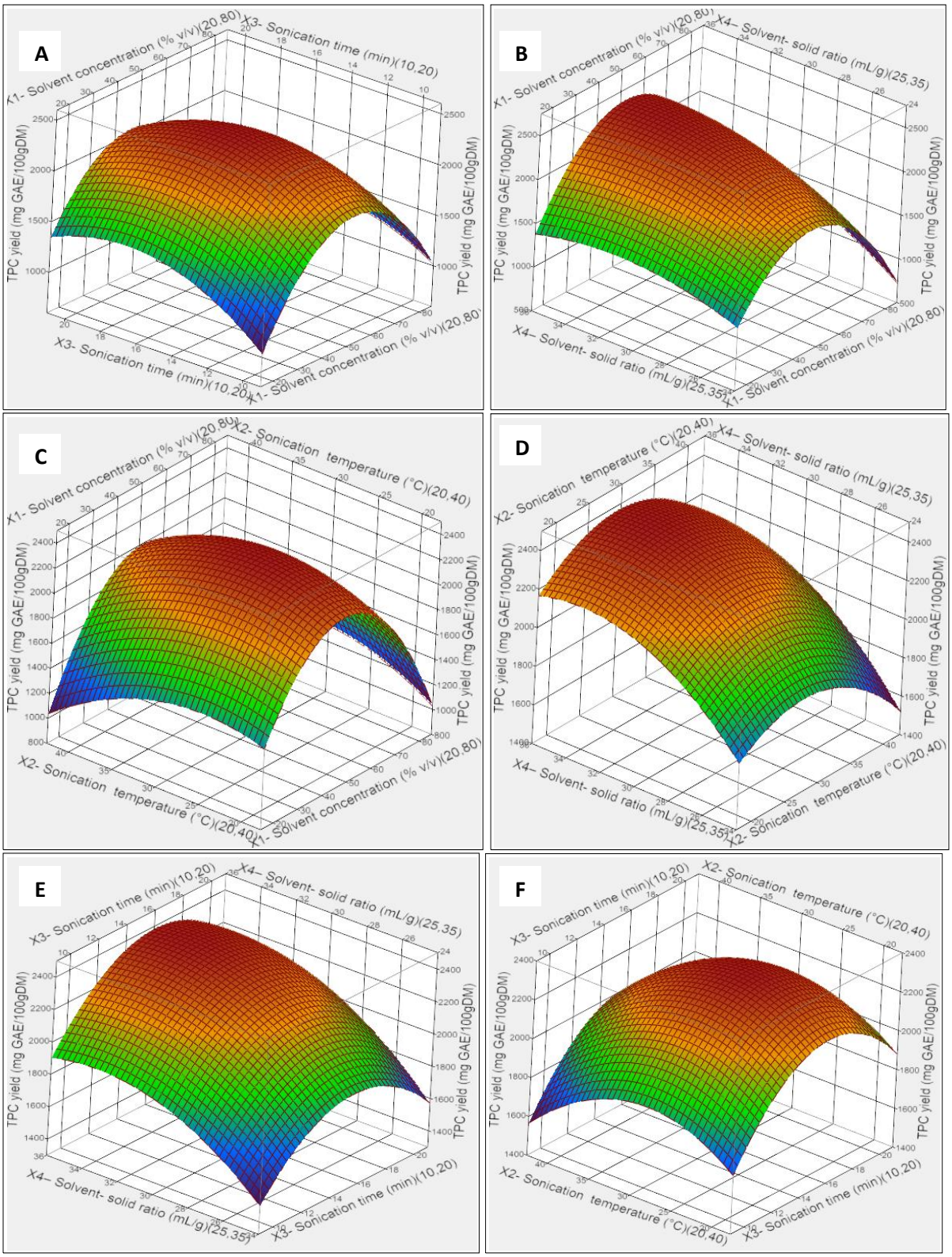


Figure 38 : Response surface analysis for the total phenolic yield from *Z.lotus* seeds with UAE with respect to sonication time and ethanol concentration (a); solvent-to-solid ratio and ethanol concentration (b); sonication temperature and ethanol concentration (c); solvent-to solid ratio and sonication temperature (d); solvent-to-solid ratio and sonication time (e); sonication temperature and sonication time (f).

Fig. 38(c-d-f) shows that the interaction effect of temperature with other factors on TPC yield that was very limited and stable as demonstrated in the equation model. Thus, only the Fig.38c showed a postive influence of both ethanol and temperation on the TPC extraction and the decrease of TPC recovery from the other figures is mainly associated to their thermal degradation at higher temperature. However, Fig.38(b-d-e) shows that maximum extraction TPC yield was for 2100 mgGAE/100g when using a ratio about 30 (mL/g, v/w) over a range of temperature and time factors, in contrary in interaction with ethanol concentration which was very significant as confirmed also as positive effect in the previous table. The increase in TPC was deemed by the effects of acoustic cavitation that contribute to the formation and rupture of cavitation bubbles and then facilitate the mass transfer of the process, while, TPC strated to decrease after higher increase in ratio which may affect the dispersion of the ethanol under ultrasound energy density **[161].** Finally the interaction effect of both temperature and time sonication was indicated in Fig. 38 (f), the longeur sonication time at a midlle temperature give a maximum TPC yield, while, higher temperature decrease significatively the TPC recovery. This combination favorise the degradation of phenolic compounds which confirmed the results of Table 24.

The prediction values for the optimal TPC extraction were verified experimentally. Optimal conditions resulted in ethanol concentration 50.16%, sonication temperature 29.01°C, sonication time 15.94 min and solvent-to-solid ratio 34.1/1 mL/g with a predicted TPC yield of 2406.0835 ± 79.87 mgGAE/100g. UAE was carried out at these optimal conditions obtaining a TPC yield of 2383.1024 ± 0.87 mgGAE/100g, very close to the value predicted by the model (Table 24). The extraction process used in the present work give higher TPC than using conventional extraction method (172.08 to 328.65 mgGAE/100g) **[27].** The present work suggests that UAE method is an efficient alternatives to other extraction techniques for extracting and maximising polyphenols from *Zls* in short extraction time and jujube seeds is a non negligeable source of polyphenols.

II. 2.3. Biological Activities

II. 2.3.1.Antioxidants activity of Zls extract

The imbalance between the production of reactive oxygen species (ROS) and the biological system's antioxidant defenses is defined as the oxidative stress. This proved in several studies to develop a lot of diseases. In order to protect our human body from these diseases, the antioxidants are showed to be effective for neutralization of free radicals **[162].** However, to

determine whether the UAE extraction precess impact the biological functions, the antioxidant effects of jujube seeds extracts were examined and evaluated by DPPH radical scavenger and FRAP (Table 25). The DPPH scavenging assay of the *Zls* extract revealed a significant highest activity with lowest EC_{50} value (0.39 µg/mL) than that of Tunisian *Z. lotus* leaves and fruits extract using methanol (0.10 ± 0.001 and 0.31 ± 0.005 mg/mL) **[56],** Tunisian *Z. lotus* pulp and peel extracts using UAE with an EC_{50} of 0.28 mg/mL **[57],** from *Z. mucronata* roots (0.029 ± 0.05 mg/mL) **[163]** and from Korian *Z. jujuba* seeds (mechu and sanzoin) (0.3 and 0.1 mg/mL), respectively **[139]**. Overall, this study showed that *Zls* extracts exhibited a high antioxidant effet in comparison to some *Zizyphus* species using both UAE and conventional methods. Furthermore, *Zls* extract by UAE exhibited a significant iron-reducing power (1670.42 ± 6.5 mg/100g) and was found to be higher that those obtained by Wang, Cheng **[58]** with 471.6 ± 30.8 mg/100g from *Z. jujuba cv. Zaowangzao*.

Table 25: Biological activities of *Z. lotus* seeds extract using UAE. Results are expressed as means ± standard deviation.

Factors	Ultrasound extraction
Sonication time (min)	15.94
Ethanol concentration (%)	50.16
Sonication temperature (°C)	29.01
Solvent solid/ratio (mL/g)	34.1
Results	
Recovery of total phenolic TPC (mg GAE/100 g)	2383.1024 ± 0.87
DPPH scavenging EC_{50} (µg/mL)	0.39 ± 0.00
FRAP (mg GAE/100 g)	1670.42 ± 6.5
AChE assay IC_{50} (mg/mL)	0.93 ± 0.01
HMGR assay (%) (for 100 µg/mL)	45.41
HepG2 cells IC_{50} (mg/mL)	3 ± 0.5
MCF-7 cells IC_{50} (mg/mL)	<0.05± 0.0

Thus, this paper revealed the effectiveness of ultrasound method for extraction of polyphenols from jujube seeds with significant antioxidant activities in comparison to conventional methods that gave lower recovery and activity which could be attributed to the mecanic cavitation of ultrasound due to the acoustic bubbles which results to ennhanced desired vompounds without altering its quality. The inhibition of DPPH as well as the iron chelating effect are probably due to the significant jujube content in photochemical substances especially in phenolic compounds, the latter having powerful reducing effects of oxidation. In

addition, from other jujube extracts that contain other compounds namely ascorbic acid, tocopherol and pigments, were found to present synergic effects between them and contribute to the total antioxidant activity of this extract and therefore the trapping of free radicals. While, this suggested that not only phenolic compounds present in the jujube extract can act as antioxidants but also other compounds may be responsible for this activity **[96, 164, 165].** In our case, the antioxidant activity using DPPH and FRAP methods from *Zls* extract is in perfect agreement with other research showing that there can be correlation between the phenolic content and the antioxidant capacity **[166].**

II.2.3.2. AChE inhibition of Zls extract

The antiacetylcholinesterase activity of the *Zls* extract are presented in the Table 25. The rapid breakdown of AChE following the stoping of the transmission of nerve impulses to cholinergic synapses is well controlled by the role of AChE. One of the ways deemed effective used against AD is more particularly based on the inhibition of AChE, which makes it possible to maintain the levels of acetylcholine for the transmission of nerve impulses **[167].** The anticholinesterase activity of jujube extract at different concentrations exhibited a significant inhibitory activity with an IC_{50} value of 0.93 ± 0.01 mg/mL. These results are higher than that obtained from *Zizyphus oxyphylla* extracts using *n*- butanol which showed a maximum inhibitory effect with an IC_{50} value of 9.58 ± 0.08 mg/mL **[147].** This demonstrated the good effect of ethanol/water used for extraction of *Zls* which exhibited a good inhibition of AChE which can be applicable against AD. Ethanol concentration is considered as a crucial factor in UAE due to cavitation phenomena enhancing solvent penetration into jujube extract **[9].** Morever, the *Zls* extract is approximatively in the same range than Tunisian *Zls* extract found by Tlili, Hanen **[168]** using acetone for extraction by (0.85 mg/mL). However, in comparison to other plants that used acetone also, our extract had a highest AChE inhibitory effect than that obtained by Tlili, Hanen **[168]** from *Herniaria fontanesii* and *Hyoschyamus albus* with an IC_{50} value of 1 and 1.17 mg/mL, respectively. Thus, our results suggested that extraction using ultrasounds under different conditions mainly ethanol as solvent extraction is an important parameter to take into account in AChE tests. Major medicinal plant extracts showed some level of inhibitory activity against the AChE. This could be attributed to the phytochemicals mostly phenolic compounds present in the extract and their possible synergistic interaction effect **[148-151, 169].** To the best of authors knowledge, this is the first report on in vitro inhibition of the AChE enzyme by *Zls* extracts under the effect of ultrasound

extraction where the interest of its application against Alzheimer's disease or alleviation of severe constipation.

II.2.3.3. HMGR inhibition of Zls extract

The HMGR inhibition by the *Zls* extract is demonstrated, at the concentration of 100 µg/mL *Zls* ethanolic extracts showed an activiy of 45.41% as a HMGR inhibitors which is higher than the acetone and ethanol extracts from lichen *U. complanata* with 2.22 and 21.48%, respectively at the concentration of 60 µg/mL **[170].** Similarly, *Peganum arma* and *Tencrium polium* from aerial parts extracts showed a value of 28.5 and 28.8% of HMGR inhibition which are lower than our sample **[152].** However, *Zls* extract showed a significant HMGR inhibitory effects than seeds extracts of some species, mainly *Cannabis sativa* (7.4%), *Cuminum cyminum* (26%) and *Pimpinella anisum* (10.5%) **[152].** Several bioactive compounds mainly polyphenols, saponins, alkaloids and triterpenes were found to have a good hypolipidemic activity against HMGR **[154, 155, 157].**

Thus, jujube extract was found as a modest HMGR inhibitors which is due to the presence of some bioactivie compounds that may be responsible for this enzyme inhibition like all polar plant extracts found in literature **[154, 155].** The properties of our sample phytochemicals make them possible antihyperlipidemia applications by fiting the enzyme active site. Furthermore, other *Zizyphus* species (*Z. mauritiana*) leaves extracts were used previously for the treatment of fatty liver and atherosclerosis by reducing cholesterol and triglyceride and levels **[156].** Among some studies that have shown the effect of polyphenols in inhibiting the action of HMG-CoA, Islam, Sharma **[171]** have demonstrated that curcumin, tetrahydrocurcumin, epigallocatechin-3-gallate and kaempferol among all the other polyphenols tested can occupy the HMG-CoA binding site on the NADP+ site which utilizes two molecules of nicotinamide adenine dinucleotide phosphate-oxidase (NADP[H]), thus can plays the role of competitive inhibitors of substrate binding to enzyme that can block the electron transfer on the substrate HMG-CoA. Compelling effect of these compounds and major phenolic compounds in general indicates the importance of their uses for the cholesterol-lowering in order to the maintenance of cardiovascular health **[172, 173].**

II.2.3.4. Anti-proliferative activity of Zls extract

The *in-vitro* evaluation of *Zls* extracts on cytotoxicity effects were analysed. The toxicity of extracts was tested in the human cell lines $HepG_2$ and MCF-7 using 5 serial concentrations

ranging from 0.05 to 1 mg/mL of extract in order to calculate the cell viability. The IC_{50} values which confirm the concentration of extracts that killed 50% of the cells was obtained from dose-response curves. The phenolic compouds of *Zls* studied were revealed to be non-toxic towards only to $HepG_2$ cell line because the value is higher than 0.1 mg/mL. This value is considered as limit of toxicity to human cell lines **[174].** In contrary, *Zls* extract analysed against toxicity in MCF-7 cells showed an IC_{50} value lower than 0.05 mg/mL which is considered toxic to human cell lines. The value of 1 mg/mL which correspond to the maximum concentration present in the UAE extract inhibited 70.84% and 26.21% for MCF-7 and $HepG_2$ cells, respectively. Thus, *Zls* extract exhibited no significant activity against $HepG_2$ cells. In contrary, it exhibited a significant activity against MCF-7 cells. These findings can be due to the main bioactive compounds contained in *Zls* extract that may include flavonoids, tannins, alkaloids, terpenoids and saponins as observed previously from other jujube species and from other part of *Zizyphus lotus* which showed a strong antiproliferative activity against $HepG_2$ and MCF-7 cells **[40, 140].**

In addition, Hoshyar, Mohaghegh **[175]** demonstrated that *Z. jujube* fruits extract was found to exhibit activity against MCF-7 cells with an IC_{50} value of 1.8 mg/mL after 24 h which is higher value than that of *Zls* extract (IC_{50} less than 0.05 mg/mL after 24 h). Also, Klaab, Almalki, and Hassan **[176]** demonstrated the cytotoxic action of *Z. jujuba* extract and Tamoxifen drug, each individually and in combination. The results indicated a reduction inviability and high potent inhibitory effect toward the proliferation of MCF-7 cells, this effect may be due to cell apoptose. However, the cytoxicity effect reported from *Zls* extracts against $HepG_2$ cells was significantly higher than that reported from mung bean sprouts extracts obtained using maceration method with an IC_{50} of 14.04 ± 1.5 mg/mL. This extract also contained polyphenolic compounds **[177].** The effective cytotoxicity toward hepatocellular $HepG_2$ and beast MCF-7 could be related to the presence of the some secondary metabolites, some of them are considered the major class of jujube polyphenols, represented from 89 to 94% of the total phenolic contents mainly flavan-3-ols such as monomer (as (-)-epicatechin, gallocatechin gallate, and (+)-catechin), dimer (procyanidine B2) and polymeric proanthocyanidins in *Zls*, which were applicated in the inhibition of cell proliferation in different cancer types **[25].** As well as, Rached, Barros **[40]** concluded that quercetin-3-*O*-rutinoside found in other parts of *Z. lotus* which represents 50% of jujube flavonoids offers a plausible explanation of the observed cytotoxicity.

The present research data suggests that in some cases the ultrasound extraction can positively influence the extraction yield of TPC and also the antitumor activity against the tested cell lines. This is can be related to the mechanical acoustic effects of ultrasound which causes the rupture of the cell wall allowing mass transfer and therefore increase the recovery process of TPC but following the sonication time (15.94 min) which is a very sensitive parameter in extraction procedure. To the best of our knowledge, no study has, as yet, been carried out on the effects of ultrasounds on phenolic extracted from *Zls* and cytotoxicity toward hepatocellular $HepG_2$ and beast MCF-7 of the studied UAE extract from *Zls.*

Chapter III. Valorization of *Zizyphus lotus* endocarp for the recovery of Total Phenolic Compounds using optimized microwave and ultrasound process

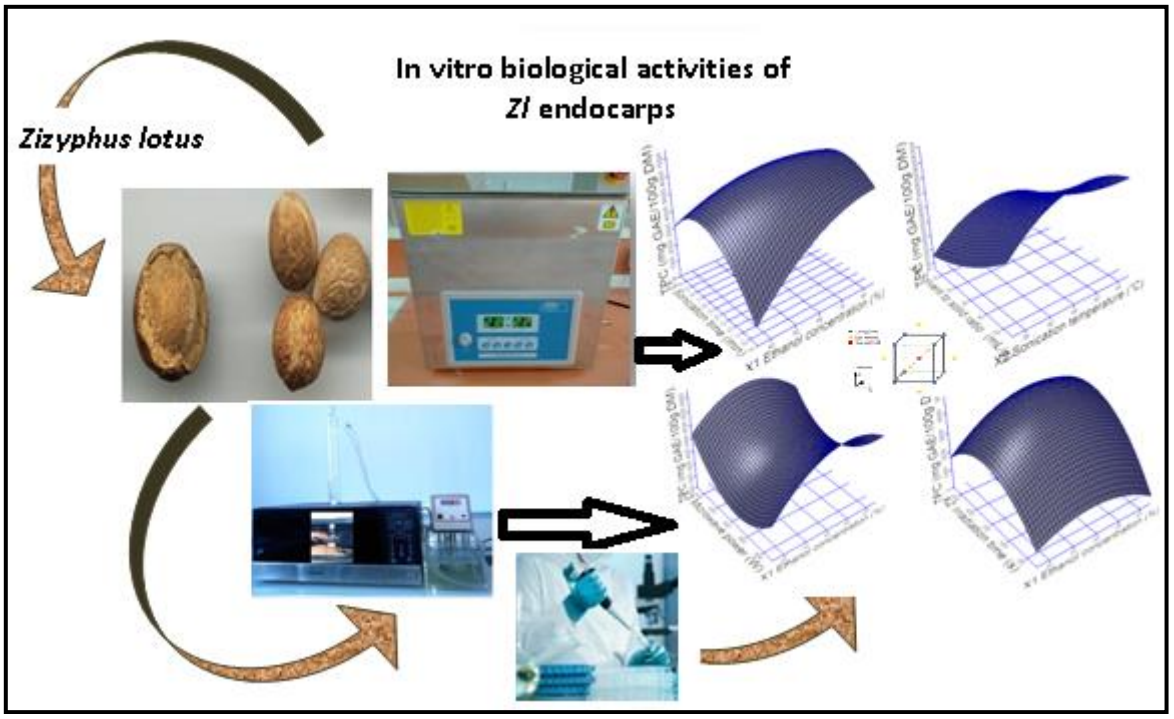


Figure 39 : Graphical abstract of microwave and ultrasound assisted extraction of polyphenols from jujube endocarps.

Abstract

This part was aimed to optimize the extraction conditions of total phenolic contents (TPC) from *Zizyphus lotus* endocarps by microwave and ultrasound procedures using response surface methodology (RSM) in order to obtain maximum extraction yields. The optimal conditions for jujube extracted by MAE X_1, X_2 and X_3 were : ethanol 45.07%, 210 s and 500 W, respectively. However UAE jujube extracts was for X_1,14.26 min, X_2, 80% ethanol, X_3, 43.14°C and X_4,35 mL/g. The experimental value of TPC yields fr both MAE and UAE were for 5568.59 ± 361.11 and 10087.08 ± 231.81 mg GAE/100 g which are in close agreement with the predicted values indicating the success of RSM model. Results showed that optimized *Zle* extract exhibited a high inhibitory effects on some biological activities including DPPH free radical scavenging, FRAP, AChE, HMGR inhibitory and antiproliferative effects. The liquid chromatography-high resolution tandem mass spectrometry (LC/MS-MS) profile revealed 30 active compounds. The *Zle* extract was found to contain significant major compounds comprised terpenes (41.38% from all secondary metabolites) where tensagenin was the major one 48.77% from all terpenes and 20.18% from all secondary metabolites. In the meanwhile, organic acids were for 52.37% of all primary metabolites where majors compounds are N-[2-[2-(2-aminoethylamino)ethylamino]ethyl]-2-[1-[2-(2-aminoethylamino)ethyl]-4,5-dihydroimidazol-2-yl]acetamide and 3-(decyloxy)-2-hydroxypropyl prop-2-enoate representing 32.49 and 35% from all organic acids), respectively. The findings demonstrated the beneficial application of green methods for an increase extraction of TPC amounts from *Zle* extracts that could be valorized in food and pharmaceutical industries.

Keywords : *Zizyphus lotus*, mircowaves, response surface methodology, phenolic compounds, LC/MS-MS, biological activities.

III.1.Optimizing phenolic extraction conditions by UAE method

Modeling and Fitting the Model Using RSM

The experimental design and subsequent response allied to TPC are summarized in Table 26, with results from TPC recovery varying in the range of 2033,67- 9998,04 mg GAE/ 100g DM.

Table 26: Box–Behnken design with the observed responses and predicted values of TPC from endocarp using UAE.

Run	X_1 - Ethanol concentration (% v/v)	X_2 - Sonication temperature	X_3 - Sonication time (min)	X_4 -Solvent-solid ratio (mL/g)	Experimental	Predicted
1	60(0)	45(0)	10(-1)	25(-1)	6247,51	6350,44
2	60(0)	35(-1)	15(0)	25(-1)	6514,15	6411,35
3	40(-1)	45(0)	15(0)	25(-1)	5082,60	5215,09
4	80(1)	45(0)	15(0)	25(-1)	9998,04	10003,85
5	60(0)	55(1)	15(0)	25(-1)	7991,26	7904,06
6	60(0)	45(0)	20(1)	25(-1)	6937,25	6886,02
7	60(0)	35(-1)	10(-1)	35(0)	2888,15	2786,87
8	40(-1)	45(0)	10(-1)	35(0)	2684,49	2686,91
9	80(1)	45(0)	10(-1)	35(0)	5796,39	5891,79
10	60(0)	55(1)	10(-1)	35(0)	4963,63	4888,29
11	40(-1)	35(-1)	15(0)	35(0)	2033,67	1999,89
12	80(1)	35(-1)	15(0)	35(0)	5975,57	5975,51
13	60(0)	45(0)	15(0)	35(0)	6330,74	6359,23
14	60(0)	45(0)	15(0)	35(0)	6416,22	6359,23
15	60(0)	45(0)	15(0)	35(0)	6330,74	6359,23
16	40(-1)	55(1)	15(0)	35(0)	5810,24	5734,92
17	80(1)	55(1)	15(0)	35(0)	4935,25	4893,64
18	60(0)	35(-1)	20(1)	35(0)	3035,68	3206,04
19	40(-1)	45(0)	20(1)	35(0)	4084,00	3968,95
20	80(1)	45(0)	20(1)	35(0)	3920,49	3898,41
21	60(0)	55(1)	20(1)	35(0)	3561,48	3757,79
22	60(0)	45(0)	10(-1)	45(1)	4156,68	4132,53
23	60(0)	35(-1)	15(0)	45(1)	3400,78	3468,33
24	40(-1)	45(0)	15(0)	45(1)	5238,30	5327,53
25	80(1)	45(0)	15(0)	45(1)	3710,57	3673,11
26	60(0)	55(1)	15(0)	45(1)	4545,63	4628,78
27	60(0)	45(0)	20(1)	45(1)	3063,93	2885,62

As demonstrated by Jacotet-Navarro, Rombaut **[178]**, the regression coefficients of the intercept, linear, quadratic, and interaction terms was calculated using the square technique

(Table 26). Notably, the linear parameters, namely ethanol concentration, sonication temperature and liquid–solid ratio ($p < 0.0001$), followed by sonication time ($p = 0.0007$) significantly affected the extraction content of phenolic compounds extract from *Z.lotus* endocarp. Otherwie, all the quadratic terms X_1^2, X_2^2, X_3^2 , X_4^2 were highly significant at the level $p < 0.001$.

Table 27 : Analysis of variance (ANOVA) for the experimental results obtained by using UAE.

Parameter[a]	Estimated coefficients	Standard error	DF[b]	Sum of squares	F-value	Prob > F
Model	6359,2335	78,5157	14	84590271	326,7067	<0,0001*
Linear						
X_1-Ethanol	783,58399	39,25785	1	7368046	398,3988	<0,0001*
X_2 -Temperature	663,29047	39,25785	1	5279451	285,4660	<0,0001*
X_3 - Time	-177,8346	39,25785	1	379502	20,5201	0,0007*
X_4 - Ratio	-1554,577	39,25785	1	29000519	1568,092	<0,0001*
Quadratic						
X_1^2	-628,2351	58,88678	1	2104956	113,8174	<0,0001*
X_2^2	-1080,002	58,88678	1	6220825	336,3672	<0,0001*
X_3^2	-1619,478	58,88678	1	13987775	756,3352	<0,0001*
X_4^2	323,90202	58,88678	1	559533	30,2546	0,0001*
Interaction						
X_1X_2	-1204,224	67,99659	1	5800621	313,6463	<0,0001*
X_1X_3	-818,8536	67,99659	1	2682085	145,0234	<0,0001*
X_1X_4	-1610,795	67,99659	1	10378644	561,1853	<0,0001*
X_2X_3	-387,418	67,99659	1	600371	32,4627	<0,0001*
X_2X_4	-83,06634	67,99659	1	27600	1,4924	0,2453
X_3X_4	-445,6228	67,99659	11	794319	42,9497	<0,0001*
Lack Of Fit			10	217058,54	8,9119	0,1050
Pure Error			2	4871,23		
Total Error			12	221929,77		
R^2					0,997383	
R^2 Adjusted					0,99433	
CV%	2.70					
RMSE	135,9932					
Corr.Total			26	84812201		

Regarding TPC yield, the interaction of ethanol concentration with sonication temperature (X_1–X_2), with sonication time (X_1–X_3), with liquid to solid ratio (X_1–X_4), and that of sonication temperature with sonication time (X_2X_3) followed by that of sonication time with liquid to solid ratio (X_3X_4) were highly significant ($p < 0.0001$), while sonication temperature

with liquid-to-solid ratio (X_2X_4) didn't exhibit any significant effect (p = 0,2453). As represented in Table 27, the analyses of variance (ANOVA) for the adequacy of the selected mathematical models justified the obtained significant terms which played a dominant role in *Zizyphus* endocarp extraction by ultrasound.

Based on the significant terms, the second order polynomial equation was generated to describe the empirical relationship between the TPC (Y_1) for the UAE efficiency and operational conditions (ethanol concentration, sonication temperature and sonication time and solid/liquid ratio) in terms of coded values (Eq. 15).

$$Y1 = 6359{,}23 + 783{,}58\,X1 + 663{,}29\,X2 - 177{,}83\,X3 - 1554{,}57\,X4 - 628{,}2351\,X12 - 1080{,}00\,X22 - 1619{,}47\,X32 + 323{,}90\,X42 - 1204{,}22\,X1X2 - 818{,}85\,X1X3 - 1610{,}79\,X1X4 - 387{,}41\,X2X3 - 445{,}62\,X3X4 \quad (15)$$

The p-values were used to verify the significance of independent factors and their interactions, where p-value < 0.001 indicated high significance of the response model and p-value < 0.05 indicated significance. Our results show that the model represented the data satisfactorily where p < 0.0001 which means that the model was statistically highly significant and suggests that the UAE of *Z.lotus* endocarp could be well described with the appropriate model. The Eq. (15) shows that both ethanol concentration and sonication temperature have significant positive linear effects and significant negative quadratic effects on TPC of *Z.lotus* endocarp extract, whereas, sonication time have both significant negative linear and quadratic effects. On the other side, solid/liquid ratio have significant negative linear effect and its quadratic interaction have significant positive effects. Morever, all parameters indicates that their interaction have significant negative effects on TPC of *Z.lotus* endocarp.

The adjusted coecient of determination (R_2adj) and the coecient of determination (R_2) were 0,997383 and 0,99433, respectively, which are very high and implied that the sample variations of 99.43% for the UAE efficiency of *Z.lotus* endocarp phenols were attributed to the independent variables, and only 0.57% of the total variations could not be explained by the model, indicating a very good degree of correlation between experimental and predict values of the TPC yield. Also, the value of F-value for the lack of fit was not significant (p > 0.05), thus confirming the validity of the model. In addition, the low value of coefficient of variance (2.70%) clearly indicated that the model was reproducible and reliable as shown by several authors**[179].** All these results indicate that the model could work well for the prediction of TPC in the *Z.lotus* endocarp extracts.

Response Surface Analysis (RSA) for UAE

To provide a better understanding of the interaction between factors, the 3D response surface plot was constructed (Figure 40) using Equation (15). The graphs were generated by plotting the response using the z-axis against two independent variables, while keeping the other independent variable at the fixed level. Figure 40A–C shows the interactions between the ethanol concentration and each of the three other factors, namely sonication temperature, sonication time and liquid-to-solid ratio, respectively, on the recovery of TPC. As shown, an increase of ethanol concentration from 40% to 80% (v/v), or sonication temperature from 35 to 55 °C and sonication time from 10 to 25 min resulted in a rapid enhancement of TPC with a maximum of 5500 mg GAE/ 100g DM being recovered at 55°C with 80% of ethanol and at maximum yield of 3000 mg GAE/ 100g DM in 25 min.

The highest concentration of ethanol showed a maximum extraction of TPC yield which means that it required high sonication intensity to generate the cavitation bubbles and a good solubilisation of extracts on ethanol at more then 60%, as it was obtained by several authors using UAE for extraction of antioxidants **[10]**.

Figure 40B shows that maximum recovery (3000 mg GAE/ 100g DM) was achieved for 70% (v:v) of ethanol, the increase of ethanol concentration didn't infuence positively the solid/liquid ratio. This confirm the results of Table 27 and the equation. This can be explained by a rapide dissolution of the solvent at higher concentration on tissues of *Z.lotus* endocarp which increase the extraction yield and didn't need a very higher ratio to dissolve. The response surface plot for the significative interactive effect of irradiation time and liquid-to–solid ratio on the response value is shown in Figure 40D. The increase of both sonication temperature and liquid-to–solid ratio decrease the TPC yield and a maximum TPC yield was obtained at 42 °C (2500 mg GAE/ 100g DM). These results confirm those reported in the litterature **[57]**. The interaction between sonication time and liquid-to–solid ratio was demonstrated in Figure 40E. The best content (3500 mg GAE/ 100g DM)was found with the solid–liquid ratio of about 30 mL/g. This diminished the supply of ultrasonic energy density at higher increase in ratio. The yield of TPC constantly improved with the increase of both sonication temperature and sonication time slightly, reaching a maximum (4000 mg GAE/ 100g DM) when X_2 and X_3 became 55 °C and 15 min, respectively (Figure 40F).

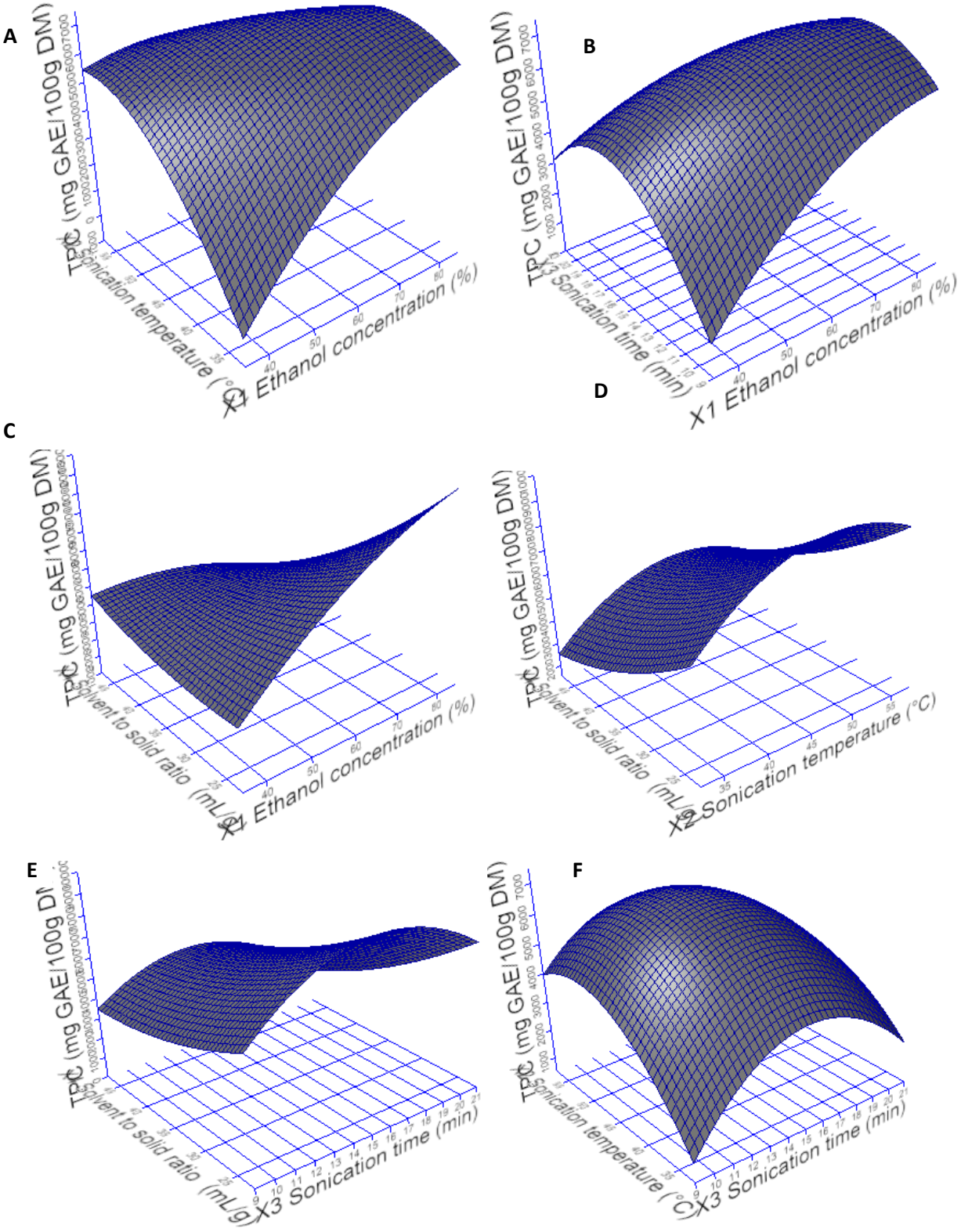


Figure 40 : Response surface analysis for the total phenolic yield from *Z.lotus* endocarp with UAE with respect to irradiation time and ethanol percentage(A); solvent-to-solid ratio and ethanol percentage (B); microwave power and ethanol percentage (C); solvent-to-solid ratio and microwave power (D); solvent-to-solid ratioand irradiation time (E); microwave power and irradiation time (F).

Validation and Verification of the Predictive Model for UAE

According to the result of response surface and prediction by this built model, the optimal conditions were thus obtained for the following conditions: ethanol at 80% (v/v), 43.15 °C sonication temperature, 14.26 min sonication time, and a liquid-to-solid ratio of 25 mL/g. To ensure that the predicted result was not biased to the practical value, experimental rechecking was performed using these deduced optimal conditions. The value of predicted extraction yield of TPC using UAE was for 10087.08 mg GAE/100g DM, that was consistent with the experimental yield of mg GAE/100g DM (Table 28). As we can see, the experimental and the predicted values are not different and their correlation indicates that the response of regression model is adequate to reflect the expected optimization for the extraction of phenolic compounds from *Z.lotus* endocarp.

Table 28 : Biological activities of *Zle* endocarp extract using UAE. Results are expressed as means ± standard deviation.

Factors	Ultrasound extraction
Sonication time (min)	14.26
Ethanol concentration (%)	80
Sonication temperature (°C)	43.14
Solvent solid/ratio (mL/ g)	25
Results	
Recovery of total phenolic TPC[a] (mg GAE[b]/100 g)	10087.08 ± 231.81
Recovery of total flavonoids TFC[c](mg QE[d]/100 g)	453.21± 2.83
Recovery of condensed tannins TTC[e] (mg CE[f]/100 g)	2315.04± 0.4
DPPH[g] scavenging EC_{50} (µg/mL)	0.75 ± 0.01
FRAP activity (mg $_{GAE}$/100g)	221.62 ± 1.91
HepG2 cells IC_{50} (mg/mL)	1.61 ± 1.48
MCF-7 cells IC_{50} (mg/mL)	3.23 ± 2.36

Optimal conditions for yield optimization were 80% ethanol, 14.26 min and 43.14°C and 25 mL/g (Table 28). The recovery of TPC extracted with microwaves through RSM model showed a significantly higher value (10087.08 ± 231.81GAE mg/ ml) than those obtained from UAE of jujube sarcocarp using ethanol 70% (31.76 ± 0.08 mg/100 g) **[95].** Additionally, TPC value extracted from Spanish jujube fruits using methanol ranged between 1442 and 3432 GAE mg/100 g is significantly lower than obtained results **[25].** However, this value is very higher than TPC observed from endocarp under MAE. Furthermore, the TFC of *Zls*

extracts using UAE (Table 28) was significantly higher than those obtained from other jujubes species (453.21± 2.83 mg QE/100 g), in comparison to TFC reported from jujube sarcocarp using ethanol 70% (1 ± 0.02 mg/100 g) **[95].** While, TTC of *Zls* was for 2315.04 ± 0.4 mg CE/100 g which is lower than TTC of *Zle* under MAE. From these finding, we can suggested that UAE preserved the TPC than by unsing MAE, while TFC were not preserved by UAE.

III.1.1. Effect of UAE on biological ativities

III.1.1.1.Antioxidants activity of Zle extract

The DPPH scavenging assay of the *Zle* extract (Table 28) revealed a significant highest activity with lowest EC_{50} value (0.75 µg/mL) than that of Tunisian *Z. lotus* leaves and fruits extract using methanol (0.10 ± 0.001 and 0.31 ± 0.005 mg/mL) **[56]**, Tunisian *Z. lotus* pulp and peel extracts using UAE with an EC_{50} of 0.28 mg/mL **[57]**, from *Z. mucronata* roots (0.029 ± 0.05 mg/mL) **[163]** and from Korian *Z. jujuba* seeds (mechu and sanzoin) (0.3 and 0.1 mg/mL), respectively **[139].** Overall, *Zle* extracts exhibited a high antioxidant effet in comparison to some *Zizyphus* species using both UAE and conventional methods. Furthermore, *Zle* extracted by UAE exhibited a lower iron-reducing power (221.62 ± 1.91 mg/100g) than that obtained by Wang, Cheng **[58]** with 471.6 ± 30.8 mg/100g from *Z. jujuba cv. Zaowangzao*.

III.1.1.2.Anti-proliferative activity of Zle extract

The *in-vitro* evaluation of *Zle* extracts on cytotoxicity effects were analysed (Table 28). The phenolic compouds of *Zle* studied were revealed to be non-toxic towards the studies cell lines **[174].** The maximum inhibition by the UAE extract was for 3.23 ± 2.36 and 1.61 ± 1.48 mg/mL for MCF-7 and $HepG_2$ cells, respectively. These findings can be due to the main bioactive compounds contained in *Zle* extract that may include flavonoids, tannins, alkaloids, terpenoids and saponins as observed previously from other jujube species and from other part of *Zizyphus lotus* which showed a strong antiproliferative activity against $HepG_2$ and MCF-7 cells **[40, 140].** In addition, Hoshyar, Mohaghegh **[175]** demonstrated that *Z. jujube* fruits extract was found to exhibit activity against MCF-7 cells with an IC_{50} value of 1.8 mg/mL after 24 h which is lower value than that of *Zle* extract. The effective cytotoxicity toward hepatocellular $HepG_2$ and beast MCF-7 could be related to the presence of the some secondary metabolites which were applicated in the inhibition of cell proliferation in different cancer types **[25].**

III.2. Optimizing phenolic extraction conditions by MAE method

Modeling and Fitting the Model Using RSM

Based on a Central Composite Design (CCD) using RSM, through a 2^3factorial experimental design, to optimize the Microwave-Assisted Extraction (MAE) of phenolic compounds from *Z.lotus* endocarp,was investigated. The experimental values of TPC extracts at various experimental conditions are presented in Table 29. In this study, only the solvent concentration, irradiation time and microwave power were studied, while the solid/liquid ratio were fixed at optimum that was found in UAE study. Each factor, containing three levels, was chosen from a series of single factor experiments. The model presented the total of 17 experiments.

Table 29: Central composite design with the observed responses and predicted values of TPC from endocarps using MAE.

Run	X1- Ethanol concentration (% v/v)	X2- Irradiation time (s)	X3- Irradiation power (W)	TPC yield (mg GAE/100gDM)	
				Experimental	Predicted
1	20(-1)	60(-1)	300(-1)	2926,44	2917,54
2	60(1)	60(-1)	300(-1)	3129,89	3192,43
3	40(0)	150(0)	300(-1)	5001,87	5061,78
4	20(-1)	240(1)	300(-1)	3240,21	3194,72
5	60(1)	240(1)	300(-1)	4835,68	4767,62
6	40(0)	60(-1)	400(0)	2778,56	2750,58
7	20(-1)	150(0)	400(0)	3255,08	3443,19
8	40(0)	150(0)	400(0)	4483,56	4375,14
9	40(0)	150(0)	400(0)	4599,39	4375,14
10	40(0)	150(0)	400(0)	4599,39	4375,14
11	60(1)	150(0)	400(0)	3949,38	4039,73
12	40(0)	240(1)	400(0)	3873,23	4179,67
13	20(-1)	60(-1)	500(1)	2955,08	2953,53
14	60(1)	60(-1)	500(1)	2597,80	2573,69
15	40(0)	150(0)	500(1)	5054,77	5273,32
16	20(-1)	240(1)	500(1)	4368,70	4236,53
17	60(1)	240(1)	500(1)	5215,43	5154,71

Eq. (16) , show the relationship between the independent variables for the extraction of total phenolic compounds of *Z.lotus* endocarp.

$$Y2 = 4375{,}14 + 298{,}26\,X1 + 714{,}54\,X2 - 633{,}68\,X12 - 910{,}02\,X22 + 792{,}40\,X32 + 324{,}50\,X1X2 + 251{,}45\,X2X3 \quad (16)$$

As shown in Table 29, ANOVA results indicated that positive linear effect of both ethanol concentration (X_1) and irradiation time (X_2) on TPC of jujube extracts is significant ($p < 0.001$) such as their interaction(X_1–X_2), and their quadratic terms. According to Eq. (16) the positive coefficients for both X_1, X_2 indicate positive influences that may increase the responses (TPC).

However, microwave power (X_3) only didn't exhibit any significant effect for linear and interaction effect (X_1–X_3) with ethanol concentration (p = 0,1698 and 0,0719, respectively), but it dit as a quadratic effect (p= 0,0006), also interaction with irradiation time (X_2X_3) where p = 0,0140. As represented in Table 30, the analyses of variance (ANOVA) confirm the adequacy of the mathematical models obtained from *Z.lotus* endocarp extraction by microwaves.

Table 30 : Analysis of variance for the experimental results obtained by using MAE.

Parameter[a]	Estimated coefficients	Standard error	DF[b]	Sum of squares	F-value	Prob > F
Model B_0	4375,1487	93,51739	9	12580382	29,2652	<0,0001*
Linear						
X_1 - Ethanol	298,26591	69,11143	1	889625,5	18,6255	0,0035*
X_2 - Time	714,54773	69,11143	1	5105784,6	106,8963	<0,0001*
X_3- Power	105,76839	69,11143	1	111869,5	2,3421	0,1698
Quadratic						
X_1^2	-633,6829	133,5192	1	1075861,7	22,5246	0,0021*
X_2^2	-910,0203	133,5192	1	2218781,8	46,4531	0,0002*
X_3^2	792,40541	133,5192	1	1682315,1	35,2215	0,0006*
Interaction						
X_1X_2	324,50313	77,26893	1	842418,3	17,6371	0,0040*
X_1X_3	-163,6815	77,26893	1	214333,2	4,4873	0,0719
X_2X_3	251,45931	77,26893	1	505854,3	10,5907	0,0140*
Lack Of Fit			5	325403,21	14,5528	0,0655
Pure Error			2	8944,08		
Total Error			7	334347,30		
R^2					0,974111	
R^2 Adjusted					0,940826	
CV%	5.55					
RMSE	218,5495					
Corr.Total			16	12914729		

The ANOVA analysis have been used to determine the adequacy of the model. However, the response surface quadratic regression model showed that the experimental and predicted

models were highly significant ($p < 0.001$) with highly F-value (29,26), at the same time, a high proportion of variability was explained by the RSM models for TPC as indicated by the values of R-squared (0,97 and 0,94 for R^2 and R^2adj, respectively) which were close to 1 that indicates the good correlation of the model. Additionally, the F -value of lack-of fit statistics was not significant ($p > 0.05$), thus confirming the validity of the model. Thus,it can be concluded that the model could be used for the prediction of TPC from *Z.lotus* endocarp.

Response Surface Analysis (RSA) for MAE

The influence of the three parameters on TPC of jujube endocarp is depicted in Figure 41. Our results indicate that, yield of TPC increased (1800 mg GAE /100g DM) at longeur irradiation time (240 s) as shown in Fig.41A, with increasing ethanol concentration up to 40% and then it remained significantly constant. Therefore, Figure 41B shows in general, the highest value of TPC (4500 mg GAE /100g DM) was achieved at a microwave power of 500 W and 60% of ethanol. Similar results have been obtained by Chew, Khoo **[180]** the 40% ethanol extract of *Centella asiatica* extracts. As we can observe in Figure 41C that the interaction between microwave power and irradiation time was not significant to the TPC yield as demosntated previously on Eq and Table 29.

It is very important to notice taht the increase in microwave power at longeur irradiation time, leads to the decrease of the response which affect negatively the extraction yeild of TPC from *Z.lotus* endocarp (2000 mg GAE /100g DM at 60 s). Our results are simillar to those of **[181]** which demonstrated that there is a strong relation between the microwave power and irradiation time which is based on the function shape as seen that the TPC yield of *Vitisvinifera L. cv. Ahmar Bou-Amar* seeds and skin decrease at the conditions seen in our case. To conclude, juste a moderate microwave power will suffice to increase extract phenols from *Z.lotus* endocarp by MAE which means confire the solubility of the target analytes, the diffusionrate, and the mass transfer of desired compounds. Others show that higher microwave power can affect the quality of the phenolic compounds extracts with causing its degradation of thermo labile ones **[182].** Additionally, high power may encourage solvent loss through evaporation**[183].**

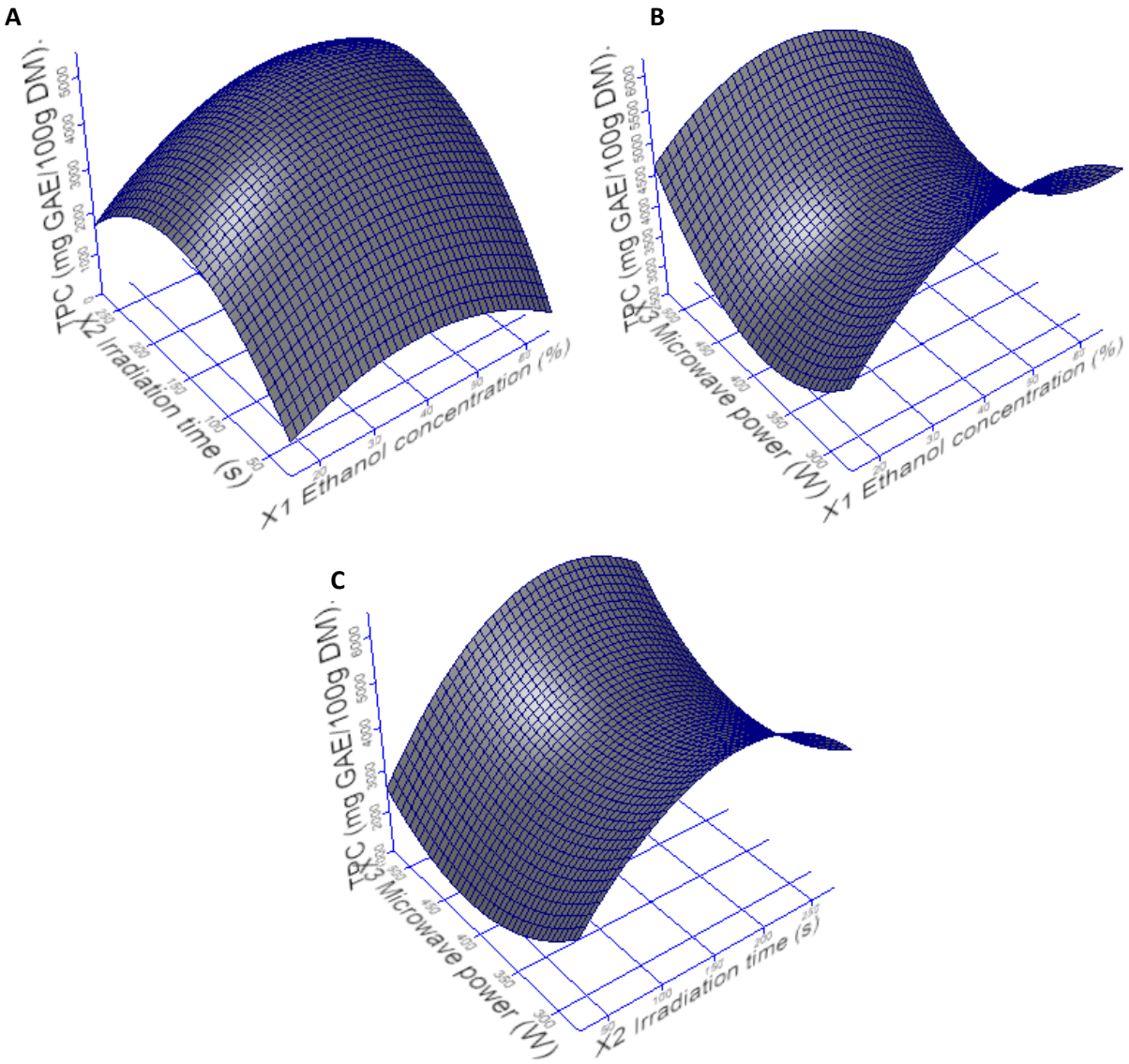


Figure 41 : Response surface analysis for the total phenolic yield from *Z.lotus* endocarp with MAE with respect to irradiation time and ethanol percentage(A); solvent-to-solid ratio and ethanol percentage (B); microwave power and ethanol percentage (C).

Validation and Verification of the Predictive Model

According to the result of response surface and prediction model, the optimal conditions were thus obtained for the following conditions: ethanol at 45.07% (v/v), 201.83 s irradiation time, and 500 W microwave power. The value of predicted extraction yield of TPC using UAE was for 5568.58 mg GAE/100g DM, that was consistent with the experimental yield of mg GAE/100g DM (Table 30). The validity of those predicted optimal values of TPC for *Z.lotus* endocarp extracts, was also experimentally confirmed which were very close to the values predicted by the models.

III.2.1. Effect of experimental coditions on TPC extraction yield

Optimal conditions for yield optimization were 45.07% ethanol, 3.3 min and 500 W and 30 mL/g (Table 31). The recovery of TPC extracted with microwaves through RMS model showed a significantly higher value (5568.59 ± 361.11 GAE mg/ ml) than those obtained from UAE of jujube sarcocarp using ethanol 70% (31.76 ± 0.08 mg/100 g) **[95].** Additionally, TPC value extracted from Spanish jujube fruits using methanol ranged between 1442 and 3432 GAE mg/100 g is significantly lower than obtained results **[25].** Furthermore, the TFC of *Zls* extracts using MAE was significantly higher than those obtained from other jujubes species (781.6 ± 5.11 mg QE/100 g), in comparison to TFC reported from jujube sarcocarp using ethanol 70% (1 ± 0.02 mg/100 g) **[95].** Similarly, two different Chinese jujubes that were around 122.1 to 319.5 and 65.1 to 158.6 mg/100 g, respectively **[31, 96].** While, TTC of *Zls* was for 8350.46 ± 7.22 mg CE/100 g which was very higher in comparison to polymeric proanthocyanidins extracted from *Zizyphus jujuba* fruits obtained by Wojdyło, Carbonell-Barrachina **[25]** that showed a value between 939 and 2548 mg/100 g in depends on cultivars. In addition, Gao, Wu **[97]** compared proanthocyanidin extracts from several *Zizyphus* varieties, among those containing the highest content about 413.7 ± 23.1 mg/100 g from *Zizyphus jujuba cv. Zaowangzao* which is significantly lower than our extracts.

Table 31. Phenolic yields of jujube endocarp samples and some biological activities under optimum extraction conditions by MAE.

Factors	Microwave extraction
Irradiation time (min)	3.3
Ethanol concentration (%)	45.07
Microwave power (W)	500
Solvent solid/ratio (mL/ g)	30
Results	
Recovery of total phenolic TPC[a] (mg GAE[b]/100 g)	5568.59 ± 361.11
Recovery of total flavonoids TFC[c](mg QE[d]/100 g)	781.6 ± 5.11
Recovery of condensed tannins TTC[e] (mg CE[f]/100 g)	8350.46 ± 7.22
DPPH[g] scavenging EC_{50} (mg/mL)	0.64 ± 0.01
FRAP activity (mg $_{GAE}$/100g)	262.99 ± 0.03
HepG2 cells IC_{50} (mg/mL)	0.96 ± 2.96
MCF-7 cells IC_{50} (mg/mL)	0.57 ± 3.9

The variation results in TPC, TFC and TTC from our study using MAE and others compared in literature is related to many factors such as family, geographical conditions, varieties of plants, extraction method and environemental effects **[31, 65, 98, 99].** This study provides a successful RSM optimization study of polyphenols extraction conditions from *Zle* with combination to microwave process in less time and solvent consumption due to its heating microwave energy. Howerver, it will be more interesting to know the different phytochemicals present in the *Zle* extracts and evaluation each of them are responsible for some biological activities studied in this work.

III.2.2. Identification of phenolic compounds from *Z. lotus* endocarps

The content of bioactive compounds extracted from *Zle* obtained by LC-MS/MS analysis in the negative and positive mode (Figure 42) were demonstrated in Table 32. The identification of phytochemicals was carried out using Data Analysis program from Bruker, pubchem, metlin and others references on the *Zizyphus* genus were used to find the chemical structures. Results indicated the presence of 39 biomolecules based on the exact mass and on the fragmentation patterns. The heatmap was made to compare the intensities of identified compounds and to visualize the most higher amount as shown in Figure 42.

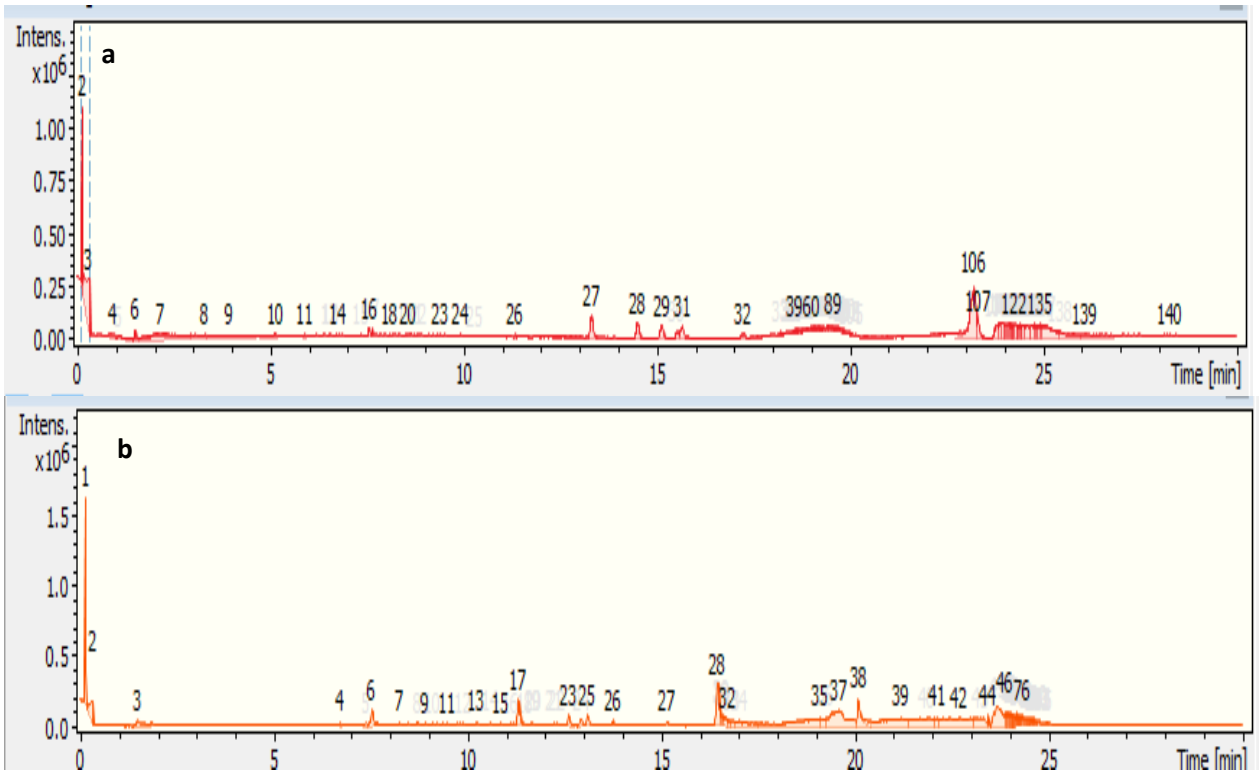


Figure 42 : Total ionic chromatogram and ionic chromatograms extracted from ions identified in *Z. lotus* endocarps : (a) LC-MS/MS (-) and (b) LC-MS/MS (+).

The results obtained during the screening phytochemical by khnowing their intensities (Figure 43) showed that the secondary metabolite are 26.60% of all compounds detected in *Zle* samples. Where terpenes presented 45.61% from all secondary metabolites in which tensagenin was the gave a highest intensity of all terpenes and represents 48.77% from all terpenes and 22.25% from all secondary metabolites. Followed by some alkaloids reprensenting 16.12% of all secondary metabolite. However, flavonoids present in endocarp extracts are very lower in comparison to other jujube parts, from our sample it is fro 11.74% of all secondary metabolites where ampelopsin A was the major compound (51.56% of total flavonoids). *Zle* is not very used like other used parts of jujubes for the treatment of several diseases due to their lower content of phenolic compounds especially flavonoids most known substances for their antibacterial, antioxidant and antifungal effect agents which is not the case for fruits, seeds and leaves **[1, 2].**

a. Characterization of flavonoids and derivative

Some flavanones were detected at different retention times 1.06, 7.57, 7.58 and 8.46 min with a $[M-H]^-$ ions at m/z of 341.1043, 469.1288, 531.1220 and 431.1041, respectively. These compounds were tentatively identified as Luteolin 5,7,3',4'-tetramethyl ether, ampelopsin A, apigenin-7-methyl ether 5-(6''-malonylglucoside) and kaempferol-7-rhamnoside, respectively. This last was previousely detected in jujube plant **[3].**

b. Characterization of terpenes

Three sesquiterpenes were observed at 14.49, 15.48, 18.14 with m/z of 309.1698, 397.2205, 253.2139 in negative mode. These compounds were assigned as farformolide B, stephanol and geranyl ethyl butirate, respectivly **[4].** Morever, one compound at Rt = 16.49 min, which showed a m/z of 449.2976 in postive mode was tentatively identified as tenasogenin. From Figure 43 we observed clearly that terpenoids are present with high intensities in *Zle* extracts and arround 45.61% of all secondary metabolites in which tensagenin are 48.77% of all terpenoids. This compound was very used due to its antidiabetic effect and identified as a pregnane ester from *Marsdenia tenacissima* **[5, 6].** In addition, one peak at Rt = 7.46 min with a m/z of 479.1598 was assigned as paeoniflorin (monoterpene glycoside). These compounds were never been detected in jujube endocarps.

Compound of *Z. lotus* endocarp extract	Intensity
Indole derivative	
Luteolin 5,7,3',4'-tetramethyl ether	
3-benzoyl-2-phenylnaphtho[2,3-b]furan-4,9-dione	
Glucose	
Pentapetide (Pro-Tyr-Pro-Arg-Ser)	
Tripeptide (Ser-Pro-Asn)	
Tetrapeptide (Leu-Val-Asp-Ala)	
Tetrapeptide (Pro Leu Asp Ser)	
Tetrapeptide (Asp-Val-Ser-Asp)	
Paeoniflorin	
7-Decyl-3-methoxy-2-(2,3,4-trimethoxyphenyl)-4H-1-benzopyran-4-one	
Tetrapeptide (Asp-Val-Ser-Asp)	
Ampelopsin A	
Apigenin-7-methyl ether 5-(6''-malonylglucoside)	
Kaempferol-7-rhamnoside	
Imidazole-4-Carboxamide	
N-[2-[2-(2-aminoethylamino)ethylamino]ethyl]-2-[1-[2-(2-aminoethylamino)ethyl]-4,5-dihydroimidazol-2-yl]acetamide	
1-(1,8-diamino-3,6,10,13,16,19-hexazabicyclo[6.6.6]icosan-3-yl)propan-2-one	
1-Octyl-3-(Piperidin-1-Ylmethyl)-5-M-Tolyl-1H-Indole	
4-Gingerol	
Farformolide B	
2-Hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy)propyl] phenyl]propan-1-one	
N-propan-2-yl-5-[[1-(propan-2-ylamino)tetrazol-5-yl]diazenyl]tetrazol-1-amine	
Aspartic Acid	
Phenyl 3,5-di-tert-butyl-4-hydroxybenzoate	
Stephanol	
Methyl 3,4,5-tris(2-propoxyethoxy)benzoate	
Stearyllisomaltse	
3-(decyloxy)-2-hydroxypropyl prop-2-enoate	
Tenasogenin	
Triethylene glycol monododecyl ether	
Acetic acid--11-methyltridecan-1-ol (1/1)	
T-Boc-Aminooxy-PEG3-Amine	
Geranylethyl butirate	
(8)-Gingerol	
2,4-Diethoxy-2-methoxy-6,10-dimethylundecane	
3-Hydroxybicyclo(4.2.0)octa-1,3,5-triene-7,8-dione	
Imidapril	

Figure 43 : Heatmap of the chemical profile and of *Z. lotus* endocarps. Mean values refer to colors from minimum displayed in beige to maximum represented with dark brown.

c. Characterization of alkaloids

Alkaloid group was characterised in this study and observed with a negative mode at 1.05, 13.15 min with m/z of 387.1099 and 415.3124, respectively were assigned as indole derivatives. These compounds were detected previousely from other plants and known as one of antimalarial, antioxidant and antiiflammatory agents **[7].** Morever, compound observed at 17.11 with m/z of 319.2808 $(M+H)^+$ was attributed to quinoline derivative. This was previously detected in some *Zizyphus* species as a quinoline alkaloids which is one of most known as antioxidant and anti-aging agent **[8, 9].**

d. Characterization of other compounds

The analysis showed the detection of two gingerol with a deprotonated molecular at 13.29 and 18.42 with a m/z of 265.1444 and 321.2068, respectively. These compound are originated from *Zingiber* and known with a potential anticancer, antioxidant and anti-Alzheimer effects **[10, 11].** This compound was previousely detected from jujubes **[12-15].** The mass spectrometric characterization of compounds at 15.76, 16.47 and 19.02 with 441.2473, 287.2185 and 303.2864, respectively were attributed to methyl 3,4,5-tris(2-propoxyethoxy)benzoate, 3-(decyloxy)-2-hydroxypropyl prop-2-enoate and 2,4-diethoxy-2-methoxy-6,10-dimethylundecane.

The primary metabolites contain arround 69.60% of all compounds found in our sample as shown in Figure 43. Organic acids are the major compounds with high intensities which presented 52.37% from all primary metabolites. It has been noticed that both N-[2-[2-(2-aminoethylamino)ethylamino]ethyl]-2-[1-[2-(2-aminoethylamino)ethyl]-4,5-dihydro imidazol-2-yl]acetamide and 3- (decyloxy)- 2-hydroxypropyl prop-2-enoate were found as the major abundant compounds with 18.29 and 16.98% of all primary metabolites and 35, 32.49% from all organic acids, respectively. These compounds were never been reported from *Z. lotus*. Thus, it is used under another structure of 2-propenoic acid from jujubes and showed a significant effect as antioxidant, antiinflammatory and anticancer against breast cell lines **[16, 17].** In addition, peptides are present with low intensities in comparison to other primary substances with 8.61% of *Zle* extracts and 12.37% of all primary metabolites. Jujube extracts have been previously found with high protein content due to th seeds part which is known by its functional properties (emulsifying activity, foaming capacity, emulsifying stability, water retention and solubility) **[18].** Therefore, as most potentially valuable compound which play

an important antioxidant, anticholinesterase roles that can be used as functionnal food **[19, 20].** However, at date no report have been studied the peptides of jujube endocarps.

a. Characterization of sugars

The examination of chromatograms obtained at both positive and negative modes indicated the presence of some sugars at 1.11 and 16.45 min with m/z of 179.0535and 595.4116 which were clearly attributed to glucose and stearyllisomaltase **[21].**

b. Characterization of peptides

The mass spectrometric characterization of compounds observed at 6.78, 7.20, 7.22 and 7.43 with m/z of 619.3104, 317.1460, 417.2346 and 431.2139 $(M+H)^+$ were identified tentatively as tetrapeptide and pentapeptide. In negative mode, one pick was found at retention time of 7.55 min with m/z of 433.1562 and identified as tetrapeptide. Additionaly, peak at 15.36 min in postivie mode with m/z of 301.1376 was attributed to aspartic acid. These compounds were found in several vegetable plants.

c. Characterization of other compounds

The mass spectrometric characterization of compounds at 1.07, 7.48, 13.12, 15.11 and 20.09 in positive and negative modes with m/z of 377.0797, 483.2735, 371.3229, 353.1955 and 149.0216 were assigned as 3-benzoyl-2-phenylnaphtho[2,3-b]furan-4,9-dione, 7-Decyl-3-methoxy-2-(2,3,4-trimethoxyphenyl)-4H-1-benzopyran-4-one,1-(1,8-diamino-3,6,10,13,16,19 -hexazabicyclo [6.6.6]icosan-3-yl) propan-2-one, 2-Hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy) propyl] phenyl] propan-1-one and 3-Hydroxybicyclo(4.2.0)octa-1,3,5-triene-7,8-dione. However, other peaks obtained with $(M-H)^-$ showed the presence of some compounds with retention times of 11.18 and 11.32 min with m/z of 223.1315, 343.2925, respectively were identified as imidazole carboxylate derivative and N-[2-[2-(2-aminoethyl amino) ethyl amino] ethyl]-2-[1-[2-(2-amino ethyl amino) ethyl]-4,5-dihydroimidazol-2-yl]acetamide, respectively **[22].** All these compounds were reported for the first time from jujubes and can participate in the studied activities what we have noticed in the Figure 43.

Some peaks with high intensities were observed at 7.34, 12.62, 12.96, 20.11 and 23.25 min with a positive and negative mode at m/z of 447.7601, 239.1591, 111.1156, 391.2804 and 134.8914, respectively, were unknown compounds.

Table 32: Identification proposal of bioactive compounds present in the *Z. lotus* endocarp MAE by LC–MS/MS in ESI negative and positive mode. Peaks with a minus/plus superscript were analyzed in negative/positive mode. Compounds are indicated by retention time (Rt) order and numbered according to the appearance in each chromatogram, negative [M − H]− or positive mode [M + H]+. NF : not fragmented.

Rt (min)	[M - H]-/ [M + H]+	Intensity (%)	E	Fragments ion (m/z) ; intensity(%)	Molecular formula	Tentative identification	References
1.05-	387.1099	100	0	59.0099 (100), 89.0211 (81.2), 119.0318 (56.7)	$C_{21}H_{16}N_4O_4$	Indole derivative	125554521 (pubchem)
1.06-	341.1043	36.16	3.34	71.0094 (100), 158.0790 (50.9), 119.0293 (47.3)	$C_{19}H_{18}O_6$	Luteolin 5,7,3',4'-tetramethyl ether	49500 (metlin)
1.07-	377.0797	19.82	5.9	387.1090 (100), 96.9567 (41.2), 44.9941 (32.9), 112.9824 (13.6)	$C_{25}H_{14}O_4$	3-benzoyl-2-phenylnaphtho[2,3-b]furan-4,9-dione	421759 (metlin)
1.11-	179.0535	22.82	-8.37	71.0094 (100), 44.9939 (80.6), 136.0506 (74.6)	$C_6H_{12}O_6$	Glucose	5793 (pubchem)
6.78+	619.3104	41.25	15.3	103.0376 (100), 45.0331 (46.9), 191.0892 (23.6), 323.1692 (16.3)	$C_{28}H_{42}N_8O_8$	Pentapetide (Pro-Tyr-Pro-Arg-Ser)	264985 (metlin)
7.20+	317.1460	100	-5.6	106.0641 (100), 132.0430 (25.4), 225.0997 (14.6)	$C_{12}H_{20}N_4O_6$	Tripeptide (Ser-Pro-Asn)	19282 (metlin)
7.22+	417.2346	60.02	5.4	45.0326 (100), 89.0587 (43.8), 133.0833 (20.8), 177.1095 (18.8), 144.0757 (17)	$C_{17}H_{30}N_4O_7$	Tetrapeptide (Leu-Val-Asp-Ala)	182319 (metlin)
7.34+	447.7601	74.55	-	45.0329 (100), 89.0587 (46.7), 166.0878 (18.3), 133.0842 (17.3)	-	Unknown	-
7.43+	431.2139	40.48	-0.7	45.0328 (100), 103.0380 (91.6), 59.0485 (44.4), 151.0648 (25.4), 132.0429 (21.5)	$C_{18}H_{30}N_4O_8$	Tetrapeptide (Pro Leu Asp Ser)	203134 (metlin)
7.46-	479.1598	15.44	-8.2	249.0639 (100), 91.0362 (82.2), 59.0096 (37.8)	$C_{23}H_{28}O_{11}$	Paeoniflorin	68046 (metlin)
7.48+	483.2735	79.81	1.3	45.0330 (100), 89.0587 (38.9), 133.0846 (34.6), 177.1107 (17)	$C_{29}H_{38}O_6$	7-Decyl-3-methoxy-2-(2,3,4-trimethoxyphenyl)-4H-1-benzopyran-4-one	853802 (metlin)
7.55-	433.1562	100	-2.3	91.0365 (100), 249.0642 (86.9), 59.0099(26.7)	$C_{16}H_{26}N_4O_{10}$	Tetrapeptide (Asp-Val-Ser-Asp)	126582 (metlin)
7.57-	469.1288	32.89	1	249.0639 (100), 91.0364 (88), 59.0094 (31.9), 341.1104 (12.9)	$C_{28}H_{22}O_7$	Ampelopsin A	417152 metlin
7.58-	531.1220	14.25	16.34	96.9568 (100)	$C_{25}H_{24}O_{13}$	Apigenin-7-methyl ether 5-(6"-malonylglucoside)	49440 (metlin)
8.46-	431.1041	23.1	-13.2	105.0161 (100), 75.0049 (43.3)	$C_{21}H_{20}O_{10}$	Kaempferol-7-rhamnoside	449600 (metlin)
11.18-	223.1315	21.77	-0.8	166.0611 (100), 151.0368 (59.5), 195.1341 (43.4), 130.9801 (13.1)	$C_9H_{16}N_6O$	Imidazole-4-Carboxamide	135460287 (Pub Chem)
11.32+	343.2925	100	0.9	240.2300 (100), 57.0691 (17.9)	$C_{15}H_{34}N_8O$	N-[2-[2-(2-aminoethyl amino) ethyl amino] ethyl]-2-[1-[2-(2-amino ethyl amino) ethyl]-4,5-dihydroimidazol-2-yl]acetamide	59846977 (PubChem)
12.62+	239.1591	100	10.1	57.0693 (24.2), 111.0397 (15.9)	$C_{10}H_{18}N_6O$	Unknown	586850(metlin)
12.96+	111.1156	85.45	-	69.0688 (100), 87.0026 (28.2)	-	Unknown	-
13.12+	371.3229	100	3.3	268.2610 (100), 57.0690 (16.3)	$C_{17}H_{38}N_8O$	1-(1,8-diamino-3,6,10,13,16,19-hexazabicyclo[6.6.6]icosan-3-yl)propan-2-one	126608456 (Pub Chem)
13.15-	415.3124	89.4	-1.4	44.9942 (100), 102.0529 (62.7), 311.3065 (11.3)	$C_{29}H_{40}N_2$	1-Octyl-3-(Piperidin-1-Ylmethyl)-5-M-Tolyl-1H-Indole	49783395 (PubChem)

13.29-	265.1444	100	0.5	96.9565 (100), 79.9533 (20.4)	$C_{15}H_{22}O_4$	4-Gingerol	263539 (metlin)
14.49-	309.1698	100	2.9	96.9565 (100), 79.9536 (36)	$C_{17}H_{26}O_5$	Farformolide B	371151 (metlin)
15.11-	353.1955	100	-0.84	96.9567 (57.3), 79.9536 (27.6)	$C_{19}H_{30}O_6$	2-Hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy)propyl] phenyl]propan-1-one	138525818(pubchem)
15.36+	301.1376	100	-7.5	56.9657 (26.6), 184.9159 (13.1)133.0806 (12.8)	$C_8H_{20}N_4O_8$	Aspartic acid	44147333 (PubChem)
15.48-	397.2205	100	3.77	96.9568 (38.5)	$C_{21}H_{34}O_7$	Stephanol	101289689 (pubchem)
15.76-	441.2473	39.93	4.7	96.9569 (40.6), 79.9537 (14.2)	$C_{23}H_{38}O_8$	Methyl 3,4,5-tris(2-propoxyethoxy)benzoate	924314 (metlin)
16.45+	595.4116	25.72	10.76	309.1997 (100)	$C_{30}H_{58}O_{11}$	Stearyllisomaltase	9894835 (pubchem)
16.47+	287.2185	37	11.1	69.0690 (100), 111.1157 (54.3), 55.0534 (22.1)	$C_{16}H_{30}O_4$	3-(decyloxy)-2-hydroxypropyl prop-2-enoate	920161 (metlin)
16.49+	449.2976	20.67	-17.5	69.0692 (100), 111.1160 (58.2), 127.0049 (24.1), 145.0161 (19.3), 181.0370 (15.2)	$C_{26}H_{40}O_6$	Tenasogenin	101277354 (pubhem)
17.06+	319.2808	46.77	20.35	45.0329 (100), 89.0588(72.1), 71.0848 (61.3), 57.0686 (56.5)	$C_{20}H_{34}N_2O$	Quinoline derivative	100310492 (pubchem)
18.14-	253.2139	21.50	11.9	116.9262 (100), 99.9212 (75.2), 44.9931 (52)	$C_{16}H_{28}O_2$	Geranylethyl butirate	6437648 (pubchem)
18.42-	321.2068	18.80	0.9	96.9567 (100), 79.9536 (17.7)	$C_{19}H_{30}O_4$	(8)-Gingerol	71735 (metlin)
19.02+	303.2864	32.13	9.8	69.0692 (100), 111.1154 (97.2), 45.0332 (38.2), 57.0690 (29.8)	$C_{18}H_{28}O_3$	2,4-Diethoxy-2-methoxy-6,10-dimethylundecane	706719(metlin)
20.09+	149.0216	15.47	11.7	65.0382 (100), 121.0276 (66.5), 93.0322 (22.4), 66.0407 (14.7)	$C_8H_4O_3$	3-Hydroxybicyclo (4.2.0)octa-1,3,5-triene-7,8-dione	393922(metlin)
20.11+	391.2804	100	-	149.0218 (100), 57.0692 (28.1), 71.0849 (26), 167.0317 (15.8)	-	Unknown	-
23.25-	134.8914	100	-	99.9223 (100)	-	Unknown	-

III.2.3. Biological Activities

Some biological activities of *Zle* extracts under MAE process were looked for as represend in the Table 31.

III.2.3.1.Antioxidants activity of *Zle* extract

The antioxidant activity was evaluated with DPPH· radical scavenger as shown in Table 31. The *Zle* extracts that exhibited high content of phenolic compounds with a remarkably significant antioxidant effet with EC_{50} value of 0.64 ± 00.01 mg/mL which is very higher than sarcocarp of *Z. jujuba* (8% of DPPH inhibition) **[25]**. However, these values are very lower than other *Zizyphus* species from other parts, Choi, Ahn **[139]** obtained from Korian *Z. jujuba* seeds (mechu and sanzoin) a highest DPPH· activity than obtained results (0.3 and 0.1 mg/mL respectively). Our results gave a lower antioxidant effects than results obtained by Hammi, Jdey **[57]** that reported an EC_{50} of 0.28 mg/mL for Tunisian *Z. lotus* pulp and peel extracts using UAE. Regarding the iron-reducing power FRAP assay for the *Zle* extracts, our results

showed a significant value of 262.99 ± 0.03 mg GAE/100 g. Our finding are significantly lower than those of Wang, Cheng **[58]** (471.6 ± 30.8 mg CE/100 g) obtained from *Z. jujuba cv. Zaowangzao*. The antioxidant capacity of *Z. lotus* extracts have also been evaluated in previous works **[57, 99, 140, 141].**

Morever, we can suggest that the solvent found in this sutdy have strong effect on antioxidant activity wich is in agreement to Kim and Son **[95].** This activity value is depending on the phenolic compounds contain in the extracts, as well as the extraction method. As demonstarted by Wojdyło, Carbonell-Barrachina **[25],** Dzoyem and Eloff **[142],** the presence of flavonoids, such as flavan-3-ols and flavonols in jujube extracts and particularly hydroxyl group position can act as proton donating and contribute to increasing radical scavenging activity. However, beacause of the absence of some compounds from this part of jujube plant which probably have a good antioxidant potential. We can resume that these lower antioxidant activities are mainly to the absence of the some actif compounds in endocarp part.

III.2.3.2.Anti-proliferative activity of Zle extract

The *in-vitro* evaluation of *Zle* extracts on cytotoxicity effects were analysed and represented as IC_{50} values which confirm the concentration of extracts that killed 50% of the cells was obtained from dose-response curves. The phenolic compouds of *Zle* studied were revealed to be non-toxic towards only to $HepG_2$ cell line because the value is higher than 0.1 mg/mL. This value is considered as limit of toxicity to human cell lines **[174].** The value of 0.96 ± 2.96 and 0.57 ± 3.9 mg/mL which correspond to the maximum inhibition present in the UAE extract for $HepG_2$ and MCF-7 cells, respectively. These findings can be due to the main bioactive compounds contained in *Zle* extract that may include alkaloids, terpenoids and saponins as observed previously from other jujube species and from other part of *Zizyphus lotus* which showed a strong antiproliferative activity against $HepG_2$ and MCF-7 cells **[40, 140].** In addition, Hoshyar, Mohaghegh **[175]** demonstrated that *Z. jujube* fruits extract was found to exhibit activity against MCF-7 cells with an IC_{50} value of 1.8 mg/mL after 24 h which is higher value than that of *Zle* extract. The effective cytotoxicity toward hepatocellular $HepG_2$ and beast MCF-7 could be related to the presence of the some secondary metabolites, some of them are considered the major class of jujube polyphenols, represented from 89 to 94% of the total phenolic contents mainly flavan-3-ols such as monomer (as (-)-epicatechin, gallocatechin gallate, and (+)-catechin), dimer (procyanidine B2) and polymeric proanthocyanidins in *Zls*, which were applicated in the inhibition of cell proliferation in

different cancer types **[25].** As well as, Rached, Barros **[40]** concluded that quercetin-3-*O*-rutinoside found in other parts of *Z. lotus* which represents 50% of jujube flavonoids offers a plausible explanation of the observed cytotoxicity. The present work data suggests that in some cases the ultrasound extraction can positively influence the extraction yield of TPC and also the antitumor activity against the tested cell lines.

Chapter IV. Compraison between optimized microwave and ultrasound-assisted extraction of Total Phenolic Compounds from *Zizyphus lotus* L parts using Response Surface Methodology and its biological effects

Abstract

This work covers the parameter of extraction of phenolic compounds from pulp, seed and endocarp under both MAE and UAE techniques combined with RSM model (BBD and CCD) in order to evaluate different parameters affecting extraction procedures. The optimized extracts obtained by only MAE were characterized by LC-ESI-MS/MS to identify the bioactive compounds present in the jujube. Besides, an *in vitro* evaluation of their antioxidant, antiacethylcholinesterase and antitumor activities were described and compared for all parts of *Z. lotus* including pulp, seed and endocarp obtained at our previous optimized conditions that come from not yet published papers. Results showed that MAE was the best method giving much compound yeilds with higher biological activities in comparison to UAE. These can help to understand the relationship between polyphenols of different *Z. lotus* parts and their biological activities through green extraction techniques which could have a therapeutic applications in the future.

Key words : MAE, UAE, seed, pulp, endocarp and biological activities.

IV.1. Comparison between ZLP, ZLS and ZLE on bioactive compounds

The choice of extraction method is a very critical point to take into account in the extraction of phenolic compounds from plants with preserving its biological propreties. Many advantages and disadvantages of extraction processes were demonstrated mainly environmental friendliness, safety, extraction yield, production cost and complexity **[9].** The TPC, TFC and TTC recovery obtained from three parts of jujube by both MAE and UAE methods are represented in Table 32 and Figure 43.

Table 32 : TPC, TFC and TTC recovery extracted by UAE and MAE from different parts of *Z. lotus*.

	ZLP		ZLS		ZLE	
	UAE extracts	MAE extracts	UAE extracts	MAE extracts	UAE extracts	MAE extracts
TPC (mg $_{GAE}$/100g)	4818.72 ± 770.02^{b}	7394.53 ± 529.33^{a}	2406.083 ± 79.87^{c}	6852.48 ± 1020.06^{b}	10087.08 ± 231.81^{a}	5568.59 ± 361.11^{c}
TFC (mg $_{QE}$/100g)	1098.76 ± 78.46 ^{a}	1013.38 ± 72.95 ^{a}	489.50 ± 0.38^{b}	499.21 ± 0.59^{c}	453.21± 2.83^{c}	781.6 ± 5.11^{b}
TTC (mg $_{EC}$/100g)	10339.50 ± 0.1^{b}	14200.54 ± 2485.51^{b}	15787.10 ± 0.10^{a}	25000.74 ± 1.28^{a}	2315.04± 0.4^{c}	8350.46 ± 7.22^{c}

The MAE gave a higher yield for TPC, TFC and TTC recovery from seed extracts. Whereas, no significant differences for TFC recovery of ZLP observed between MAE (1013.38 ± 72.95 mg $_{QE}$/100g) and UAE (1098.76 ± 78.46 mg $_{QE}$/100g). Similar observation than ZLS for both TPC and TTC yields was noticed where MAE was the best. While for TPC, only ZLE obtained by UAE (10087.08 ± 231.81 mg $_{GAE}$/100g) gave value supperior to that of MAE (5568.59 ± 361.11 mg $_{GAE}$/100g), this was different in ZLE with TFC and TTC which were higher by MAE than UAE. On the basis of these results, MAE should be choosed as method of choice based on the extraction time and lower solvent consumption.

There was an overall higher TPC recovery of the ZLE (10087.08 ± 231.81 mg $_{GAE}$/100g) compared to that of pulp and seed by both UAE and MAE (Table 32). As there was no report on endocarp of jujubes, these results are mainly due to the effect of cavitation phenomenea collapsing bubbles of ultrasound method which positively influence the phenols recovery, hence exposure to a favorable conditions sush as temperature, solvent concentration (80%, low purity) and solvent to solid ratio.

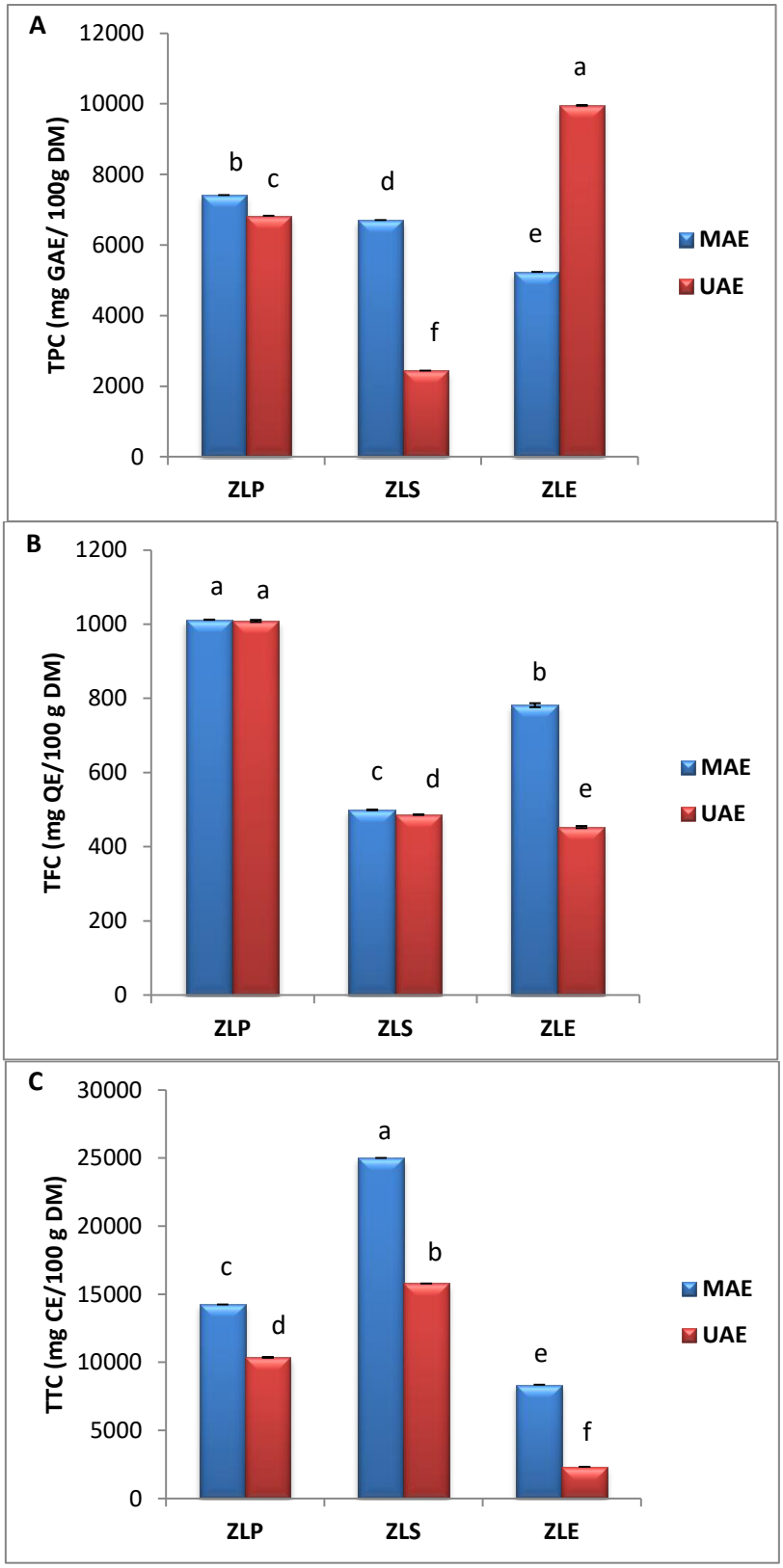


Figure 44 : Total phenolic (A), flavonoid (B) and condensed tannin (C) contents of *Zizyphus lotus* extracts obtained by UAE and MAE processes. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).

These conditions may probably related to the good mass transfer conditions optimized during extraction which increase significatively the permeability of jujube extract tissues that facilitated the solvent penetration into the inner part of the material **[184, 185].** However, the longer period (> 14 min) of exposure of polyphenols extract to the ultrasonic waves could decrease the yield and destruction of cells by formation of free radicals. As well as, temperature and power are promoter of quality and extraction yeild but longer exposure might react negatively (case of seed and pulp by UAE extracts) **[186].**

As a resume, jujube plant possess different types of bioactive compounds such as TPC, TFC and TTC **[140].** It was seen that MAE process showed the highest polyphenols recovery values implying its extraction effectienty. However, when jujubes extracted by UAE were analyzed, only the TPC values obtained from ZLE was significantly different to other UAE extracts and higher than MAE from all parts. The extraction part of *Z. lotus* showed that the UAE process makes a difference in term of extracted phenolics, thus, lower TPC value of ZLE by MAE could be related to the breakdown of phenolic compounds under the optimized conditions that was not seen using Folin–Ciocalteu test, as demonstrated by Nayak, Dahmoune **[12],** Pingret, Fabiano-Tixier **[187]** who reported degradation of bioactive compounds associated to microwave and ultrasound process. Additionnaly, the presence of synergic effects between phenolic compounds present in our extracts and non-phenolic compounds such as sugar, fatty acids that might have lowered the TPC in ZLE by MAE.

IV.2. Comparison between ZLP, ZLS and ZLE on antioxidant activities

The antioxidant capacity of jujube extracts obtained from UAE and MAE process was evaluated by DPPH and FRAP methods as represented in Table 33 and Figure 45. As represented in the Table 33, the MAE increased significatively the antioxidant effects of ZLP, ZLS and ZLE in comparison to UAE method for both DPPH and FRAP tests. The ZLS revealed the highest antioxidant activity under UAE conditions for both DPPH and FRAP tests. DPPH scavenging assay of this extract under MAE showed again a significant higher effect with minimum EC_{50} value of 0.06 ± 0.00 µg/mL. While, ZLS comes after ZLP on ability to reduce ferric iron (Fe^{3+}) in presence of phenolic compounds extracted under MAE (2039.60 ± 8.43 mg/mL). This order The order in terms of radical scavenging activity remains the same as that of TTC where ZLS contain much more compounds than that of ZLP and ZLE. It can be suggested that the antioxidant effect in jujube plant can be related in particular to the condensed tannins. This was in agreement to Wojdyło, Carbonell-Barrachina **[25]** who

showed the higher concentration of proanthcyanidines in comparison to other phenolic compounds from jujubes.

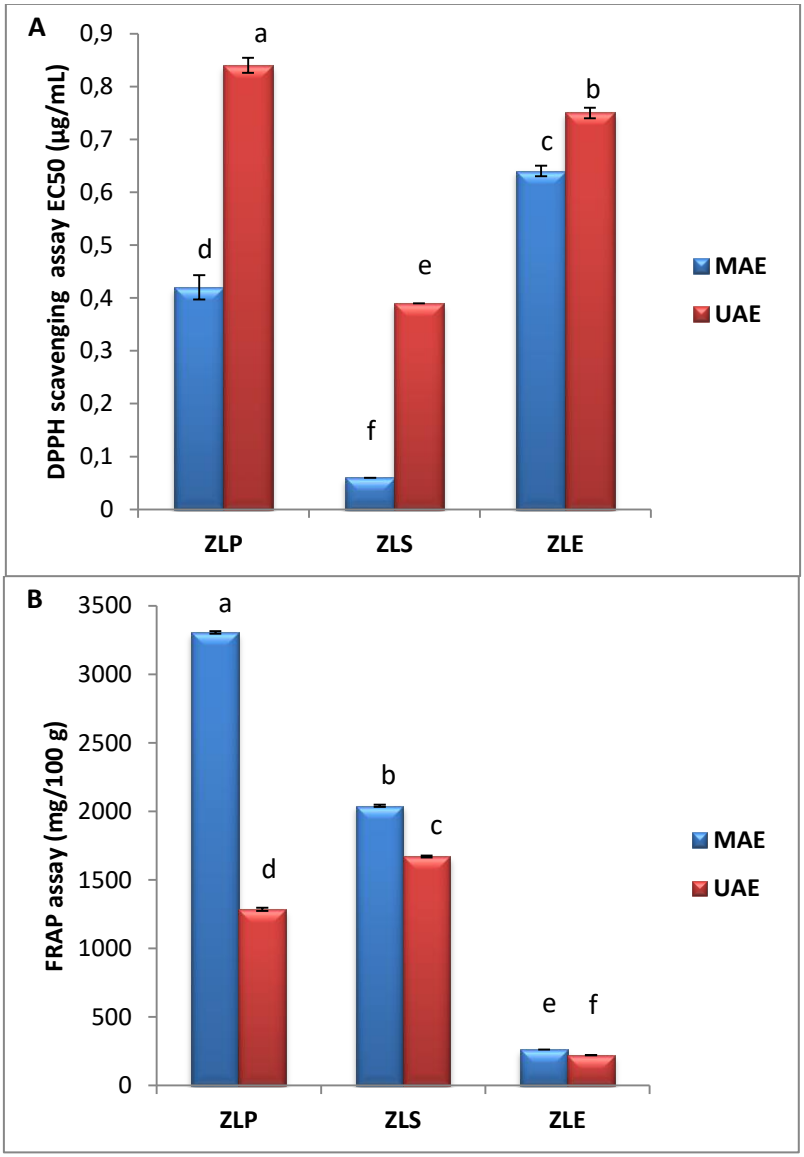


Fig.45: Antioxidant activity by DPPH (A) and FRAP assay (B) of *Zizyphus lotus* extracts obtained by UAE and MAE process. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).

However, phenolic compounds extracted by MAE obtained from ZLP had a greatest antioxidant capacity for DPPH test and showed a highest effect on FRAP test (2645.45 ± 118.67 mg/mL) in comparison to other jujube parts. This result revealed that ZLP extracts contain powerful inhibitor compounds in particular TPC and TFC as represented in the previous section. Conversely, under UAE process, the extract revealed a lowest activity by DPPH and a second order by FRAP assay where the negative effect of UAE under some

conditions appear and can be related to the longer exposure of compounds into caviation phenomena **[9]**.

Table 33: Effect of DPPH and FRAP assays for different parts of *Z. lotus* extracted by UAE and MAE.

Method	DPPH scavenging assay EC_{50} (µg/mL)			FRAP assay (mg GAE/100 g)		
	ZLP	ZLS	ZLE	ZLP	ZLS	ZLE
MAE extracts	0.42 ± 0.23 [b]	0.06 ± 0.00[a]	0.64 ± 0.01	2645.45 ± 118.67 [a]	2039.60 ± 8.43	262.99 ± 0.03
UAE extracts	0.84 ± 0.14 [b]	0.39 ± 0.00[a]	0.75 ± 0.01	801.42 ± 435.18 [b]	161670.42 ± 86.5	221.62 ± 1.91

Furthermore, the ZLE was found to have mostly non significant antioxidant propreties as it showed the very lowest value of reducing ferric iron for both MAE and UAE process and similar observation for DPPH scavenging by MAE extract and a second after ZLP for UAE extract. These differences confirme that MAE method is more effcient in the recovery of antioxidants than the herein tested UAE where it was not affect always positively could be attributed to the mecanic cavitation of ultrasound exposed for longer period of extraction where the bioactive compounds can be altereated **[188].** Thus, one of the most commonly appreciated advantages for MAE are reducing time, solvent consumption and its solubility, the heat and mass transfer kinetics of the process which come frome inside to outside, as weel as dielectric constant and molecular movement **[189, 190].** In the meanwhile, several authors confirmed that microwave extraction of polyphenolic compounds from medicinal plants can cause damge and rupture of cells due to sudden increase in heating irradiation under electromagnetic waves giving a good antioxidant capacity in comparison to UAE **[8, 10, 65, 191].**

IV.3. Comparison between ZLP, ZLS and ZLE on anticholinesterase inhibition

The AChE inhibition assay of ZLP, ZLS and ZLE were depicted in Table 34. MAE exhibited a significant effect on AChE inhibitory activity for the three jujube parts in comparison to UAE process. The AChE inhibitory effect is in the increasing order of ZLS<ZLP<ZLE. The ZLS gave 53.87 ± 1.82% of inhibition at concentration of 10 mg/mL, showing a relatively higher AChE effect compared to ZLP and ZLE with a value of 25.00 ± 3.81, 10.28 ± 0.1%, respectively. However, by using UAE, ZLS (51.07 ± 3.11%) showed again a significant different percent of AChE inhibition than that of ZLP and ZLE as represented in Table 34 and Figure 45.

Results suggested that jujube seeds are good AChE inhibitor and could be exploited against treatment of several desease mainly Alzheimer's disease and alleviation of severe constipation. Because of their anticholinesterase propreties obtained by ZLS, an evaluation of jujube seeds at different concentrations have been realised. Results revealed an IC_{50} value of 0.88 ± 0.02 and 0.93 ± 0.01 mg/mL fro both MAE and UAE, respectively. These activity levels are in the same range than that of Tunisian *Z. lotus* extract studied by Tlili, Hanen **[168]** using maceration method and acetone for extraction (IC_{50} of 0.85 mg/mL). Thus, ZLS gave higher AChE inhibitor than that obtained from *Zizyphus oxyphylla* extracts using *n*- butanol (IC_{50} of 9.58 ± 0.08 mg/mL) **[147].** Whereas, solvent nature from jujube plat, especially in ZLS can be an excellent AChE candidate that have strong inhibitory effect

Table 34: Effect of AChE assay for different parts of *Z. lotus* extracted by UAE and MAE.

Method	AChE assay (%) (for 10 mg/mL)		
	ZLP	ZLS	ZLE
MAE extracts	25.00 ± 3.81	53.87 ± 1.82	10.28 ± 0.1
UAE extracts	19.66 ± 0.16	51.07 ± 3.11	7.72 ± 0.04

Regarding the extraction process, ZLS was observed to be more effective by MAE with maximum AChE inhibitory activity than with UAE preserving quality, confirming results of polyphenols recovery in terms of yield and its ablility to extract more recovery by MAE, as demonstrated by several authors that the AChE inhibition of many plant matrix were proportional to their phytochemicals mainly polyphenols which have a positive correlation between them **[148-151].**

At the mean time, lowest AChE inhibitory values were obtained from ZLE of UAE at 7.72 ± 0.04% for 10 mg/mL. These differences may be attributed to some parameters during extraction including solvent type and concentration, irradiation, temperature and choice of method. Our finding are in good agreement to Grapevine leaves extracted by MAE than same extract by UAE with a 13.68% AChE inhibition for 0.5 mg/mL sample using water **[192].** Thus, AChE inhibitory activity using ethanol in water from ZLS is much better.

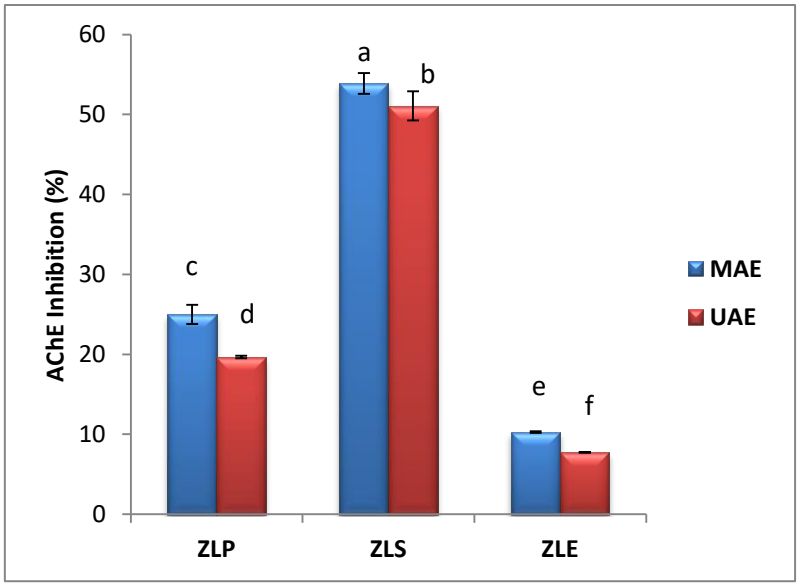


Fig.46: Percentage of AChE ihnibitory activity of *Zizyphus lotus* extracts (10 mg/mL of extract). Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).

IV.4.Comparison between ZLP, ZLS and ZLE on antioproliferative activity

The antiproliferative activity of jujube phenolic extracts was evaluated by measuring the cytotoxic effect on $HepG_2$ (human hepatocellular liver carcinoma cell) and MCF-7 (breast cell) lines as represented in Table 35 and Figure 46. The $HepG_2$ and MCF-7 cell lines were analysed for the determination of toxic effect of food components as demonstrated by several authors **[193].** The recovery of phenolic extracts for ZLP, ZLS and ZLE that killed 50% of the cells (IC_{50}) was determined from dose–response curves.

Table 35: Effect of HepG2 and MCF-7 cells tests for different parts of *Z. lotus* extracted by UAE and MAE.

Method	$HepG_2$ cells IC_{50} (mg/mL)			MCF-7 cells IC_{50} (mg/mL)		
	ZLP	ZLS	ZLE	ZLP	ZLS	ZLE
MAE extracts	< 0.02± 0.77 22	0.94 ± 1.92 51.20	0.96 ± 2.96 23.74	<1.36± 5.48 29.47	0.53± 2.33 71.33	0.57 ± 3.9 <13.91
UAE extracts	< 3.99 ± 3.36 13.64	3 ± 0.5 26.21	1.61 ± 1.48 <13.40	<2.04± 1.81 7.42	<0.05± 0.0 70.84	3.23 ± 2.36 8.74

The antiproliferative effect on $HepG_2$ cell lines as observed to be maximum in ZLP extracted by MAE with lower IC_{50} value in comparison to ZLS and ZLE for MAE extracts. Similar observation was noticed by UAE extracts following the order ZLP<ZLE<ZLS. However, jujube extracts evaluated against MCF-7 cell lines showed a lower IC_{50} value again

by ZLP followed by ZLS and ZLE for both MAE and UAE methods. By comparing both cell lines, the IC_{50} values for MCF-7 cells were lower than those of $HepG_2$ cell lines for three extracts obtained by MAE process. While, for UAE process only ZLP and ZLS revealed a lower IC_{50} values for MCF-7 cells than $HepG_2$ cells. The higher concentration value up to 0.1 mg/ml were observed for ZLS and ZLE obtained by MAE and UAE methods which revealed to be non-toxic towards the two lines except for ZLS by UAE which showed lower value (<0.05 ± 0.0 mg/mL). The limit of toxicity to human cell lines was fixed at 0.1 mg/mL **[174].** These suggested that the jujube extracts excerce a significant higher effect against MCF-7 than $HepG_2$ cells.

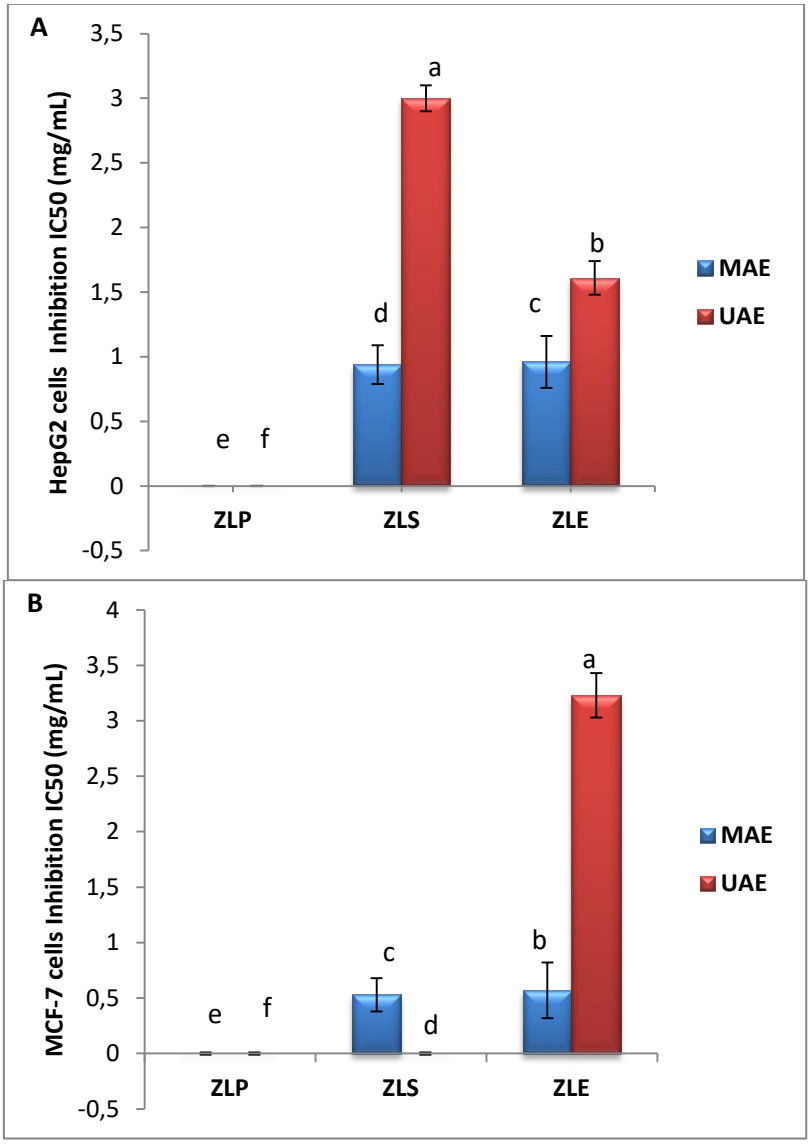


Fig.46 : Cytotoxic effect against HepG2 (A) and MCF-7 cell lines (B) assay of *Zizyphus lotus* extracts obtained by UAE and MAE process. Different superscript letters (a,b, c, d, e, f) correspond to values in the same extraction condition that can be considered statistically different ($p < 0.05$).

IV.5. Phytochemical profile of different jujube parts

Based on the better values of the biological activity of the extracts, the previous identification of the compounds in the all jujube extracts obtained by MAE process from the pulp and peel, seed and endocarp were carried out by LC-HRMS/MS. The mass was acquired in positive and negative mode as represented in the previous sections. The chromatograms for all jujube samples were shown in the previous sections. The mass analysis and the intensity of the several compounds in the MS chromatograms allowed the establishment of the heatmap shown in Tables 36 and 37. The aqueous extract from the seed samples had a higher number of compounds (47 compounds) than the extract of the plup samples (34 compounds) followed by endocarp extracts (30 compounds). On the other side, the pulp extract had compounds with retention time at 1-1.3 min, some compounds in a much higher intensity than the other samples.

By analyzing the intensities of each peak in a heatmaps. There are 38 common compounds in all extracts by negtive and positive modes, but to explain the differences in these extracts, the activities of only some compounds present all jujube parts were identified (previous sections). The highest intensity was refered to the compound with m/z of 387,1094 in $[M - H]^-$ which was more intense in the ZLP than ZLS and ZLE and refered to indole derivatives as represented in the table 36. Additionaly, 4-gingerol (m/z : 265.1444) was found to be present in the three jujube parts and more higher in both ZLP and ZLS (25%) than in ZLE. However, at m/z of 309.1698, all extracts showed approximatively similar intensities which is attributes to farformolide. Similarly for 2-hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy)propyl] phenyl]propan-1-one at m/z of 353.1955. While, from the positive mode only one highest intensity was observed in blue color from the heatmap and it was identified as isosakuranetin-7-O-rhamnoside with m/z of 342,1651, this compound was only detected in ZLS.

The compounds identified indicated that there are four main groups of chemical structures, organic acids, small peptides, flavonoids and terpenes. Other compounds that do not belong to this type, compounds are represneted with small intensities. Generaly, from primary metabolites, the ZLE was found to be more higher than ZLS<ZLP with 69.60, 55.84 and 44.87% , respectively from all jujube compounds. However, peptides are prensent in all jujube parts with 12.37, 22.95 and 31.30 % for ZLE, ZLS and ZLP, respectively from all primary metabolites. Organic acids are the main compounds reprensenting the primary

metabolites with 71.06 and 52.37% for ZLS and ZLE, respectivley, from which 3-(decyloxy)-2-hydroxypropyl prop-2-enoate was the major compound. While, from it was absent in ZLP extract. Thus, secondary metabolites are abundant in the following order ZLP<ZLS<ZLE with 55.12, 39.62 and 33.39% from all jujube compounds. Flavonoids are present in all jujube extracts with a higher intensities in ZLP that represented 80.75% from all secondary metabolites where 5-hydroxy-7-O-neryflavanone was the major compound (47.11% from all flavononoids). However, the ZLS represents 44.97% of flavonoids from all secondary metabolites, from which kaemferol-3-robinobioside was the major compound with 16.21% of this classe. Thus, ZLE which has a lowest recovery of flavonoids (11.74% from all secondary metabolites) has shown ameplopsin A as the most active one with 51.56% of this classe. Regarding other compounds, terpenes which are absent in ZLP, were detected with higher intensities from ZLE (41.38%) than ZLS (18.93%) from all secondary metabolites, as shown in the heatmaps at m/z of 449.2976, tensagenin was the major compound of terpenes with 67.97 and 48.77% of all terpenes from ZLS and ZLE, respectively.

Table 36. Heatmap representing the intensity of all compounds present in jujube samples by LC–MS/MS in ESI negative mode. Intensity: 175000; 100.

	[M - H]-	*Zizyphus lotus* samples				M-H	*Zizyphus lotus* samples		
N°	m/z	Pulp	Seed	Endocarp	N°	m/z	Pulp	Seed	Endocarp
	1-1,3 min					**2-2,4 min**			
1	179,0534				5	117,0158			
	341,1045					169,0107			
	387,1094					**5- 5,8min**			
	388,1126				6	352,0984			
	683,213					353,1023			
	729,2173				7	593,1409			
	730,2212					**5,9- 6,6 min**			
	1025,321				8	340,1507			
	1,5-1,6 min					376,1276			
2	96,9566					386,1554			
	98,9529					438,1161			
	115,0006				9	573,2268			
	126,902					**7- 7,7 min**			
	133,0112				10	753,1896			
	134,015					783,1999			
	160,8394					813,2094			
	162,8354				11	341,1098			
	191,0182					433,1555			
	209,0276					531,121			
	223,0438				12	469,1318			
	262,0531					**13,3- 13,5 min**			
	439,0699				13	265,1446			
	469,0201					333,1316			
	475,1209					**14,4- 14,6 min**			
	561,143				14	309,1701			
	732,1954					377,1565			
	781,1782					**15,1- 15,7 min**			
	1,7-1,9 min				15	353,1961			
3	112,9824					421,183			
	139,0008				16	337,1988			
	191,0169					397,221			
	341,1052				17	393,1753			
	377,0817					**17,1- 17,4 min**			
	379,079				18	337,2013			
	431,1351					**22 -23,3 min**			
	525,1019				19	134,89			
	533,1276					136,8888			
	683,2139				20	115,9174			
	711,2035					118,9894			

	857,2238			146,9629	
	539,1305			168,9864	
4	549,1583			212,9762	
	601,1195				

Table 37. Heatmap representing the intensity of all compounds present in jujube samples by LC–MS/MS in ESI positive mode. Intensity: 175000; 100.

	[M + H]+	*Zizyphus lotus* samples				M+H	*Zizyphus lotus* samples		
N°	m/z	Pulp	Seed	Endocarp	N°	m/z	Pulp	Seed	Endocarp
	1-1,9 min				16	435,1706			
1	163,0582					483,7519			
	272,0619					**11,3-11,5 min**			
	325,1057				17	343,2907			
	345,0974					365,2723			
2	130,0846								
	160,0944					**12,5-12,8 min**			
	307,079				18	239,1584			
	434,1589					319,1387			
	2-2,3 min					455,3279			
3	254,158					**13-13,8 min**			
					19	199,167			
	3,2-3,5 min					239,159			
4	120,0793					455,3291			
	166,0842				20	371,3228			
	217,1018				21	256,2972			
	5,6-5,8 min					**15,1-15,4 min**			
5	205,0879				22	281,1692			
	371,2225					399,1739			
	388,5					**16,4-16,9 min**			
6	227,101				23	287,2187			
	415,2483					304,245			
	432,2747					306,1921			
	437,23					309,2003			
	453,2035					325,1739			
	6-7 min					449,2976			
7	342,1651					595,4113			
8	476,2999					**17-17,5 min**			
9	525,2807				24	275,2551			
	541,2549					309,2017			
10	385,2379					319,2808			
	407,2195					341,2623			
11	569,3068					385,2884			

	611,1518			429,3138	
				20,1-20,45	
12	446,2903			**min**	
13	495,2712		25	149,0218	
	619,2916			167,032	
	7-8 min			279,1563	
14	317,1391			391,2801	
15	417,1595			413,2618	
	447,1559				

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General Conclusion

General Conclusion

The scientific ambition of this research work has to enhance and promote the medicinal plants of Algeria in order to facilitate the access of populations to improved traditional remedies at lower cost. But also by the need to promote a Ramnaceae plant from Algeria, *Zizyphus lotus* L, by researching some compounds for any possible therapeutic uses. The aim is to strengthen the phytochemical knowledge of these plant extracts and to highlight specific tracers for this plant in order to meet the current demand of the food and pharmaceutical industries, which require new suitable formulations from natural origin. This study can have a significant impact on the economic level for the sectors using these new ingredients, as well as on the environmental level.

The response surface methodology made it possible to study the effects of the main operating parameters in order to identify the parameters that have the most influence on microwave and ultrasonic extraction performance. The positive effects of the sonication temperature, irradiation and sonication time, microwave power, solvent / solid ratio and the ethanol rate observed during the extraction from ZLP, ZLS, ZLE according to UAE and MAE were also confirmed in the case of ZLPS extraction using MAE. However, sonication temperature as well as very high irradiation powers are not suitable for the extraction of polyphenols and polysaccharides (degradation of the polyphenols of ZLP, ZLS and ZLE has been observed at more than 63 ° C, 80% ethanol, 25min, 67 mL / g per UAE and over 600W, 60% ethanol, 210s, 47 mL / g per MAE, while 600W, 27mL / g and 40min should not be exceeded for the case of polysaccharides using MAE).

The models proposed make it possible to describe the extraction process of antioxidants from different jujube parts in different operating studied areas, depending on the part of the plant studied and according to the extraction method chosen. These experimental models also make it possible to predict the optimal operating conditions for extracting polyphenols and polysaccharides within the framework of various technological constraints. The development of more efficient methods for extracting macro and micromolecules from plant materials is needed in order to increase commercial appeal. The mathematical models generated by the RSM design proved to be appropriate for predicting the responses chosen in each experimental study. The present study reveals quite good recoveries of total phenolics from Algerian jujube plant by a combination of relatively short times of microwave and

ultrasound treatments. The highest phenolic content in the extracts was obtained for 7394.53 ± 529.33 mgGAE/100g DM using microwave irradiation from ZLP and with ultrasounds from ZLE with 10087.08 ± 231.81 mgGAE/100g DM. However, the eco-extraction using microwaves showed a good extraction efficiency for both phenolics and polysaccharides from *Zizyphus lotus* L pulp and peel. It is clearly evident that a proper combination of a modern (microwave and ultrasound) treatment along with a conventional (maceration) treatment yields a desirable degree of efficiency in extraction operation by increasing mass transfer and possibly enhancing the production of both polysaccharide and phenolic compounds.

Numerous epidemiological studies strongly suggest that the consumption of fruits and vegetables decreases the incidence of progressive diseases such as cancer, cardiovascular and neurodegenerative diseases, which develop in relation to the installation of oxidative stress. These beneficial effects are in part attributed to antioxidant, cholesterol-lowering and anti-cancer micronutrients, as factors of choice in the strategy of prevention or improvement of the consequences of these pathologies. The natural antioxidants having the main function of countering the toxic effects of activated oxygen derivatives and of helping to maintain cellular integrity. In this case, the development of high added value ingredients from *Zizyphus lotus L* for food and pharmaceutical applications is the objective of our work.

Jujube aqueous extract composition was determined by HPLC-DAD and LC-MS / MS. To the best of our knowlege, some of phenolic compounds have not been reported before in Zizyphus species. The main components identified were phenolic acids and flavonoids derivatives. The great valorization potential of *Zizyphus lotus* L has been confirmed, since in vitro studies have been carried out on all the polyphenolic extracts obtained by MAE and UAE to study the potentially complementary molecular mechanisms of action which can support the effectiveness of the plant. in humans. Among these, inhibition of acetylcholinesterase and HMGR, antioxidant and free radical scavenging effect and cytotoxicity have been identified. Phenolic extracts show powerful antioxidant effects, polysaccharide extracts on the other hand have shown powerful anti-inflammatory effects comparable to those of profennid. The current results will be useful in assessing the value of jujube extracts in the treatment of neurodegenerative diseases such as Alzheimer's, a major complication associated with mitochondrial dysfunction, oxidative stress and inflammation-induced damage in neurons. They are particularly suitable for developing a nutraceutical for the management of cancer or hypercholesterol or for additional therapy.

To Sum up, phenolic and polysaccharide extracts obtained can be used by exploiting their natural antioxidant properties (food additives, colorants, etc.) in order to replace their synthetic antioxidants. What is even more interesting is to think about the health of the consumer by giving him a nutraceutical product. This is why the molecular action of jujube extracts has been able to support its effectiveness in humans thanks to the antihypercholesterolemiens, antiacetylcholineserase effects which can be used on an industrial scale as pharmaceutical agents or non-negligible nutraceuticals alongside their antioxidant effect.

Perspectives

Besides our experimental studies, the encouraging results of this thesis will undoubtedly lead to further work to elucidate mechanism of action of some phenolics and polysaccharides from *Zizyphus lotus* extracts on absorption and metabolism by studying their bioavailability after oral administration, by implementing analytical techniques in biological fluids, as well as their probable modes of action in the treatment of Alzheimer's disease. It is highly recommended to develop an innovative process for the manufacture of microparticles formed from at least one bioactive component (model phenolic compounds) from this plant which would make it possible to combat various degenerative pathologies associated with oxidative stress. To also conduct a study on the action of the evolution of the structure of microparticles produced during gastrointestinal digestion in order to understand the impact of the formulation on gastric stability and their release in the intestine and to evaluatue if extracts showed a high bioavailability which confirm that their compounds could permeate the intestinal barrier to act as a therapeutic agent for cholesterol and Alzheimer disease by lowering cholesterol uptake by human cell lines and odd the synthesis of cholesterol by studing the inhibition of enzyme that plays a key role in cholesterol metabolism and which is a drug target of Alzheimer disease (ACAT).

Appendix

Appendix 1: The various apparatus used for analysis.

Apparatus	Brand
Ventilated oven	Memmert, Modell 100-800, Schwabach, Germany
Grinder	WH model 8100 Basic, China
Spectrophotometer	SHIMADZUFTRI 8400
Microwave apparatus	NN-S674MF, Samsung, Malaysia
Ultrasound apparatus	J P.SELECTA 195W 50/60HZ, Spain
Fourier transform infrared	IRAffinity-1S Shimadzu, Japan
Elite LaChrom® VWR Hitachi liquid chromatography	VWR, USA
Column oven L-2300	VWR, USA
Diode array detector (DAD) L-2455	VWR, USA
EZChrom Elite®	Hitachi Japan
Liquid chromatography-high resolution tandem mass spectrometry (LC–MS-MS)	Germany
Ultimate 3000 RSLCnano system	
Quadrupole time-of-flight (QqToF) Impact II mass spectrometer equipped with an	Thermo Fischer Scientific, Idstein, Germany
electrospray source	Bruker Daltonics, Bremen, German

Appendix 2: Solvents and chemicals used for investigations.

	Chemicals	Provider
Extraction of polyphenols	Acetone (99.98% purity)	VWR Prolabo (Fontenaysous- Bois, France)
	Ethanol (96.9% purity)	VWR Prolabo (Fontenaysous- Bois, France)
	Methanol (99.80%)	VWR Prolabo (Fontenaysous- Bois, France)
	Distilled water	BBBS Lab
Extraction of polysaccharides	Ethanol (96.9% purity)	VWR Prolabo (Fontenaysous- Bois, France)
	Hexane (80% purity)	VWR Prolabo (Fontenaysous- Bois, France)
	Lead acetate	Biochem Chemopharma (Montreal, Quebec)
	Bidistilled water	LGVRNAQ Lab
Quantification of phenolic compounds	Folin-Ciocalteu	Biochem Chemopharma (Montreal, Quebec)
	Sodium carbonate (Na_2Co_3)	Biochem Chemopharma (Georgia,USA)
Quantification of flavonoid contents	Chloride aluminium ($AlCl_3$, $6H_2O$)	Sigma Aldrich co (St. Louis, MO, USA)
Quantification of condensed tannin contents	Hydrochloric acid (HCl)	Sigma Aldrich co (St. Louis, MO, USA)
	Vanillin ($C_8H_8O_3$)	Sigma Aldrich co (St. Louis, MO, USA)
	Disodium hydrogen phosphate (Na_2HPO_4)	Sigma Aldrich co (St. Louis, MO, USA)
Quantification of sugars	Trichloroacetic acid (TCA)	Biochem-chemopharma (UK)
	Phenol	Biochem-chemopharma (UK)
	Sulfuric acid	Biochem-chemopharma (UK)
Standards	Gallic acid	Sigma Aldrich co (St. Louis, MO, USA)
	Catechin	Prolabo (Loire, France)
	Trolox	Prolabo (Loire, France)
	Quercitin	Prolabo (Loire, France)
	Ethylenediaminetetraacetic acid (EDTA)	Prolabo (Loire, France)

	Profenidin	Sigma Aldrich co (St. Louis, MO, USA)
	Glucose	Sigma Aldrich co (St. Louis, MO, USA)
Antioxidant assays	Ferrozine	Sigma–Aldrich Chemie GmbH (Steinheim, Germany)
	1,1-Diphenyl-2 picrylhydrazyl radical (DPPH)	Sigma–Aldrich Chemie GmbH (Steinheim, Germany)
	2,20-Azino-bis(3-ethylbenzothiazoline-6- sulphonic acid) (ABTS)	Sigma–Aldrich Chemie GmbH (Steinheim, Germany)
	Ferric chloride ($FeCl_2 \cdot 6H_2O$)	Biochem-chemopharma (UK)
	Potassium ferricyanide $K_3Fe(CN)_6$	Biochem-chemopharma (UK)
	Trichloroacetic acid (TCA)	Biochem-chemopharma (UK)
	Sodium dihydrogen phosphate (NaH_2PO_4)	Biochem-chemopharma (UK)
Antiinflammatory assays	The red blood cells suspension (RBCs)	Bouira hospital from healthy human volunteers in heparinic tubes
	Bovin serum albumin (BSA)	VWR Prolabo (Fontenaysous- Bois, France)
	Tris-HCL	Biochem-chemopharma (UK)
	Phosphate buffer	Biochem-chemopharma (UK)
	NaCl	Biochem-chemopharma (UK)
Anti-Acetylcholinesterase assays	DTNB	PubChem CID: 6254
	Acetylthiocholine iodine	PubChem CID: 74629
	Acetylcholinesterase ACHE tris	149 U/mg solid, 241 U/mg protein
	Buffer	PubChem CID: 6503
	Trypsin	
	Phosphate-buffered saline (PBS) & Fetal bovine serum (FBS)	Verviers, Belgium
	Hank's balanced salt solution (HBSS) with and without phenol red, glutamine, a mixture of penicillin and streptomycin (Pen-Strep)	Lonza (Verviers, Belgium) and VWR International
Anticholesterolemien assays	Standard cholesterol	Sigma-Aldrich, Barcelona, Spain
	HMG-CoA reductase assay kit	Sigma-Aldrich, Barcelona, Spain
	Highperformance liquid chromatography (HPLC)-grade	Merck (Darmstadt, Germany)
	Trifluoroacetic acid	Merck (Darmstadt, Germany)
Cytotoxicity assays	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Verviers, Belgium
	Dulbecco's modified Eagle medium (DMEM)	Verviers, Belgium
	Roswell Park Memorial Institute (RPMI) medium 1640	Verviers, Belgium
	Human hepatocellular liver carcinoma cell lines	
	Human breast cell lines	$HepG_2$ ATCC#HB-8065
		MCF-7ATCC#HTB-22

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ORIGINAL ARTICLE

Response Surface Methodology Optimization of Microwave-Assisted Polysaccharide Extraction from Algerian Jujube (*Zizyphus lotus* L.) Pulp and Peel

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Abstract

Purpose The active ingredient recovery from the vegetable is a very attractive research field for the development of a sustainable economy; to revalue the jujube fruit (*Zizyphus lotus*) polysaccharide (*ZLPS*), an optimized green microwave-assisted method was used for the recovery and enrichment of the antioxidants present in a distilled water extract.

Methods A series of 17 experiments including microwave power, irradiation time, and liquid-to-solid ratio independent parameters was designed by the response surface methodology to optimize the recovery of the polysaccharide extract.

Results The optimal conditions were as follows: 600 W, 40 min, and 26.69 mL/g. Under these conditions, the experimental extraction yield was 13.98 ± 1.55% which is very close with the predicted value (14.08%), and this demonstrated the validation of the extraction model proposed. The polysaccharide extract exhibited a significant scavenging activity against $ABTS^{\cdot+}$ (70.45%), DPPH*·(66.02%), and FRAP (A = 0.63) with a very important anti-inflammatory activity using a protein denaturation method that showed a maximum inhibition of 95.33% at 200 μg/mL. Additionally, the membrane stabilization method showed a significant action and protection of human red blood cells (85.76%) in hypotonic-induced lysis solution and 86.45% in heat-induced lysis solution.

Conclusion This study demonstrated the possibility of exploiting the microwave process to obtain extracts remarkably enriched with invaluable antioxidants from the jujube matrix. The operation time is short, and the antioxidant and anti-inflammatory activities of the distilled water extract were preserved.

Keywords *Zizyphus lotus* fruit · Polysaccharide · Response surface methodology · Microwave-assisted extraction · Anti-inflammatory and antioxidant activities

Introduction

There is a large variety of aromatic plants in Algeria with numerous interests including therapeutic practices. For instance, the abundant *Zizyphus* belongs to the Rhamnaceae family. This seasonal medicinal plant is extremely salt-tolerant and particularly distributed in the north, the center, and the south of the country [1]. The *Z. lotus* pulp has a very important nutritional value by containing sugars as major compounds of the biomass [2].

Polysaccharides are macromolecules with high molecular weight, which dissolve easily in water under the influence of numerous conditions. They also possess several advantages, such as various uses in food technology, thickeners, emulsifiers, stabilizers, as well as their modulation of some rheological properties [3, 4]. Several authors have shown the richness

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of several jujube species in polysaccharides, as well as their important biological properties, such as antioxidants, immunological, anti-inflammatory, anticancer, hepatoprotective, anticoagulant, and hypoglycemic properties [5, 6].

In general, several factors can influence the efficiency of polysaccharide extraction and their biological activities, mainly the extraction time, the extraction power, and the water to raw material ratio. Thus, the effects of these parameters can be interactive or independent [3, 7]. Microwave-assisted extraction (MAE) has been very used recently for the extraction of polysaccharides from plant materials which can palliate to the inconvenience of traditional methods as the longer extraction time and the low extraction yield. The MAE process is well applied due to its molecular rotation and power energy which gives a rapid increase of polysaccharides yield thanks to enhanced extraction efficiency when breaking the cells membrane after temperature increase [8].

Response surface methodology (RSM) is an effective strategy and a methodology that consists of a statistical and mathematical collection to optimize the effect of independent variables of a process and their interaction on the variables of the response. The use of RSM could lead to simplify the complexity of experimental suits. Therefore, this method can reduce the experimentation time by minimizing the number of experiments to overcome other methods requiring very long processes [9–11].

However, no report has described the optimization of MAE of polysaccharides from the jujube fruit. Hence, in the present study, we focused on the extraction of polysaccharides from *Z. lotus* fruit (pulp and peel) localized particularly in the Djelfa region and evaluation of its antioxidant and anti-inflammatory properties.

The purpose of the present study was to optimize the process for the extraction of crude polysaccharides using RSM from the pulp and peel of jujube. To study the effect of different variables, the central composite design (CCD) (3 factors and 3 levels) was employed for extraction power, extraction time, and ratio of water to the raw material that obtains a high extraction yield of *Z. lotus* fruit crude polysaccharides (*ZLPS*) (%) with antioxidant properties which were evaluated using $DPPH^{\cdot}$, $ABTS^{\cdot+}$, and FRAP radical scavenging activities. The evaluation of anti-inflammatory activity using inhibition of BSA, membrane stabilization, hypotonicity, and heat-inducing hemolysis tests were investigated.

Materials and Methods

Plant Materials and Chemicals

The *Z. lotus* fruit (pulp and peel) was harvested at a mature stage from the Djelfa region (Algeria) in August 2017. The peels were separated from the other parts of the fruit and dried at 40 °C in an air circulation oven until constant weight. The powders were sieved and stored in a dark bottle at room temperature until use. All chemicals used for assays were of analytical grade.

Microwave-Assisted Extraction of Polysaccharides

Extraction of polysaccharides from the jujube pulp and peel was performed following the procedures of several authors with some modifications [3, 12–15]. The jujube powders were first treated with hexane at 65–75 °C using Soxhlet (25 g/250 mL) for 6 h to remove lipids and then depigmented. Powders were made under the same conditions as delipidation using ethanol (80%) as a solvent to eliminate small molecules, ingredients, oligosaccharides, and colored ingredients. The obtained powders were dried and extracted by microwave irradiation following a preliminary study to determine the range of factors studied; then the optimization study was carried out to set the levels of each independent variable. Each pretreated powder was extracted with bidistilled water at a microwave power (200–600 W, X_1), an irradiation time (20–40 min, X_2), and a liquid/solid ratio (20–40 mL/g, X_3). The solution was centrifuged at 2500×*g* for 15 min, filtered, and concentrated in a rota evaporator at 50 °C for 1 h. Afterward, it was precipitated by adding 3 to 4 volumes of 80% (v/v) ethanol and left at 4 °C for 48 h. Thus, the *Z. lotus* pulp and peel polysaccharide extracts were obtained and then centrifuged (at 2500×*g* for 15 min). The sediments were recovered and lyophilized until constant weight. The yield of extraction of polysaccharide (Y) was calculated by the following formula (Eq. 1):

$$Y = (Wp/\mathrm{W}j)*100 \quad (1)$$

where Wp corresponds to the weight of dried polysaccharide powder (g) and Wj is the weight of each jujube fruit powder (g).

Polysaccharide extracts were subjected to deproteinization. It was carried out according to the method of Chen et al. [14], with some modifications. Briefly, the crude polysaccharides (sediment) were adjusted to 20 mL, with bidistilled water, and then 2% of lead acetate was added, and the solution was well homogenized. After incubation at 20 °C for 1 h, the solution was centrifuged at 3000×*g* for 20 min. Finally, precipitation was realized under the above conditions; thus, *ZLPS* extracts were deproteinized. The yield of the extracted polysaccharide is determined using Eq. 1.

Design of Experiments

Many independent factors (physical, operational, sensorial, and chemical) influence the optimization of the polysaccharide extraction process. Hence the advantage of using a

modern statistical approach involving the systematic design of experiments (DoE), which requires less time and experiences to reach a formulated food or pharmaceutical. In addition, DoE is considered to be a very effective and rentable analytical technique for the experimenter [15]. Therefore, the extraction procedure of polysaccharide compounds from a jujube powder was carried out by two sequential steps; the first step consists in a screening of independent factors to be identified and their levels influencing the extraction efficiency ("Single-Factor Experiments" section). However, a second step with an optimization procedure based on response surface methodology was performed ("Response Surface Methodology Study" section).

Single-Factor Experiments

The effect of extraction power (100, 200, 300, 400, 500, 600, 700, and 800 W, respectively), irradiation time (5, 10, 20, 30, 40, 50, and 60 min, respectively), and liquid/solid ratio (1/5, 1/10, 1/20, 1/30, 1/40, 1/50, and 1/60 mL/g, respectively) on the yield of *ZLPS* were investigated by a single-factor design as follows: one factor was changed while keeping the other factors constant, and *ZLPS* extracts were extracted as described previously.

Response Surface Methodology Study

Based on the single-factor experimental results, the variables of the extraction of polysaccharides were selected; a CCD was used (17 runs) with three independent variables (X_1, microwave power; X_2, irradiation time; X_3, liquid/solid ratio) to explore the optimal levels as shown in Table 1. For each factor, three levels coded as (+1), (− 1) with three center points coded as (0), are applied which enables to fit second-order polynomials to the experimental data points. The relation between the coded values and actual values are described in Eq. 2 [16]:

$$x = \frac{X_i - X_0}{d_A} \tag{2}$$

where x is a coded value of the variable; X_i is the actual value of variable; X_0 is the actual value of the X_i at the center point; and d_A is the step change of the variable. The general equation to predict the linear quadratic model is given in Eq. 3:

$$Y = B_0 + \sum_{i=1}^{k=3} B_i X_i + \sum_{i=1}^{k=3} B_{ii} X_i^2 + \sum_{i>1}^{k=3} B_{ii} X_i X_j + E \tag{3}$$

where Y is the response variable; 0, i, ii, and ij are the regression coefficients of variables for the intercept, linear, quadratic, and interaction terms, respectively; X_i and X_j are the independent variables (i–j); and E is an error.

In order to understand the relationship between the studied responses and the level of each factor, the experimental results are obtained from the second-order polynomial model using the 3-D surface by the JMP (Version 7.0, SAS). The existence or absence of significant linear and/or quadratic effects on the extraction efficiency of polysaccharides from jujube as well as the effect of interaction between the studied variables was marked by p value according to the analysis of the variance (ANOVA) and expressed as mean ± standard deviation (SD) for three replicates for all the experiments. Significance at $p < 0.05$ levels was determined by ANOVA followed by Duncan's multiple comparison tests.

Table 1 The three variables and levels in quadratic orthogonal rotation combination design

Independent variables	Symbol	Levels		
		− 1	0	+ 1
Microwave power (W)	X_1	200	400	600
Irradiation time (min)	X_2	20	30	40
Liquid/solid ratio (mL/g)	X_3	20	30	40

Analytical Methodology

Chemical Analysis of the Extracted Polysaccharide

The total carbohydrate content of *ZLPS* was estimated using the method of Dubois et al. [17]. The colorimetric method of Bradford [18] was used to determine the protein content of *ZLPS* using bovine serum albumin as a standard.

Antioxidant Assays

Scavenging Activity Against the DPPH· Radical The free radical-scavenging activity of extracts was measured using the method of Achat et al. [19] with some modifications. Briefly, 0.5 mL of *ZLPS* extract at different concentrations (25–200 μg/mL) was added to 1 mL of freshly DPPH· solution (0.2 mM in methanol, 99%). The mixture was incubated in the dark at 25 °C for 30 min, and the absorbance was measured at 517 nm using a UV/Vis spectrophotometer (Perkin Elmer Lambda 40 UV/Vis Spectrophotometer). Results were expressed as the percentage of inhibition of 2,2-diphenyl−1-picrylhydrazyl radical (DPPH*), calculated according to eq. 3 (Eq. 4), against ascorbic acid as a standard:

$$\%\text{Inihibition} = \lfloor (\text{Abs}_b - \text{Abs}_s)/\text{Abs}_{DPPH} \rfloor * 100 \tag{4}$$

Where: A_b is the absorbance value of the blank (DPPH· solution without extract); A_s is the absorbance of the diluted extract with DPPH* solution; A_{DPPH} is the absorbance of the DPPH* solution.

Scavenging Activity Against the $ABTS^{\cdot+}$ Radical The $ABTS^{\cdot+}$ (2,2′-azino-di [3-ethylbenzthiazoline sulphonate]) assay was performed following the method of Dahmoune et al. [9]. This assay is based on the ability of interaction of bioactive substances with $ABTS^{\cdot+}$ radical, which is used for both lipophilic and hydrophilic antioxidants, decreasing its absorbance at 734 nm [20]. The radical solution was prepared in ethanol with 7 mM $ABTS^{\cdot+}$ and 2.45 mM of potassium persulfate and stored in the dark at 27 °C for 16 h and then diluted to an absorbance of 0.7 at 734 nm with ethanol. About 120 μL of *ZLPS* extract in various concentrations (25–200 μg/mL) was added to 480 μL of a radical solution. The percentage inhibition of $ABTS^{\cdot+}$ is calculated (Eq. 5) and compared with ascorbic acid as a reference standard:

$$\%\text{Inihibition} = \left\lfloor \left(Abs_{b(t=6)} - Abs_{s(t=6)}\right)/Abs_{ABTS(t=0)} \right\rfloor *100 \quad (5)$$

where Abs_b is the absorbance value of the blank ($ABTS^{\cdot+}$ solution without extract); Abs_s is the absorbance of the diluted extract with $ABTS^{\cdot+}$ solution; Abs_{ABTS} is the absorbance of the radical solution at $t = 0$; and t is the time in min at which absorbance was read.

Ferric Reducing Antioxidant Power Activity The FRAP assay was determined according to the method described by Hammi et al. [7] with some modifications. Briefly, 625 μL of $K_3Fe(CN)_6$ (1%) and 625 μL of sodium phosphate buffer (0.2 M, pH 6.6) were mixed with 250 μL of each *ZLPS* extract in distilled water solution at different concentrations (25–200 μg/mL) and then incubated at 50 °C for 20 min. After cooling, the reaction was stopped before the addition of 625 μL of trichloroacetic acid (TCA, 10%); then mixtures were centrifuged at 2000×*g* for 10 min. After that, 625 μL of this mixture was added to 125 μL of $FeCl_3$ (1%) and 625 μL of distilled water and kept for 10 min. The absorbances were read at 700 nm against a blank using ascorbic acid as a reference standard. The greater ferric reducing capability showed the highest absorbance.

Anti-inflammatory Activities

Inhibition of Albumin Denaturation The anti-inflammatory activity of *ZLPS* extracts was investigated using inhibition of albumin denaturation technique according to Karthik [21] and Rani et al.'s [22] methods with some modifications. Briefly, the reaction mixture contained 1 mL of each extract at different concentrations (25–200 μg/mL) using distilled water as a solvent and 1 mL of 0.2% aqueous solution of bovine serum albumin (BSA) prepared in Tris-HCL (0.05 M, pH 6.8). The standard consists of 100 μg/mL of profenid solvent with 5 mL of BSA solution (0.2%). The control consists of 5 mL of BSA solution (0.2%) with 50 μL of distilled water (obtained results correspond to the total denaturation of BSA, in the absence of inhibitor substances). All samples were incubated at 37 °C for 15 min and then heated to 72 °C for 10 min. After 10 min of cooling, the turbidity was measured at 660 nm using a UV/Vis spectrophotometer. The percentage inhibition of protein denaturation was determined using the following formula (Eq. 6):

$$\%\text{Inihibition} = \left\lfloor (Abs_1 - Abs_2)/Abs_1 \right\rfloor *100 \quad (6)$$

where Abs_1 is the absorbance of the control and Abs_2 is the absorbance of the sample.

Membrane Stabilization Assay a- Preparation of Red Blood Cell

The red blood cell (RBC) suspensions were prepared after the collection of blood from healthy human volunteers in heparin tubes [23]. After centrifugation at 3000 rpm for 10 min, the tubes were washed three times with 2 volumes of normal saline (10 mM, 154 mM NaCl, pH 7.4). After washing, the volume of blood was reconstituted as 10% of erythrocyte suspension with normal saline (PBS isosaline) [22, 24].

b- Hypotonicity-Induced Hemolysis

As described by Oyedapo, Akinpelu [25]; Rani, Punitha [22], the reaction mixture consisted of 1 mL of *ZLPS* extract at different concentrations (25–200 μg/mL), 1 mL of isosaline (10 mM phosphate buffer, 154 mM NaCl, pH 7,4), 2 mL of hyposaline (10 mM phosphate buffer, 50 mM NaCl, pH 7,4), 0.5 mL of 10% RBCs prepared in isosaline. A profenid (100 μg/mL) was used as a standard anti-inflammatory drug. A 1 mL of isosaline was used as a control test instead of extracts, while drug control tests lacked red blood cells. All prepared tubes were incubated for 30 min in a water bath at 37 °C and centrifuged at 2500×*g* for 5 min. The supernatant absorbance was read at 560 nm. The inhibition percentage of hemolysis was calculated as follows (Eq. 7):

$$\%\text{Inihibition} = \left\lfloor (Abs_1 - Abs_2)/Abs_1 \right\rfloor *100 \quad (7)$$

where Abs_1 is the absorbance of positive control and Abs_2 is the absorbance of extract.

Heat-Induced Hemolysis The test of heat-induced hemolysis was carried out using a method of Rani et al. [22]. Briefly, 500 μL of RBCs suspension (10%) was added to 500 μL of extract at different concentrations (25–200 μg/mL). The control was measured with 500 μL of RBCs suspension (10%) with a saline solution. Profenid was used as a standard drug. The mixture was incubated at 56 °C for 30 min in a water bath; after cooling the temperature, all tubes containing reaction mixture were centrifuged at 2500 rpm for 5 min, and the

absorbance was measured at 560 nm. The inhibition percentage of hemolysis was calculated according to the equation below (Eq. 8):

$$\%\text{Inihibition} = \lfloor (Abs_1 - Abs_2)/Abs_1 \rfloor * 100 \quad (8)$$

where Abs_1 is the absorbance of positive control and Abs_2 is the absorbance of extract.

Infrared Spectroscopy of *ZLPS*

The polysaccharide fractions of *ZLPS* were analyzed using the Fourier transform-infrared spectrophotometer (Shimadzu FTRI-8400s) according to the method of Zhao et al. [5].

Results and Discussion

Polysaccharide Recoveries and MAE Optimized Process

Screening Factor Experiments

The effects of various extraction parameters such as the microwave power, irradiation time, and liquid/solid ratio on the polysaccharide yield were studied using single-factor experiments.

Effects of Microwave Power To study the effect of different microwave powers on polysaccharide extraction yields, the extraction process was carried out using the powers of 100, 200, 300, 400, 500, 600, 700, and 800 W. The extraction time and the liquid/solid ratio were fixed at 30 min and 50:1 mL/g, respectively. As shown in Fig. 1a, the maximum extraction yield of *ZLPS* (12.2%) was observed when the microwave power was set at 400 W and then slightly decreased for more than 600 W. These results are in agreement with those obtained by Dahmoune et al. [9] who reported that an intermediate power of 400 W gives better phenolic compound recovery and activities. Rostami and Gharibzahedi [13] suggested that the use of maximum microwave power of 400 W for extraction of polysaccharides from the jujube fruit enhances polysaccharide extract due to the heating effect and its solubility, which was observed in our results where the yield was decreased from 400 to 600 W. The higher power density may cause a degradation of extract which may conduce to polysaccharide caramelization. However, sometimes microwave treatment can contribute to the appearance of certain chemical reactions not appearing by conventional methods, but does not participate to the degradation of polysaccharide extract as demonstrated by Zhou et al. [26] who studied the effect of microwave treatment on the degradation of polysaccharide from *Porphyra yezoensis* according to the Maillard reaction of d-glucose-glycine, UV, and fluorescence reaction tests. Therefore, 200–600 W range was adopted for the RSM trials, while 400 W was kept for the next experiments.

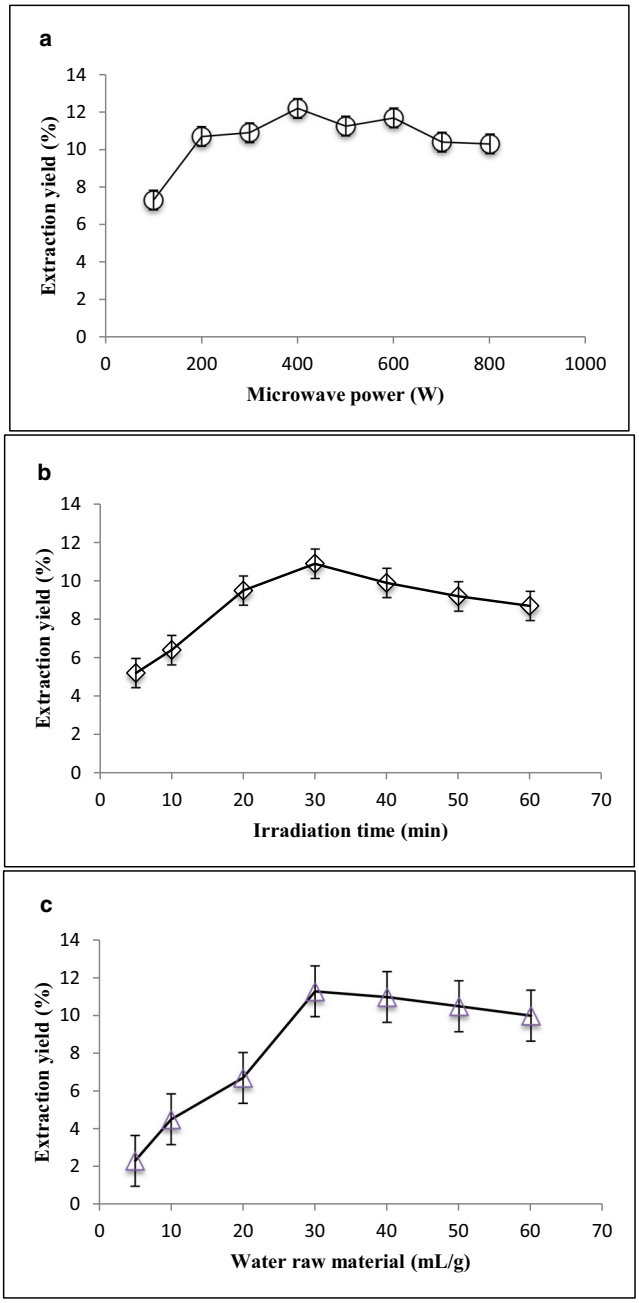


Fig. 1 Effect of microwave power (**a**), irradiation time (**b**), and water to the raw material ratio (**c**) on the extraction yield of *ZLPS* (%)

Effects of Extraction Time The extraction yield of *ZLPS* affected by irradiation time is another parameter that should be considered for the optimization step. Figure 1b showed that extraction time influenced significantly the extraction yield of *ZLPS*, when both factors (X_1, X_2) were fixed at 400 W and 50:1 mL/g, respectively. A significant increase of *ZLPS* extraction yield (10.9%) was observed from 5 to 30 min of

microwave irradiation exposure, followed by a significant decrease after 30 min. This is in agreement with Adeli and Samavati's [27] results about polysaccharide recovery from *Zizyphus lotus* using UAE showing a maximum yield between 30 and 40 min of treatment. Rostami and Gharibzahedi [13] showed also that polysaccharide extraction yield from *Z. jujube Mill.* is higher when the irradiation time is between 30 and 60 min using MAE. These observations suppose the effect of the applied extraction procedure and the studied plant type. Other work suggested also that the extraction of polysaccharides from plants was favored by a longer extraction time which let the water penetrate the raw materials using both microwaves and ultrasound as an innovative extraction method [3, 7]. The 20–40-min range was selected for the RSM trials, while 30 min was kept for the next single-factor trials.

Effect of Liquid/Solid Ratio In this present study, liquid/solid ratio was set at 5, 10, 20, 30, 40, 50, and 60 mL/g, while the other two extraction factors were fixed at 400 W and 30 min for microwave power and extraction time, respectively. Figure 1c showed that the *ZLPS* yield increased significantly from 2.3 to 11.99% by increasing the liquid/solid ratio from 5 to 30 mL/g. Thereafter, it was observed that *ZLPS* was constant until the ratio of 60 mL/g. The increase of extraction ratio implies the diffusivity of the solvent inside the cells which allows the improvement of polysaccharide desorption from them as reported by Samavati and Manoochehrizade [3]. Our results are in agreement with those obtained from jujube polysaccharide by Adeli and Samavati [27] using UAE (ratio of 24.44 mL/g) and as demonstrated by Rostami and Gharibzahedi [13] using MAE. For the liquid/solid ratio, the range of 20–40 mL/g was considered for the RSM optimization.

Response Surface Methodology Optimization of Polysaccharide Extraction

Referring to the results of the screening factor experiments approach, the major factors, namely microwave power (W), irradiation time (s) and liquid/solid ratio (mL/g) were varied according to CCD layout Tables (1 and 2). Then, the optimization of polysaccharide yield extracted from jujube powder according to these three selected parameters is performed by the RSM.

Table 3 reports the applied second-order regression model using statistical analysis. The analysis of variance (ANOVA) was used to determine the quadratic model adequacy for experimental data prediction of polysaccharide recovery using MAE. The ANOVA result showed that the higher model *F*-value (13.70) associated lower *p* values ($p < 0.0012$) indicates that most variation in the response can be explained by the regression model. The lack of fit *F*-value of 2.50 and *p* value of 0.30 showed that the lack of fit of the model was insignificant ($p > 0.05$). Additionally, the determination coefficient ($R^2 = 0.95$) revealed that 95% of the total variations are well explained by the experimental data model. To determine

Table 2 Response surface central composite design and results for extraction yield of the *ZLPS*

Run	X_1—Microwave power (W)	X_2—Irradiation time (min)	X_3—Liquid/solid ratio (mL/g)	*ZLPS* Extraction yield (%)	
				Actual	Predicted
1	200	20	20	9.40	8.86
2	600	20	20	10.20	10.79
3	400	30	20	9.90	10.03
4	200	40	20	8.00	8.46
5	600	40	20	13.70	13.04
6	400	20	30	9.70	9.19
7	200	30	30	10.70	10.45
8	400	30	30	10.80	10.76
9	400	30	30	9.80	10.76
10	400	30	30	10.70	10.76
11	600	30	30	13.10	12.83
12	400	40	30	11.10	11.09
13	200	20	40	4.80	5.58
14	600	20	40	6.10	5.76
15	400	30	40	7.50	6.85
16	200	40	40	7.60	7.13
17	600	40	40	9.32	9.97

Table 3 Analysis of variance (ANOVA) for response surface quadratic model for the extraction of *ZLPS*

Parameter	Estimated coefficients	Standard error	Degree of freedom	Sum of squares	*F*-value	Prob > *F*
Model (*ZLPS*)B_0	10,769,859	0,339,426	9	77,608,047	13,7044	0.0012*
Linear						
X_1	1.192	0.250843	1	14.208640	22.5812	0.0021*
X_2	0.952	0.250843	1	9.063040	14.4035	0.0068*
X_3	− 1.588	0.250843	1	25.217440	40.0771	0.0004*
Quadratic						
X_1^2	0.8777465	0.484614	1	2.064195	3.2805	0.1130
X_2^2	− 0.622254	0.484614	1	1.037402	1.6487	0.2400
X_3^2	− 2.322254	0.484614	1	14.448799	22.9629	0.0020*
Interaction						
X_1X_2	0.665	0.280451	1	3.537800	5.6225	0.0495*
X_1X_3	− 0.435	0.280451	1	1.513800	2.4058	0.1648
X_2X_3	0.49	0.280451	1	1.920800	3.0527	0.1241
Lack of fit			5	3.7978978	2.5041	0.3096
Pure error			2	0.6066667		
Total error			7	4.4045645		
R^2					0.946294	
R^2 adjusted					0.877244	
CV%	7.34					
RMSE	0.793236					
Corr. Total			16	82.012612		

whether is the good statistical quadratic model, the adjusted R^2 value should be close to 1 (0.87), this explained a high correlation degree as shown in Table 3. At the same time, the coefficient of the variation value (CV) was less than 10% that displayed also the perfect precision and better reliability of the experimental values and the predicted one [7, 9]. Therefore, the model is adequate and can be used to optimize experimental variables.

As shown in Table 3, the independent variables have significant effects on *ZLPS* recovery using MAE at the level of $p < 0.01$. *ZLPS* extraction was affected more significantly by water to raw material ($p = 0.0004$), followed by microwave power ($p = 0.0021$) and by irradiation time at ($p = 0.0068$). Also, quadratic liquid/solid ratio (X_3^2) and interaction (X_1X_2: microwave power and irradiation time) terms affect significantly the response ($p < 0.0001$ and $p < 0.05$ respectively), while all the other interaction terms (X_1X_3, X_2X_3) were insignificant ($p > 0.05$). Consequently, the predictive mathematical equations (Eqs. 9 and 10) are given below in terms of coded factors excluding non-significant terms (Eq. 8) and including non-significant terms, respectively ($p > 0.05$):

$$Y(ZLPS) = 10.76 + 1.19X_1 + 0.95X_2 - 1.58X_3 - 2.32X_3^2 + 0.66X_1X_2 \quad (9)$$

$$Y(ZLPS) = 10.76 + 1.19X_1 + 0.95X_2 - 1.58X_3 + 0.66X_1X_2 - 0.435X_1X_3 + 0.49X_2X_3 + 0.88X_1^2 - 0.62X_2^2 - 2.32X_3^2 \quad (10)$$

Analysis of Response Surface Plot

The effects of the operational parameters, the optimal levels of independent variables, and their mutual interaction on the polysaccharide extraction yield according to Eqs. 9 and 10 can be visualized on three-dimensional response surface profiles (Fig. 2). The plots were generated by sketching the response using the *z*-axis versus two independent variables (X_1 and X_2) while keeping the other independent variables (X_3) constant at their zero levels [28]. In summary, only the interaction between microwave power and irradiation time (Fig. 2a**)** with their corresponding linear parameters were significant, but water raw material did not influence the polysaccharide yield as an interactive factor with microwave power and irradiation time as can be observed in Fig. 2b–c (non-significant interaction effect), but it exhibited the best linear and quadratic effects.

In fact, through the 3-D response surface plot, Fig. 2a shows that the extraction yield of *ZLPS* increased rapidly with

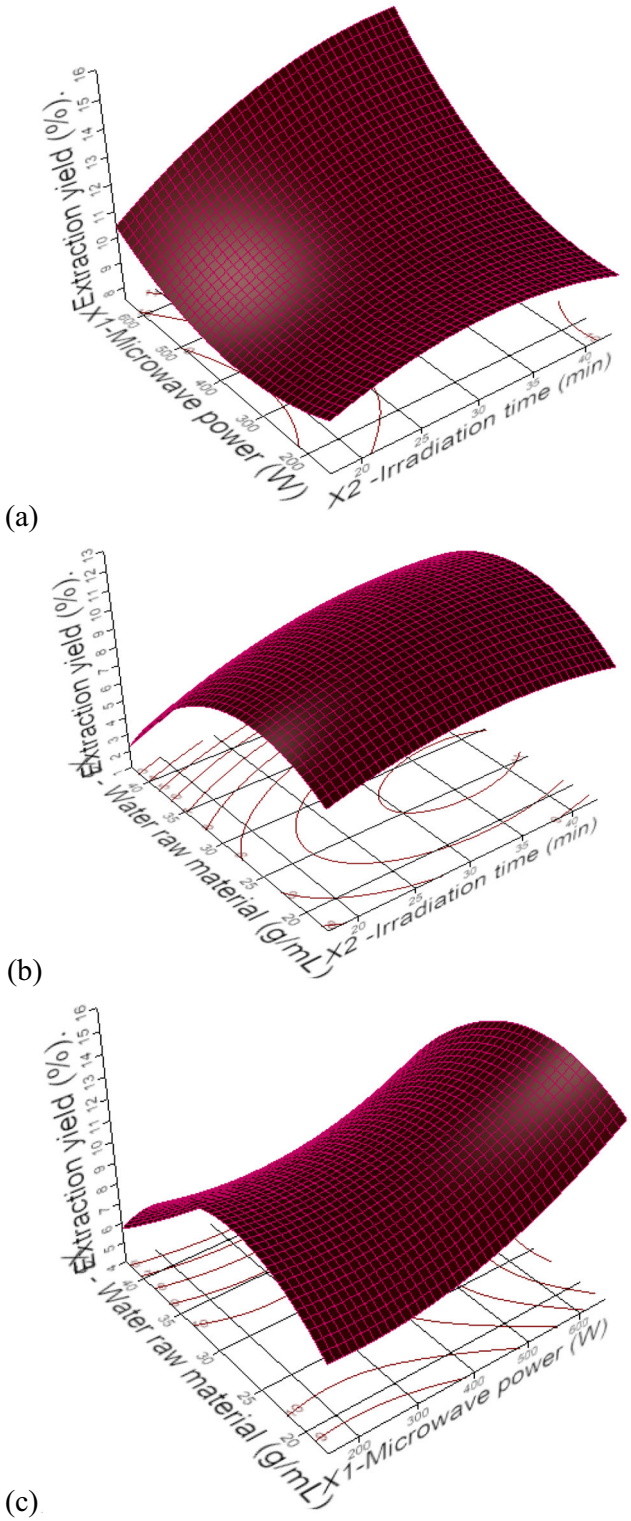


Fig. 2 The response surface plot (**a**, **b,** and **c**) indicating the effects of factors (X_1, microwave power; X_2, irradiation time; X_3, liquid/solid ratio)

the increase of microwave power from 400 to 600 W and with the increase of extraction time from 30 to 40 min. As reported by Thirugnanasambandham et al. [8], the increase of microwave power is not only for the increase of dipole rotations which leads to power degenerate inside the extract, but also the heat in the extract was reached rapidly which improves the solubility of the polysaccharides and extraction yield. This graph was consistent with results shown in Table 3, so it is evident that the interaction between microwave power and irradiation time had a very significant impact ($p < 0.05$) on the extraction efficiency of polysaccharide from *Z. lotus*.

From Fig. 2b, it can be observed that the *ZLPS* yield increased significantly from 5 to 10% with the increase of irradiation time (> 40 min) and the decrease of liquid/solid ratio from 40 to 26 mL/g. The exposure time of 40 min provided a good penetration of water into the *Z. lotus* powder which dissolved its polysaccharides, as showed by Liu, Liu [29] for jujube powders during a large time of irradiation which enhances diffusivity of the water into cells and improves desorption of the polysaccharides from the cells when liquid/solid ratio was < 30 mL/g, Chouaibi, Mahfoudhi [30] suggested that the extraction yield decreased with the increase of liquid/solid ratio which is probably due to the breakage of cell membranes and its enhancing in the raw material. This confirmed and explained that the mutual interaction between these two variables was insignificant ($p = 0.12$).

As shown in Fig. 2c, it can be seen that the maximum extraction yield of the *ZLPS* could be reached when the liquid/solid ratio decreases from 40 to 30 mL/g to 600 W. The significant increase in liquid/solid ratio can affect certain rheological properties of the polysaccharide extract by affecting the extent of its gelatinization because of the increase in the amount of the solvent entering the mixture, and thus the extraction efficiency can be decreased by affecting the transfer of the mass of the raw material [13].

Validation and Verification of the Predictive Model

From the model (Eq. 8), the optimal conditions of polysaccharide yield using MAE were as follows: microwave power 600 W, irradiation time 40 min, and liquid/solid ratio 26.69 mL/g. Under the optimal conditions, the model predicted a maximum response of 14.08 ± 1.55%.

To confirm the predictability of the established model, three additional experiments under the optimum extraction conditions were carried out. The *ZLPS* yield was 13.98 ± 1.55% which is closed with the predicted value (14.08 ± 1.55%), which was found to be not significantly different than the predicted one ($p > 0.05$) using a paired *t*-test, suggesting that the model had high suitability of the optimizing extraction conditions for *ZLPS* recovery (Table 4).

Chemical Analysis of Crude Polysaccharide

According to Table 5, the crude *ZLPS* extract produced under the optimal MAE conditions revealed high carbohydrate content and low amount of protein. Total sugars before and after

Table 4 Predicted and experimental values of the response yield of *ZLPS* (%) at optimum conditions

Optimum condition			Extraction yield of *ZLPS* (%)		
Microwave power (W)	Irradiation time (min)	Liquid/solid ratio (mL/g)	Experimental	Predicted	Desirability
600	40	26.69	13.98 ± 1.55%	14.08%	0.987

deproteinization were 98.92 ± 0.12 and 92.35 ± 0.52% respectively, while total proteins after deproteinization were 3.2 ± 0.23%. These values are approximatively the same for *ZLPS* extract obtained by Chouaibi et al. [31] from Tunisia region, who found 93.15% of total sugars from purified polysaccharide, with a very small amount of protein (2.31%). In addition, a comparable result was obtained from Tunisian *Zizyphus lotus* pulp and peel which showed 97.92% of total sugars and absence of protein contents [7].

Infrared Spectrum Analysis

The Fourier transform-infrared spectroscopy (FTIR) is used to identify the characteristic organic groups in polysaccharides. The FTIR spectrum of *ZLPS* extract was depicted in Fig. 3, and the results were analyzed in three characteristic regions: O–H stretching band envelope (3200–3600 cm^{-1}), C–H (methyl) stretching band envelope (2800–3000 cm^{-1}), and the fingerprint region envelope (700–1800 cm^{-1}) [32].

The band at 1071 cm^{-1} was attributed to the stretching vibration of the C–O–C glycosidic bond and side group C–O–H link bonds [33]. Large absorption bands were detected around 1550 cm^{-1}, 1400 cm^{-1}, and 1309 cm^{-1} for C–H band stretching, and these were due to asymmetric and symmetric bending vibrations [34], which may confirm the presence of uronic acid [35]. In addition, the peak at 2933 cm^{-1} was referred to C–H absorption stretching vibrations. A very large absorption band was shown around 3349 cm^{-1} which is a characteristic of the hydroxyl group (O–H) due to the inter- and intra-molecular interaction of the polysaccharide chains [35]. Our results are in agreement with those obtained by Hammi et al. [7] for the *Z. lotus* species obtained from Tunisia.

Table 5 Quantitative analysis of sugars and protein in *ZLPS*

Polysaccharide extract	
Total sugars before deproteinization (%)	98.92 ± 0.12
Total sugars after deproteinization (%)	92.35 ± 0.52
Total proteins after deproteinization (%)	3.2 ± 0.23

Biological Activities of *ZLPS*

Antioxidant Activity

DPPH* Scavenging Activity *ZLPS* extracts scavenged strongly DPPH* radicals in a dose-dependent manner (Fig. 4a); i.e., the percentage of inhibition increased significantly with the increase of sample concentration. The results indicated also that ascorbic acid used as a standard presented higher scavenging activity than *ZLPS* extract. At the highest concentration of 200 μg/mL, the scavenging effect was 66.02 ± 0.89% and 86.49% for *ZLPS* and ascorbic acid, respectively. Our results are in agreement with those obtained by Hammi et al. [7] for the Tunisian *Z. lotus* pulp and peel using UAE. Wu et al. [36] reported that the height value of antioxidant activity induced by polysaccharide extract could be explained by the presence of uronic acid. In addition, antioxidant activities against DPPH· of crude polysaccharide from *Z. jujuba cv. Jinsixiaozao* (CZSP) fruit were found to be higher (60%) at a concentration of 5 mg/mL when using both hot water (CZPH) and under ultrasonication (CZPU) [37]. More recently, Rostami and Gharibzahedi [13] showed that at a concentration of 200 μg/mL, purified polysaccharides extracted from *Ziziphus Mill.* fruit using MAE exhibited obvious scavenging activity on DPPH* radical. Comparing to our results, Yuan et al. [12] showed a lower antioxidant activity against DPPH· at 200 μg/mL (43.1%) of a polysaccharide extract from

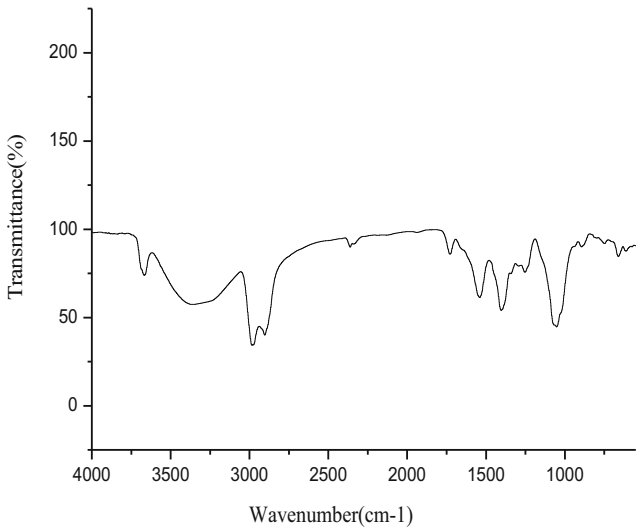


Fig. 3 FTIR spectrum of the *ZLPS* extract

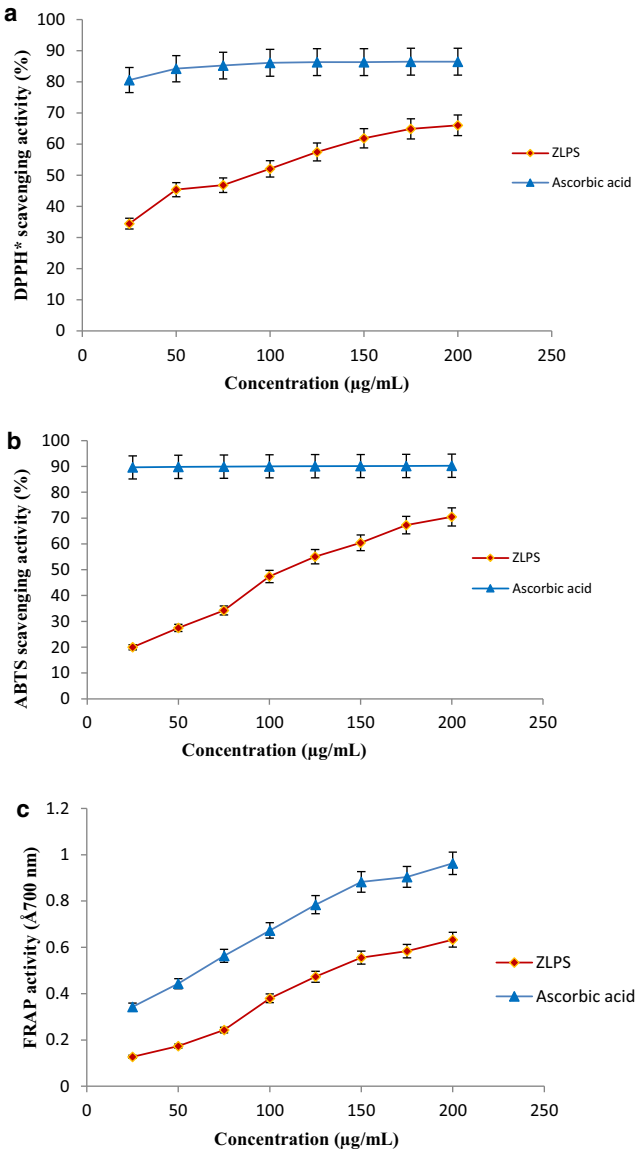


Fig. 4 Comparison of DPPH*· radical scavenging activity (**a**), $ABTS^{\cdot+}$ scavenging activity (**b**), and FRAP scavenging activity of *ZLPS* and positive control (ascorbic acid) in the various concentrations

Ziziphus jujuba Mill. var. spinosa. Our results imply that *ZLPS* might act as an electron or hydrogen donator to scavenge DPPH* (Fig. 4a**)** with an IC_{50} of 0.68 ± 0.10 mg.

$ABTS^{\cdot+}$ Scavenging Activity As shown in Fig. 4b, *ZLPS* extract exhibited the higher antioxidant capacity to reduce $ABTS^{\cdot+}$ radical with dose-dependent effect. At the highest tested concentration of 200 µg/mL, $ABTS^{\cdot+}$ inhibition was 71% and 90% for *ZLPS* and ascorbic acid, respectively. Our results are higher than those obtained by Lin et al. [38] for *Z. jujuba* polysaccharide extracted from seeds which demonstrated an $ABTS^{\cdot+}$ scavenging activity of 33.41% at a concentration of 5 mg/mL.

FRAP Scavenging Activity The ability of polysaccharide extracts to reduce the Fe^{3+}/ferricyanide complex was evaluated by following the formation of blue complex which absorbs at 700 nm [39]. Higher absorbance at 700 nm corresponds to a higher reducing capacity. Figure 4c showed clearly that *ZLPS* extract and ascorbic acid reduce significantly the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) with a dose-dependent effect. Our results were higher than those obtained by Hammi et al. [7] for the same species using UAE. Results obtained by Rostami and Gharibzahedi [13] showed that *Zizyphus* extracts exhibited less reducing power activity using MAE (0.65 at 700 nm) compared with our extracts obtained with optimal conditions using MAE as well.

These results suppose that the potential capacity of *ZLPS* to donate atom and/or electron to the radical species. This implied the possible implication of these molecules to counteract oxidative damage which may occur in our organism and cause several pathogeneses.

Anti-inflammatory Activities

Polysaccharides from natural sources have been reported to have multiple biological activities including antioxidant and anti-inflammatory properties with no toxic effect [40]. Several methods have been used in pharmacology for drug preparations especially chemicals. More recently, medicinal plant preparations with a high potential for anti-inflammatory drugs have been used. These techniques include inhibition of protein denaturation, stabilization of the erythrocyte membrane, lysosomal membrane stabilization, decoupling of oxidative phosphorylation, and fibrinolytic and platelet assay aggregation [41, 42]. In the present work, inhibition of albumin denaturation and stabilization of erythrocyte membranes exposed to both heat and hypotonic that induced lyses were studied due to its simplicity and reproducibility.

Table 6a shows that the *ZLPS* extracts prevent significantly and strongly albumin denaturation and the highest concentration tested of 200 µg/mL gives an inhibition of 95.33 ± 0.57% which is very close to the profenid as anti-inflammatory drug standard giving an inhibition of 95% with 100 µg/mL. Mizushima and Kobayashi [43] suggested that the denaturation of proteins is well documented and is caused by inflammation and rheumatoid arthritis. However, the main mechanism action to protect against protein denaturation was NSAIDs (non-steroidal anti-inflammatory drugs). The ability of *ZLPS* extract to bring down thermal denaturation of protein is possibly a contributing factor for its anti-inflammatory activity. Additionally, the anti-inflammatory activity of *Zizyphus* extract may be due to the presence of therapeutically active polysaccharide, as demonstrated by Mzoughi et al. [44], who have shown that many polysaccharides exhibited anti-inflammatory activity and it may be a potential therapeutic agent for the inflammatory disorder [45].

Table 6 Effect of *ZLPS* on heat-induced protein denaturation (a), hypotonicity-induced hemolysis (b), and heat-induced hemolysis (c)

Concentration (µg/mL)	% Inhibition					
	(a)		(b)		(c)	
	ZLPS	Profenid	*ZLPS*	Profenid	*ZLPS*	Profenid
25	12.22 ± 1.53		10.63 ± 0.14		10.09 ± 1.92	
50	37.33 ± 0.57		16.38 ± 0.00		21.22 ± 0.30	
75	53 ± 0.70		22.97 ± 0.01		25.26 ± 0.06	
100	63.66 ± 0.57	85 ± 0.00	36.81 ± 0.85	85 ± 2.32	37.44 ± 0.88	88 ± 1.09
125	73 ± 1.41		54.97 ± 0.01		43.13 ± 0.07	
150	80,66 ± 0.57		56.06 ± 0.01		54.05 ± 0.10	
175	88 ± 1.37		60.80 ± 0.07		61.72 ± 0.01	
200	95.33 ± 0.57		85.76 ± 0.56		86.45 ± 0.12	

To confirm the membrane-stabilizing action of *ZLPS* extracts, experiments were performed on the erythrocyte's membrane. Table 6b–c demonstrate that *ZLPS* extracts inhibited the hemolysis of erythrocytes induced by both hypotonic solution and heat.

The whole plant extract exhibited a minimum membrane stability of 10.63 ± 0.14% and a maximum activity of 85.76 ± 0.56% (Table 6b). The standard anti-inflammatory drug (profenid) at 100 µg/mL exerted a maximum membrane stability of 85 ± 2.32%.

Moreover, Table 6c shows the membrane stability of 10.09 ± 1.92% and 86.45 ± 0.12% as a minimum and maximum percentage activity using different concentrations respectively. The response of the red blood cells was also monophasic and biphasic to the fractions. The standard anti-inflammatory drug (profenid) at 100 µg/mL exerted a maximum membrane stability of 88 ± 1.09%. The activities of the extracts/fractions were approximatively higher than that of the standard drugs even at lower concentration ranges.

Conclusion

In this study, all statistical indicators support that RSM is a successful tool to describe the microwave process in extracting the water-soluble polysaccharide from *Z. lotus* for the following tested parameters: power (200–600 W), time (20–60 min), and liquid/solid ratio (20–60 mL/g). The optimal extraction parameter analyzed by CCD is as follows: microwave power 600 W, irradiation time 40 min, and the ratio of water to raw material 26.69 mL/g. Under these optimal conditions, the experimental yield of *ZLPS* was 13.98 ± 1.55% which is in good agreement with the predicted value of 14.08%. *ZLPS* extract displayed stronger $DPPH^{*}$, $ABTS^{\cdot+}$, and FRAP radical scavenging activities. *ZLPS* also displayed significant anti-inflammatory effects on BSA protein denaturation and stabilization of erythrocyte membranes exposed to both heat- and hypotonic-induced hemolysis. The polysaccharide extracted from *Z. lotus* pulp and peel should be explored as a novel antioxidant and anti-inflammatory agent to be used in functional foods or medicines. However, the MAE method can be also recommended to extract other plant polysaccharides to achieve better qualitative and quantitative yields.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Ultrasound Assisted Extraction of Phenolic Compounds from a Jujube By-Product with Valuable Bioactivities

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Abstract: Jujube plant is a potential source of polyphenols with biological propreties. The purpose of this study was to investigate the application of ultrasound technique for extracting phenolic compounds (TPC) from seeds of *Zizyphus lotus* under optimization conditions based on response surface methodology. A maximum TPC, total flavonoids content (TFC), and total condensed tannins content (TTC) of 2383.10 ± 0.87 mg GAE/100g, 486.50 ± 0.38 mg QE/100g and 15,787.10 ± 0.10 mg CE/100g, respectively obtained under ethanol concentration 50.16%, sonication temperature 29.01 °C, sonication time 15.94 min and solvent-to-solid ratio 34.10:1 mL/g. The optimized extract was then evaluated for its antioxidant, antiacethylcholinesterase, antihypercholesterolemia, and antiproliferative activities. The results showed that ultrasound method is a green and safe method that can be used to effectively extract TPC from jujube seeds. The biological activity of *Zizyphus* extract exhibited a very good antioxidant against DPPH (EC_{50} = 0.39 µg/mL) and FRAP (1670.42 ± 6.5 mg/100 g). Additionally, it possesses acetylcholinesterase (AChE) inhibitory effect (IC_{50} = 0.93 ± 0.01 mg/mL) and HMGR inhibition (45.41%) using 100 µg/mL. The extract significantly inhibits cell proliferation on the MCF-7 and $HepG_2$ tumor cell lines with an IC_{50} values of <0.05 and 3 ± 0.55 mg/mL, respectively. Therefore, the ultrasound method can be considered a method for obtaining a significant anticancer activity with respect to the lines and therefore makes it possible to recover a maximum of phenolic compounds in less time with an AChE and HMGR inhibitory activity. Thus, it can be suggested that *Zls* extract is a promising fruit for the development of supplementary dietary due to its potential behaviour as nutraceutical.

Keywords: jujube; ultrasound; response surface methodology; polyphenols; biological activities

1. Introduction

The production of free radicals (FR) is associated with many physiological and biochemical processes that have taken place in the human body and their over production leads to the appearance of

many cardiovascular diseases which can cause significant oxidative damage to biomolecules, namely, lipids, proteins, or DNA [1]. Due to the release of FR such as the superoxide radical, the hydroxyl radical, hydrogen peroxide, and lipid peroxide radicals, antioxidants are capable to inhibit the oxidation of other molecules and preventing cell death. In contrast, several authors have shown that FR scavenging are associated with the mixture of nutrients consumed regularly by humans and have shown that a high intake of antioxidants results in the reduction of these diseases [2].

The alteration of cerebral cholinergic neurotransmission, more precisely the basal nucleus of Meynert which is deficient in cholinergic neurons are the main causes of progressive loss of attention and memory, in other words, Alzheimer's disease (AD) [3]. In the treatment of this disease acetylcholinesterase (AChE, E.C. 3.1.1.7) inhibition has been used [4]. AChE is an enzyme localized in the neurosynaptic gaps [5] and neuromuscular junctions [6]. As a result of this inhibition process the neurotransmitter acetylcholine remains in the neurosynaptic gap for a longer period of time, keeping the person having AD in a more active mental state [7–9].

The β-Hydroxy-β-methylglutaryl coenzyme A reductase (HMGR, E.C. 1.1.1.88), a rate-limiting enzyme in cholesterol biosynthesis, which catalyzes the reductive deacylation of HMG-CoA to mevalonate pathway, the metabolic pathway that produces cholesterol and other isoprenoids [10,11]. The high risk of coronary heart diseases or hyperlipidemia were found to be caused by elevated serum-blood cholesterol levels [12]. Decreasing total cholesterol levels is significantly regulated by the HMGR inhibitor enzyme. Furthermore, to prevent hypercholesterolemia, statins are very used as antihyperlipidemic drugs which bind into the active site of HMGR natural substrate [13]. However, some reports mentioned the disadvantages of this commercial drug due the side effects that it may have in vivo. In adverse, others demonstrated the importance of exploitation of natural compounds that may act as statins in order to have a lowering cholesterol levels by inhibition of HMGR within the cell [12].

Cancer, known as the disease of the century, is the primary cause of death in the globe after cardiovascular diseases [14,15]. One of the means of intervention is the inhibition of the proliferation cancer cell. The dietary consumption of polyphenols from herbs is associated with anticancer activity which has been suggested by epidemiology and considered to promote cancer reduction [16,17]. Therefore, several recent studies have focused on the development of new anti-tumor agents from natural products [15,18].

Jujube is a thorny tree that belongs to the genus *Zizyphus* of the Rhamnaceae family. It is native to China, and distributed widely in the Europe, America, and Maghreb [19]. However, this plant contains a large quantity of primary metabolites with significant nutritional properties, namely proteins (19.11 ± 0.03%), lipids (32.92 ± 0.29%), and sugars (40.87 ± 0.39%) from which most studies on this plant are concentrated [20]. Among these substances, polyphenols from jujube, secondary metabolites of plants, are important determinants of the nutritional and organoleptic qualities of *Zizyphus lotus* [21,22]. Several bioactive compounds extracted from jujubes have attracted the attention of many researchers due to their important biological actions, including antioxidant [19,23], enzyme inhibitory [24,25], antiproliferative and cytotoxic effects [24,26], anti-inflammatory, and other effects [27–29], which can be used in the food, pharmaceutical, and cosmetic industry.

Several studies reported that innovative techniques are one of the most rapid methods for extraction of target compounds from plant materials. Among these, one of the simplified method in manipulation is ultrasound-assisted extraction (UAE) that was very used on extraction of phenolic compounds from different materials due to its high reproducibility and very appreciated for its reduction in solvent consumption [30,31]. Response surface methodology (RSM) is used as an effective statistical method for optimizing complex processes that reduce the number of experimental trials which is very used to evaluate interactions between multiple factors and the response variables that influence the results [14,32].

The diversity of the experiments made on the genus of *Zizyphus* indicates the biological potential of its bioactive components mainly polyphenols, proteins, and polysaccharides for all possible pharmaceutical

and nutraceutical applications. To date, no data have evaluated the effect of ultrasound on polyphenols extracted from *Zizyphus lotus* seeds (*Zls*) and evaluation of its antioxidant, antiacetylcholinesterase, antihypercholesterolemia, and antiproliferative properties. The overall aim of this research work was to find out optimal extraction parameters (ethanol concentration, time, temperature, and solid/liquid ratio) for the valorization of phenolic compounds extracted from jujube seeds using green extraction technology under RSM model through the investigation of some biological effects of UAE extracts. This could be one of the economical solutions to valorize vegetable biomass in order to protect consumer and environment by consuming natural products and avoiding the generation of some industrial wastes. Additionally, it is one of the promotional strategies to meet the challenge in the 21st century through its uses on the development of new functional and nutraceutical ingredients in food and pharmaceutical industries with a high nutritional value.

2. Materials and Methods

2.1. Plant Materials and Chemicals

Jujube samples (*Zizyphus lotus* L.) were collected from Djelfa province of Algeria. Jujube seeds were cleaned and separated manually. Seeds were dried at 40 °C for 24 h in an oven (Memmert, Modell 100-800, Schwabach, Germany). The dried seeds were milled into fine powder (<250 µm) which were frozen stored at 4 °C until analyses.

Folin–Ciocalteu, gallic acid were from Sigma Aldrich Co. (St. Louis, MO, USA). 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Potassium ferricyanide ($C_6N_6FeK_3$), ferric chloride ($FeCl_2{\cdot}6H_2O$), trichloroacetic acid, and sodium dihydrogen phosphate (NaH_2PO_4) were bought from Biochem-chemopharma (UK). Acetylcholinesterase (AChE), acetylcholine iodide (AchI), 5-5′ -dithiobis (2-nitrobenzoic acid) (DTNB), and HMG-CoA Reductase assay kit were obtained from Sigma (Barcelona, Spain). Roswell Park Memorial Institute (RPMI) medium 1640, Hank's balanced salt solution (HBSS) with and without phenol red, glutamine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsin, Dulbecco's modified Eagle medium (DMEM), glutamine, Pen-Strep (penicillin and streptomycin mixture), FBS (fetal bovine serum), and phosphate-buffered saline (PBS) were bought from Lonza (Verviers, Belgium). Magnesium chloride hexahydrate, hydrogen peroxide were purchased to PanReac (Barcelona, Spain). All other chemicals used in this study were from Sigma Aldrich.

2.2. Ultrasound Assisted Extraction

One gram of powder material was dissolved with the extracting solvent, introduced in an amber glass vial, and placed in a ultrasonic bath (J P.SELECTA 195W 50/60HZ, Barcelona, Spain) at 20 kHz. As previously stated, the suspension was exposed to ultrasonic waves allowing the appearance of cavitation pellets (Table 1). This last being very sensitive, was controlled by external frozen water circulation. After extraction, the extract was filtered through Whatman No. 1 paper and kept in shaded flasks at 4 °C for further analysis.

2.3. Response Surface Methodology (RSM)

In current study, a Box–Behnken design (BBD) experimental design with a total of 27 experiments based on the response surface methodology (RSM) was used to evaluate the effect of the independent variables including X_1- Solvent concentration (% *v*/*v*), X_2- Sonication temperature (°C), X_3- Sonication time (min), and X_4- Solvent- solid ratio (mL/g). The concentrations are indicated in Table 1 and used for each test as indicated in Table 2, Total Phenolic Content (TPC) was determined and used for estimating the parameters in the following equation. After estimating these parameters, the prediction of the of

the TPC yields on jujube seeds extracts was performed following the second order experimental model according to Equation (1).

$$y = B_0 + \sum_{i=1}^{k} BiXi + \sum_{i=1}^{k} BiiX^2 + \sum_{i>1}^{k} BiiXiXj + E \quad (1)$$

where y represents the predicted response which is TPC yield; βo is a constant coefficient; βi, βii, βij are regression coefficients for linear, quadratic, and interactive terms, respectively; and Xi and Xj represent the coded independent variables while E is error. The significance and suitability of the model was analyzed by the variance (ANOVA) using JMP.

2.4. Analytical Determinations

2.4.1. Total Phenolic Content (TPC)

The TPC yield was determined for *Zls* extracts according to the color method based on Folin–Ciocalteu reaction [19]. The results were expressed as mg gallic acid equivalent per 100 g of dry matter (mg GAE/100 g).

Table 1. Box–Behnken design with the observed responses and predicted values of Total Phenolic Content (TPC) from *Z.lotus* seeds using ultrasound-assisted extraction (UAE).

Run	X_1— Ethanol Concentration (% *v*/*v*)	X_2— Sonication Temperature	X_3— Sonication Time (Min)	X_4— Solvent-Solid Ratio (mL/g)	Experimental	Predicted
1	50	30	10	25	1643.19249	1683.34855
2	50	20	15	25	1877.93427	1895.48557
3	20	30	15	25	1525.8216	1497.67432
4	80	30	15	25	1154.14711	1117.74039
5	50	40	15	25	1760.56338	1734.20927
6	50	30	20	25	1741.00157	1774.20231
7	50	20	10	30	1956.18153	1889.99739
8	20	30	10	30	1314.55399	1308.63111
9	80	30	10	30	1377.1518	1353.84064
10	50	40	10	30	1791.86229	1786.75448
11	20	20	15	30	1658.84194	1701.38889
12	80	20	15	30	1314.55399	1382.74865
13	50	30	15	30	2230.04695	2310.90245
14	50	30	15	30	2425.6651	2310.90245
15	50	30	15	30	2276.99531	2310.90245
16	20	40	15	30	1384.97653	1410.35255
17	80	40	15	30	1408.4507	1459.47444
18	50	20	20	30	2057.90297	2036.2763
19	20	30	20	30	1674.49139	1630.96635
20	80	30	20	30	1377.1518	1316.23848
21	50	40	20	30	1885.759	1925.20866
22	50	30	10	35	1990.08868	2050.45862
23	50	20	15	35	2300.46948	2259.98739
24	20	30	15	35	1661.45018	1671.12241
25	80	30	15	35	1780.1252	1781.53799
26	50	40	15	35	2291.34064	2206.95314
27	50	30	20	35	2190.92332	2244.33794

Data were expressed as mean ± standard deviation from triplicate experiments.

Table 2. Analysis of variance (ANOVA) for the experimental results obtained by using UAE.

Parameter [a]	Estimated Coefficients	Standard Error	DF [b]	Sum of Squares	F-Value	Prob > F
Model B_0	2310.9025	42.28475	14	3,245,323.8	43.2157	<0.0001 *
Linear						
X_1—Ethanol	−67.37959	21.14238	1	54,480.1	10.1566	0.0078 *
X_2—Temperature	−53.57764	21.14238	1	34,446.8	6.4218	0.0262 *
X_3—Time	71.183272	21.14238	1	60,804.7	11.3357	0.0056 *
X_4—Ratio	209.31142	21.14238		525,735.3	98.0118	<0.0001 *
Quadratic						
${X_1}^2$	−664.7757	31.71357	1	2,356,942.5	439.4001	<0.0001 *
${X_2}^2$	−157.6356	31.71357	1	132,528.0	24.7069	0.0003 *
${X_3}^2$	−243.7076	31.71357	1	316,764.8	59.0538	<0.0001 *
${X_4}^2$	−129.108	31.71357		88,900.6	16.5736	0.0016 *
Interaction						
X_1X_2	91.940532	36.61967	1	33,812.2	6.3035	0.0274 *
X_1X_3	−89.98435	36.61967	1	32,388.7	6382	0.0302 *
X_1X_4	122.58738	36.61967		60,110.7	11.2063	0.0058 *
X_2X_3	−1.956182	36.61967	1	15.3	0.0029	0.9583
X_2X_4	27.060511	36.61967		2929.1	0.5461	0.4741
X_3X_4	25.75639	36.61967		2653.6	0.4947	0.4953
Lack of Fit			10	43,510.243	0.4172	0.8589
Pure Error			2	20,857.773		
Total Error			12	64,368.016		
R^2					0.980552	
R^2 Adjusted					0.957862	
CV%	4.11					
RMSE	73.23934					
Corr.Total			26	33,09691.8		

[a] Coefficients refer to the general model; [b] Degree of freedom; * significance.

2.4.2. Total Flavonoids Content (TFC)

The TFC yield was measured for *Zls* extracts, using methanolic aluminium chloride ($AlCl_3$) for 2% as described in Ghafar, et al. [33]. The results were expressed as mg quercetin equivalents per 100 g of dry matter (mg QE/100 g).

2.4.3. Total Condensed Tannins Content (TTC)

The TTC yield was determined using the method of Hagerman [34] and the results were expressed as mg catechin equivalent per 100 g of dry matter (mg CE/100 g).

2.4.4. Antioxidant Activity by DPPH and FRAP Assays

The radical-scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) of *Zls* extracts was measured by the methods adapted from those reported by Hammi, et al. [35]. Results were expressed as the percentage of DPPH, calculated following the equation below (Equation (2)):

$$\mathrm{DPPH\%} = [(\mathrm{A_b} - \mathrm{A_s})/\mathrm{A_{DPPH}}] \times 100 \quad (2)$$

where A_b, A_s, and A_{DPPH} were the absorbance value of blank, diluted extract, and control, respectively. The radical-scavenging activity (EC50) is defined as the concentration of *Zls* extracts that provided 50% of DPPH free radicals. However, FRAP assay was expressed in terms of antioxidants having an iron reduction capacity equivalent to that of equivalents in gallic acid of 1 mg per 100 g of dry matter (mg GAE/100 g).

2.4.5. AChE and HMGR Inhibitions Tests

Anticholinesterase activity was determined using AChE assay according to what was previously reported [36]. Briefly, 100 µL of distillated water was added to 325 µL of 50 mM Tris buffer (pH 8) and 25 µL of AChE solution. The solution was mixed and incubated for 15 min at 25 °C. Subsequently, 75 µL of AChI solution (mg/mL) and 475 µL of DTNB (1.2 mg/mL of 50 mM Tris buffer (0.1 M NaCl and 0.02 M $MgCl_2$) at pH 8) were added. The absorbance was read for 4 min with 30 s intervals and the initial velocity was calculated at 405 nm on the spectrophotometer Schimadzu UV-160A, Kyoto, Japan. A control reaction was carried out using the same procedure but with adding 100 µL of distillated water instead of 100 µL of *Zls* extract at different concentrations. All tests were done in triplicate and the percentage inhibition was calculated as

$$I\ (\%) = 100 - (Vsample/Vcontrol) \times\ 100 \tag{3}$$

where I is the percent inhibition of acetylcholinesterase, *Vsamples* the initial velocity of the extract containing reaction, and *Vcontrols* the initial velocity of the control reaction.

The inhibition of the enzymatic activity of 3-hydroxy-3-methylglutaryl reductase (HMGR) was measured using the oxidation of nicotinamide adenine dinucleotide phosphate hydrate (NADPH) in triplicate [37]. Briefly, at 0, 1, 2, 4, and 6 min, aliquots were removed and then the reaction was stopped by adding methanol (50%). The amount of NADPH wasanalyzed by HPLC–DAD using VWR-Hitachi Elite LaChrom®, Tokyo, Japan. In order to evaluate the decrease of the peak area over time, which allowed to make a linear regression and to obtain the reaction velocity, the decrease in activity was monitored at 340 nm. The activity value without inhibitor was considered to be 100%. The IC_{50} values were determined from regression curves by plotting inhibition as a function of the concentration of inhibitor.

2.4.6. Anti-Proliferative Activities on $HepG_2$ and MCF-7 Cells

The cytotoxicity of *Zls* extracts were performed using the MTT viability test, against the human tumor cell lines $HepG_2$ (ATCC#HB-8065), from human hepatocellular liver carcinoma cell lines, and MCF-7 HTB-22), from breast cancer, were cultured in DMEM supplemented with 10% FBS, 100 U/mL Pen-Strep, and 2 mM L-glutamine at 37 °C in an atmosphere containing 5% CO_2 every 48 to 72 h before reaching confluence, the medium was changed [10]. For each concentration of extract, the assays were done in 8 × 12 replicates and the cell viability percentage was calculated by the following equation (Equation (4)):

$$\begin{aligned} &Viability\ (\%) \\ &= [(Abs\ 595 - Abs\ 630\ of\ experimental\ wells)/(Abs\ 595 - Abs\ 630\ of\ control\ wells)] \times 100 \end{aligned} \tag{4}$$

2.5. Statistical Analysis

In order to see the effect of UAE obtained from the Box Behnken Design (BBD) trials, ANOVA analysis was used in this study. Each extraction trial and all the analyses were carried out in triplicate. The JMP (Version 10.0, SAS) was used to construct the BBD and to analyze ANOVA results.

3. Results and Discussion

3.1. Optimization Study by RSM Using UAE Technology

3.1.1. Model Parameter Estimation

The factorial experimental design based on a BBD using UAE method and corresponding responses for the obtaining of TPC from *Zls* extracts was investigated and presented at various conditions with a model of the total of 27 experiments in Table 2. In order to estimate experimental error measurement,

three replications at the central points were made [38]. The factors studied were ethanol concentration, sonication temperature, sonication time, and solvent/solid ratio, which is ethanol/seed quantity. These factors were used in the in the range indicated in Table 1. The parameters were estimated from the experimental TPC results and ANOVA analysis was carried out to determine the applicability of the model, Table 2.

The second order polynomial equation was generated to describe the empirical relationship between the *Zls* extract and operational conditions in terms of coded values. The mathematical models were simplified by neglecting statistically the insignificant terms ($p > 0.01$) following predictive equation:

$$\begin{aligned} Y = {} & 2310.90 - 60.37\ X_1 - 53.57\ X_2 + 71.18\ X_3 + 209.31\ X_4 \\ & -664.77{X_1}^2 - 157.63{X_2}^2 - 243.70{X_3}^2 - 129.10{X_4}^2 + 91.94X_1X_2 - 89.98X_1X_3 + 122.58X_1X_4 \end{aligned} \quad (5)$$

It can be seen that all factors influence the extraction yield as linear and quadratic effects, but the interaction effects were significant for all the four parameters, positive for ethanol concentration and sonication temperature, negative for ethanol concentration and sonication time. While, positive also for ethanol concentration and solvent–solid ratio which was highly significant then the others. On the other side, it can be seen in Equation (3) that X_2X_3, X_2X_4, X_3X_4 didn't exhibit any significant effect on extraction yield (Table 2).

However, if there is any significance of each factor, it was demonstrated by a presence of $p < 0.05$ and the contrary, it was also demonstrated *p*-values ($p < 0.001$) which indicates high significance. Very low *p*-values ($p < 0.0001$) indicated that each generated model was statistically significant and suggests that the UAE of *Zls* could be well described with those appropriate models.

The values of R-squared are close to 1 for the model (0.98 and 0.95 for R^2 and R^2, adjusted respectively), which are very high and indicates a good correlation between the experimental and the predicted values, also indicated that 98% could be explain by the model of the variation in the TPC extracts using UAE method. In addition, other parameters were insignificants as F-value for the lack of fit ($p > 0.05$) and values of coefficient of variation ($CV = 4.11$) which provide also the validity of the deduced model.

3.1.2. Effect of Experimental Conditions on TPC, TFC, and TTC Extraction Yield

The influence of the four parameters on TPC yields is shown in Figure 1.

Confirming the results of the single-factor trials, Figure 1a–c shows that the TPC yield reached a maximum level when ethanol concentration was set at medium levels (0 coded value) as it mentioned in Table 2, we can notice that the yield of TPC using UAE mainly depends on the ethanol concentration as its quadratic, interaction and linear effects were highly significant ($p < 0.01$), which showed the increase on TPC for all other parameters.

Figure 1c–f shows that the interaction effect of temperature with other factors on TPC yield that was very limited and stable as demonstrated in the equation model. Thus, only the Figure 1c showed a positive influence of both ethanol and temperature on the TPC extraction and the decrease of TPC recovery from the other figures is mainly associated to their thermal degradation at higher temperature.

However, Figure 1b–e shows that maximum extraction TPC yield was for 2100 mgGAE/100 g when using a ratio about 30 (mL/g, *v*/*w*) over a range of temperature and time factors, in contrary in interaction with ethanol concentration which was very significant as confirmed also as positive effect in Table 2. The increase in TPC was deemed by the effects of acoustic cavitation that contribute to the formation and rupture of cavitation bubbles and then facilitate the mass transfer of the process, while, TPC started to decrease after higher increase in ratio which may affect the dispersion of the ethanol under ultrasound energy density [39].

Furthermore, Figure 1f shows the interaction effect between temperature and time sonication. It is obvious that longer sonication time increases the extraction yield for polar compounds in hydro-alcoholic solvent. While, the prolonged exposures at higher temperature decrease significatively the TPC recovery due to their thermal degradation which confirmed the results of Table 2.

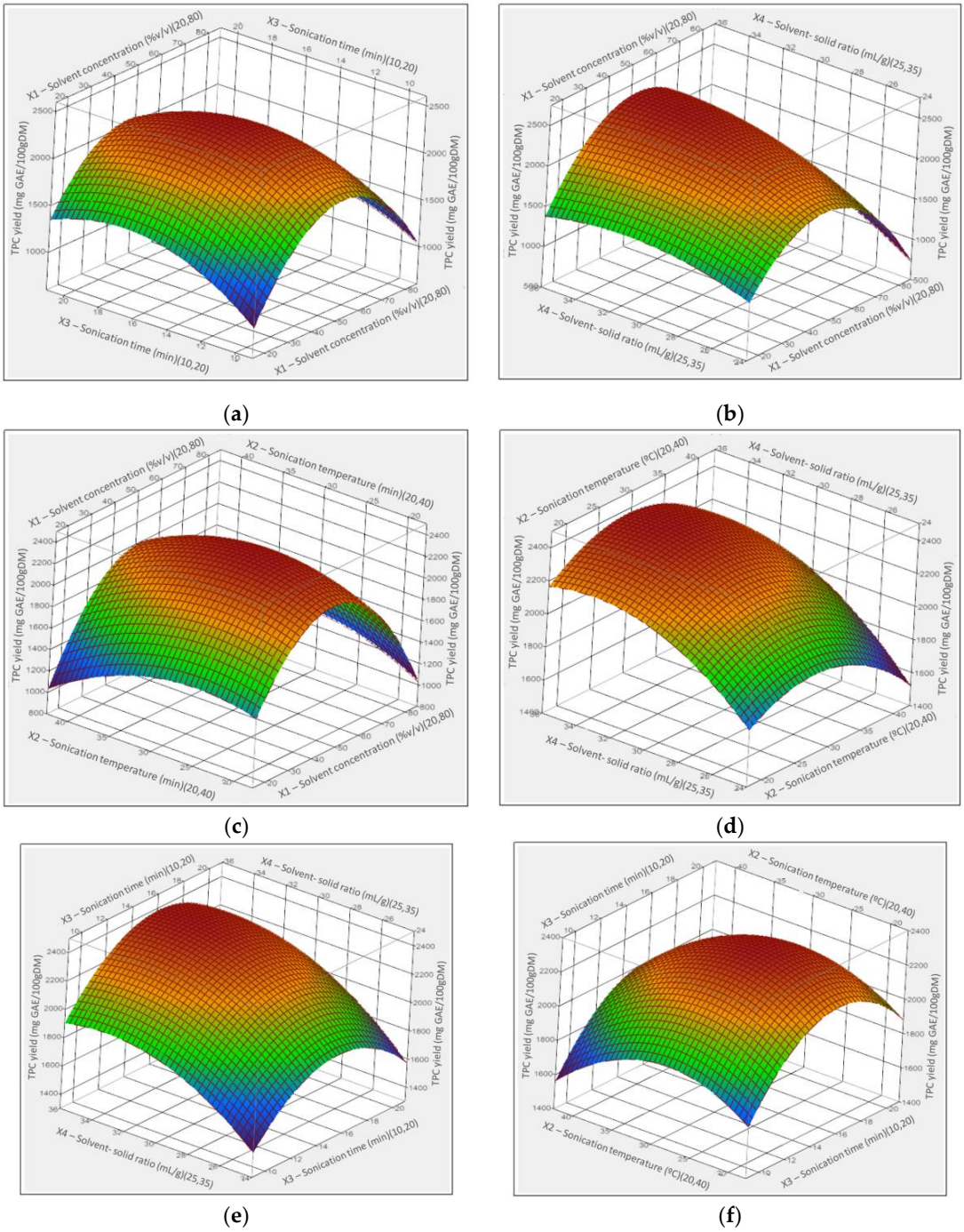


Figure 1. Response surface analysis for the total phenolic yield from *Z.lotus* seeds using UAE with respect to sonication time and ethanol concentration (**a**); solvent-to-solid ratio and ethanol concentration (**b**); sonication temperature and ethanol concentration (**c**); solvent-to-solid ratio and sonication temperature (**d**); solvent-to-solid ratio and sonication time (**e**); sonication temperature and sonication time (**f**).

The prediction values for the optimal TPC extraction were verified experimentally. Optimal conditions resulted in ethanol concentration 50.16%, sonication temperature 29.01 °C, sonication time 15.94 min and solvent-to-solid ratio 34.1/1 mL/g with a predicted TPC yield of 2406.08 ± 79.87 mgGAE/100 g (Figure 2).

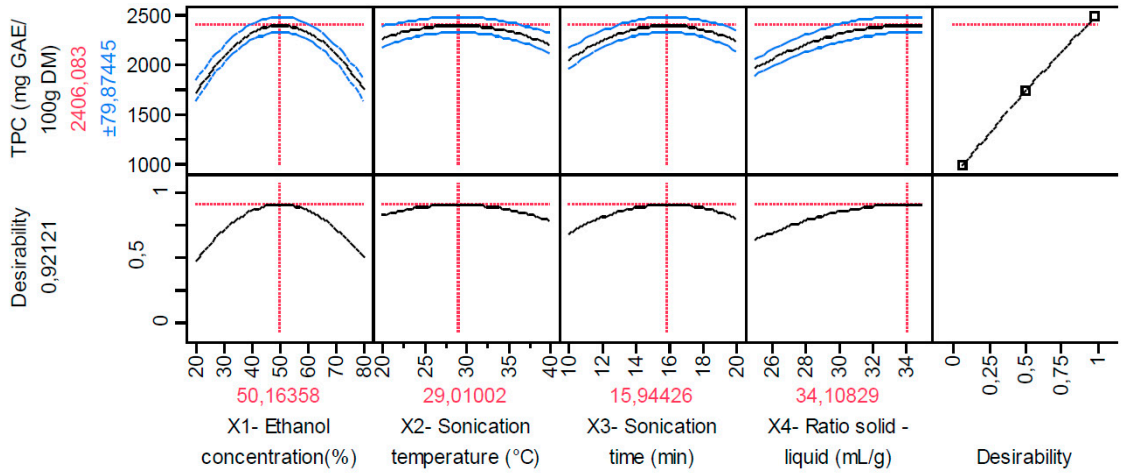


Figure 2. Prediction Profiler of *Z.lotus* seeds extract using UAE.

The UAE was carried out at these optimal conditions obtaining a TPC yield of 2383.10 ± 0.87 mgGAE/100g, very close to the value predicted by the model (Table 3). This result indicates that *Zls* showed a significantly higher TPC recovery in comparison to TPC obtained from other plants. In the meanwhile, the ethanol/water mixture showed a high extraction efficiency by ultrasound and this was in agreement with other studies showing a great effect of ethanol concentration for phenolic compounds extraction, from *Citrus limon* fruit using 63.93% of ethanol (1502.2 ± 0.88 mgGAE/100 g) [32]. Similarly, from *Pistacia lentiscus* leaves that obtaining a lower TPC value of 1420.76 ± 19.98 mgGAE/100 g [40]. More recently, Esmaeelian, et al. [41] have shown a lower TPC value from *Crocus sativus* L. corms extract than our result (100.39 mgGAE/100 g dry saffron corm) using 80% of ethanol. The extraction process used in the present work give higher TPC than Indian jujube cultivars using conventional extraction method ranging from 172.08 to 328.65 mgGAE/100 g with 80% of ethanol [42]. Similarly, higher TPC recovery than work of Al-Saeedi, et al. [43] from Oman *Zizyphus jujuba* fruit which has a content of 64.89 ± 0.44 mg GAE/100 g using methanol as extraction solvent. More recently, Noriega-Rodríguez, et al. [44] found a TPC value of 2.16 g gallic acid equivalent (GAE)/100 g from Globe artichoke (*Cynara scolymus* L.) using conventional extraction method with 75% of ethanol which is lower than jujube extract. To note, these results show the efficiency of extraction of the phenolic compounds using the hydro-alkolic solvent which makes it possible to increase the polarity, and thus the solubility of the solid ratio that increased the extraction yield beside the other factors.

However, the TFC using UAE (Table 3) was found to be significantly higher (486.50 ± 0.38 mg QE/100 g) than those obtained from *Zizyphus jujuba* seeds using ethanol 70% by ultrasound extraction method (200.01 ± 0.15 mg/100 g) [45]. Similarly, our extract showed a significantly higher TFC than *Zizyphus jujuba* fruits extract obtained at different stage of ripening, ranging from 26.7 to 48.5, 19.9 to 34.6 mg QE/100 g in "Ya Tsao", "Ta-Jan Tsao" cultivars, respectively [46].

Regarding to the TTC yields, the *Zls* extract was for 15,787.10 ± 0.10 mg CE/100 g (Table 3), which is five times higher than that of polymeric proanthocyanidins extracted from *Zizyphus jujuba* fruits, which was between 939 and 2548 mg/100 g in depends on cultivars [47]. The present work suggests that UAE method is an efficient alternative to other extraction techniques for extracting and maximizing polyphenols from *Zls* in short extraction time and jujube seeds is a non-negligible source of polyphenols.

Table 3. Biological activities of *Z. lotus* seeds extract using UAE. Results are expressed as means ± standard deviation.

Factors	Ultrasound Extraction
Sonication time (min)	15.94
Ethanol concentration (%)	50.16
Sonication temperature (°C)	29.01
Solvent solid/ratio (mL/g)	34.10
Results	
Recovery of TPC (mg GAE/100 g)	2383.10 ± 0.87
Recovery of TFC (mg QE/100 g)	486.50 ± 0.38
Recovery of TTC (mg CE/100 g)	15,787.10 ± 0.10
DPPH scavenging EC_{50} (µg/mL)	0.39 ± 0.00
FRAP (mg GAE/100 g)	1670.42 ± 6.50
AChE assay IC_{50} (mg/mL)	0.93 ± 0.01
HMGR assay (%) (for 100 µg/mL)	45.41
HepG2 cells IC_{50} (mg/mL)	3 ± 0.50
MCF-7 cells IC_{50} (mg/mL)	<0.05± 0.0

3.2. Biological Activities

3.2.1. Antioxidants Activity of *Zls* Extract

The imbalance between the production of reactive oxygen species (ROS) and the biological system's antioxidant defenses is defined as the oxidative stress. This proved in several studies to develop a lot of diseases. In order to protect our human body from these diseases, the antioxidants are showed to be effective for neutralization of free radicals [14]. However, to determine whether the UAE extraction process impact the biological functions, the antioxidant effects of jujube seeds extracts were examined and evaluated by DPPH radical scavenger and FRAP model (Table 3).

The DPPH scavenging assay of the *Zls* extract revealed a significant highest activity with lowest EC_{50} value (0.39 µg/mL) than that of Tunisian *Z. lotus* leaves and fruits extract using methanol (0.10 ± 0.001 and 0.31 ± 0.005 mg/mL) [48], Tunisian *Z. lotus* pulp and peel extracts using UAE with an EC_{50} of 0.28 mg/mL [19], from *Z. mucronata* roots (0.029 ± 0.05 mg/mL) [49], and from Korian *Z. jujuba* seeds (mechu and sanzoin) (0.3 and 0.1 mg/mL), respectively [50]. Overall, this study showed that *Zls* extracts exhibited a high antioxidant effect in comparison to some *Zizyphus* species using both UAE and conventional methods. Furthermore, *Zls* extract by UAE exhibited a significant iron-reducing power (1670.42 ± 6.5 mg/100 g) and was found to be higher than those obtained with 471.6 ± 30.8 mg/100 g from *Z. jujuba* cv. *Zaowangzao* [51].

Thus, this paper revealed the effectiveness of ultrasound method for extraction of polyphenols from jujube seeds with significant antioxidant activities in comparison to conventional methods that gave lower recovery and activity which could be attributed to the mechanic cavitation of ultrasound due to the acoustic bubbles which results to enhanced desired compounds without altering its quality. The inhibition of DPPH as well as the iron chelating effect are probably due to the significant jujube content in photochemical substances especially in phenolic compounds, the latter having powerful reducing effects of oxidation. In addition, from other jujube extracts that contain other compounds namely ascorbic acid, tocopherol, and pigments, were found to present synergic effects between them and contribute to the total antioxidant activity of this extract, and therefore the trapping of free radicals. While, this suggested that not only phenolic compounds present in the jujube extract can act as antioxidants but also other compounds may be responsible for this activity [52–54]. In our case, the antioxidant activity using DPPH and FRAP methods from *Zls* extract is in perfect agreement with other research showing that there can be correlation between the phenolic content and the antioxidant capacity [55].

3.2.2. AChE Inhibition of *Zls* Extract

The antiacetylcholinesterase activity of the *Zls* extract are presented in the Table 3. The rapid hydrolysis of ACh following the stopping of the transmission of nerve impulses to cholinergic synapses is well controlled by the role of AChE. One of the ways deemed effective used against AD is more particularly based on the inhibition of AChE, which makes it possible to maintain the levels of acetylcholine for the transmission of nerve impulses [56]. The anticholinesterase activity of jujube extract at different concentrations exhibited inhibitory activity with an IC_{50} value of 0.93 ± 0.01 mg/mL. These results are higher than that obtained from *Zizyphus oxyphylla* extracts using *n*- butanol which showed a maximum inhibitory effect with an IC_{50} value of 9.58 ± 0.08 mg/mL [57]. This demonstrated the good effect of the used method mainly the good extraction yield of many compounds from *Zls*, ethanol/water used for extraction and ultrasounds cavitation of *Zls* which exhibited a good inhibition of AChE which can be applicable against AD. Ethanol concentration is considered as a crucial factor in UAE due to cavitation phenomena enhancing solvent penetration into jujube extract [32]. Moreover, the *Zls* extract is approximatively in the same range than Tunisian *Zls* extract [58] using acetone for extraction by maceration method (0.85 mg/mL). However, in comparison to other plants that used acetone also, our extract had a highest AChE inhibitory effect than that obtained from *Herniaria fontanesii* and *Hyoschy amusalbus* with an IC_{50} value of 1 and 1.17 mg/mL, respectively [58]. Thus, our results suggested that extraction using ultrasounds under different conditions mainly ethanol as solvent extraction is an important parameter to take into account in AChE tests. Furthermore, *Fucus vesiculosus* extracts from Tagus estuary presented an IC_{50} of 840.85 µg/mL which is in well agreement than our AChE inhibitory from *Zls*. In addition, the standard galantamine showed an IC_{50} of 0.14 µg/mL that is lower than these extracts [59]. These suggests that the intestinal motility may be affected by its consumption although with a much less effect comparatively to the chemical drugs [37]. Major medicinal plant extracts showed some level of inhibitory activity against the AChE. This could be attributed to the phytochemicals mostly phenolic compounds present in the extract and their possible synergistic interaction effect [59–64]. To the best of authors knowledge, this is the first report on in vitro inhibition of the AChE enzyme by *Zls* extracts under the effect of ultrasound extraction where the interest of its application against Alzheimer's disease.

3.2.3. HMGR Inhibition of *Zls* Extract

The HMGR inhibition by the *Zls* extract is demonstrated, at the concentration of 100 µg/mL *Zls* ethanolic extracts showed an activity of 45.41% as a HMGR inhibitors which is higher than the acetone and ethanol extracts from lichen *U. complanata* with 2.22 and 21.48%, respectively, at the concentration of 60 µg/mL [12]. Similarly, *Peganumarma* and *Tencriumpolium* from aerial parts extracts showed a value of 28.5 and 28.8% of HMGR inhibition which are lower than our sample [65]. However, *Zls* extract showed a significant HMGR inhibitory effects than seeds extracts of some species, mainly *Cannabis sativa* (7.4%), *Cuminum cyminum* (26%), and *Pimpinella anisum* (10.5%) [65]. The IC_{50} of the drug simvastatin was found to be around 0.198 ± 0.015 g/mL, which is lower than the value determined for the *Zls* extract, therefore a higher activity than that observed in our extract. These are in good agreement to our previous results from *Centaurium erythraea* extracts [37]. However, this drug is a pure compound while *Zls* is a mixture of several compounds some of which may show significant activities but which are masked in the mixture [59]. Several bioactive compounds mainly polyphenols, saponins, alkaloids, and triterpenes were found to have a good hypolipidemic activity against HMGR [66–68]. Thus, jujube extract was found as a modest HMGR inhibitors which is due to the presence of some bioactive compounds that may be responsible for this enzyme inhibition like all polar plant extracts found in literature [66,67]. The properties of our sample phytochemicals make them possible antihyperlipidemic applications by fitting the enzyme active site.

Furthermore, other *Zizyphus* species (*Z. mauritiana*) leaves extracts were used previously for the treatment of fatty liver and atherosclerosis by reducing cholesterol and triglyceride and levels [69]. Among some studies that have shown the effect of polyphenols in inhibiting the action of HMG-CoA,

demonstrated that curcumin, tetrahydrocurcumin, epigallocatechin-3-gallate, and kaempferol among all the other polyphenols tested can occupy the HMG-CoA binding site on the NADP+ site which utilizes two molecules of nicotinamide adenine dinucleotide phosphate-oxidase (NADP[H]); thus, can play the role of competitive inhibitors of substrate binding to enzyme that can block the electron transfer on the substrate HMG-CoA [70]. Compelling effect of these compounds and major phenolic compounds in general indicates the importance of their uses for the cholesterol-lowering in order to the maintenance of cardiovascular health [71,72].

3.2.4. Anti-Proliferative Activity of *Zls* Extract

The in vitro evaluation of *Zls* extracts on cytotoxicity effects were analyzed. The toxicity of extracts was tested in the human cell lines $HepG_2$ and MCF-7 using 5 serial concentrations ranging from 0.05 to 1 mg/mL of extract in order to calculate the cell viability. The IC_{50} values which confirm the concentration of extracts that killed 50% of the cells was obtained from dose-response curves. The phenolic compounds of *Zls* studied were revealed to be non-toxic towards only to $HepG_2$ cell line because the value is higher than 0.1 mg/mL. This value is considered as limit of toxicity to human cell lines [73]. In contrary, *Zls* extract analyzed against toxicity in MCF-7 cells showed an IC_{50} value lower than 0.05 mg/mL which is considered toxic to human cell lines. The value of 1 mg/mL which correspond to the maximum concentration present in the UAE extract inhibited 70.84% and 26.21% for MCF-7 and $HepG_2$ cells, respectively. Thus, *Zls* extract exhibited no significant activity against $HepG_2$ cells. In contrary, it exhibited a significant activity against MCF-7 cells. These findings can be due to the main bioactive compounds contained in *Zls* extract that may include flavonoids, tannins, alkaloids, terpenoids, and saponins as observed previously from other jujube species and from other part of *Zizyphus lotus* which showed a strong antiproliferative activity against $HepG_2$ and MCF-7 cells [21,74].

In addition, *Z. jujube* fruits extract was found to exhibit activity against MCF-7 cells with an IC_{50} value of 1.8 mg/mL after 24 h which is higher value than that of *Zls* extract (IC_{50} less than 0.05 mg/mL after 24 h) [75]. The results of cytotoxic action of *Z. jujuba* extract indicated a reduction inviability and high potent inhibitory effect toward the proliferation of MCF-7 cells, this effect may be due to cell apoptose [76]. However, the cytotoxicity effect reported from *Zls* extracts against $HepG_2$ cells was significantly higher than that reported from mung bean sprouts extracts obtained using maceration method with an IC_{50} of 14.04 ± 1.5 mg/mL. This extract also contained polyphenolic compounds [77]. The effective cytotoxicity toward hepatocellular $HepG_2$ and beast MCF-7 could be related to the presence of the some secondary metabolites, some of them are considered the major class of jujube polyphenols, represented from 89 to 94% of the total phenolic contents mainly flavan-3-ols such as monomer (as (-)-epicatechin, gallocatechin gallate, and (+)-catechin), dimer (procyanidine B2), and polymeric proanthocyanidins in *Zls*, which were applicated in the inhibition of cell proliferation in different cancer types [47]. Quercetin-3-*O*-rutinoside found in other parts of *Z. lotus*, which represents 50% of jujube flavonoids offers a plausible explanation of the observed cytotoxicity [74].

The present research data suggests that in some cases the ultrasound extraction can positively influence the extraction yield of TPC, TFC, and TTC, also the antitumor activity against the tested cell lines. This is can be related to the mechanical acoustic effects of ultrasound which causes the rupture of the cell wall allowing mass transfer, and therefore increase the recovery process of TPC but following the sonication time (15.94 min), which is a very sensitive parameter in extraction procedure. To the best of our knowledge, no study has as yet been carried out on the effects of ultrasounds on phenolic extracted from *Zls* and cytotoxicity toward hepatocellular $HepG_2$ and beast MCF-7 of the studied UAE extract from *Zls*.

4. Conclusions

In this study, the effect of ultrasound on phenolic compounds extracted from jujube seeds based on the BBD model was employed to improve the extraction yield and bioactivities of *Zizyphus lotus* seeds extract. The optimal conditions of UAE were determined as ethanol concentration 50.16%,

sonication temperature 29.01 °C, sonication time 15.94 min, and solvent-to-solid ratio 34.1/1 mL/g, giving a maximum TPC yield of 2406.0835 mg GAE/100g. The *Zls* extracts exhibited a significant antioxidant effect with both DPPH and FRAP assays, with a high inhibitory effect for AChE and HMGR tests and antiproliferative capacities against MCF-7 than $HepG_2$ cell lines. However, this study proved that application of ultrasound technology was efficient for obtaining maximum yield of bioactive compounds from jujube seeds in a shorter time when compared to traditional method which can be used by exploiting the antioxidant properties (food additives, nutraceuticals, etc.), antiacetylcholinesterase, antihypercholesterolemia, and antiproliferative properties which may be useful in the development of new strategies to treat Alzheimer, hypercholesterolemia diseases, and cancer. Further studies are needed to identify active compounds after purification of extract and evaluate the cytotoxic mechanism of *Zls* extracts with in vivo models which be enable to production on an industrial scale (food, pharmaceuticals, and cosmetics).

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ORIGINAL PAPER

New bioactive constituents characterized by LC–MS/MS in optimized microwave extract of jujube seeds (*Zizyphus lotus* L.)

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Abstract

The present study was aimed to optimize the extraction conditions of total phenolic contents (TPC) from *Zizyphus lotus* seeds (*Zls*) samples by microwave procedure using response surface methodology in order to obtain maximum extraction yields. Central Composite Design was employed with three factors defined as solvent concentration (X_1), irradiation power (X_2) and microwave power (X_3) at three coded levels on the TPC recovery. The optimal conditions for X_1, X_2 and X_3 were: ethanol 60%, 210 s and 600 W, respectively. The experimental value of TPC yields was for 6709.01 ± 2.20 mg GAE/100 g which is in close agreement with the predicted value indicating the model success. Results showed that optimized *Zls* extract exhibited a high inhibitory effect on some biological activities including antioxidants and enzyme inhibition. The liquid chromatography-high resolution tandem mass spectrometry profile revealed 47 active compounds where 21 were never been detected in *Zizyphus* genus. The *Zls* extract was found to contain significant major compounds comprised 6-methyl-2-O-glucoside xanthone, jasminoside isomer, citric acid, gallocatechin, imidazole carboxylate derivative, kaempferol-3-O-robinobioside, 6-gingerol, 2-hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy)propyl]phenyl]propan-1-one, 3-(decyloxy)-2-hydroxy-propyl prop-2-enoate, tenasogenin and some small peptides. The findings demonstrated the beneficial application of microwave method for an increase extraction of TPC amounts from *Zls* extracts that could be valorized in food and pharmaceutical industries.

Keywords Jujube seeds · Microwave extraction · Phenolic compounds · LC–MS/MS characterization · Biological activities

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Introduction

Jujube (*Zizyphus lotus*. Lam) belongs to the Rhamnaceae family. In Algeria, it is widely distributed in the arid zone (Biskra, Djelfa) and north Algeria (Bejaia, Constantine, Medea and Bouira). Generally, it's in August that the different parts of *Z. lotus* are harvested just after maturation because the flowering takes place in May and June [1]. In these regions, different parts of jujubes have been used in oriental medicine for centuries to treat obesity, anemia, insomnia, bronchitis, diarrhea and to be consumed as excellent source of food for high levels of bioactive compounds with biological effects [2–4].

Among the secondary metabolites, in particular due to their structures, phenolic compounds have shown very important physiological effects, which helps protect plants from pathogen attacks [5]. On an industrial scale, due to their organoleptic properties (color and taste) it constitutes a major part of plant-derivatives foods. This class presented

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interesting biological and pharmacological activities including antiinflammatory, antitumor, antialzheimer and antioxidant activities [6, 7].

Our human body is often under attack by free radicals that are produced by metabolism of the oxygen and exogenous/environmental. Among the distinct mechanisms used against the oxidants, the inhibition of the ROS formation and their precursors are the best way used by the antioxidants. Many reports showed the effect of natural antioxidants found in plants [8, 9] and from jujubes [10–12] that had beneficial health effects.

For development of functional foods, several strategies have been used to assess the potential health benefits of herbals and nutraceuticals, one of them is the enzyme inhibitory assays [13]. Acetylcholinesterase (AChE) have become one of the most usual assays in Alzheimer's disease (AD). The transmission of nerve impulses by the acetylcholine in cholinergic synapses is terminated by AChE which increase its availability with hydrolysis of ACh to choline and acetate [14]. Several reports demonstrated the effect of some phenolic compounds on AChE activity [15, 16].

It is well known that the LDL-cholesterol contributes to several diseases unlike HDL-cholesterol which is protective. The biosynthesis of cholesterol, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) results from condensation of acetyl-CoA and acetoacetyl-coA. The rate limiting step is the conversion of HMG-CoA into mevalonate and is catalyzed by the enzyme HMGR. Phytosterols are synthesized through this pathway [17]. Several studies showed the important role of some phenolic compounds recovered in plants to reduce the free cholesterol level (hypercholesterolemia) and the perturbation of its synthesis by the activity of HMGR which is considered the best way to have a lower LDL in plasma. The biosynthetic pathway contains multienzyme in which HMGR mediates the rate-limiting step [18, 19].

Extraction is a critical step in isolating bioactive compounds from plant materials [20, 21]. Despite the diversity of studies made on the extraction of phenolic compounds from a wide range of plants including jujube of Chinese, Indian, Tunisian, Moroccan and Algerian origin with all its components, while the extraction of polyphenols using innovative methods from jujube remains marginal [22]. Recently, several studies suggested uses of this methods for extracting phenolic compounds from plants, include soxhlet extraction, ultrasound assisted extraction (UAE) [23, 24], heat reflux extraction (HRE) [25], and microwave assisted extraction (MAE) [8, 26]. However, others demonstrated another way to improve the recovery of bioactive compounds from natural matrix which is the use of microwave assisted extraction (MAE) that is totally different to the other extraction systems, it is based mainly on the scattering of heat under electromagnetic irradiation inside the sample, which accelerates the heating and allows the release of active ingredients in large quantities [27–29].

Response surface methodology (RSM) is useful for modeling quadratic models of design of experiment. Using a design of experiment based on response surface models, it can be identify and estimate if there is a minimum or maximum value inside the studied region. Three distinct levels for each continuous variable are necessary to fit a quadratic function, so standard two-value designs are not appropriate for fitting curved surfaces.

The few publications concerning the phytochemistry of *Z. lotus* seeds (*Zls*) report especially on the lipid, sugar and protein fractions. However, little is known about its phenolic profiles and other bioactive molecules. In our knowledge, nothing has been reported on the phenolic compounds characteristics of *Zls* comparing to fruit, leaves and roots in general, after work of Chouaibi et al. [30] which showed the chemical composition of the seeds and its beneficial effects as demonstrated by Abdoul-Azize [31].

For the best of our knowledge, no literature report exists on the optimization extraction of total phenolic compounds (TPC) from *Zls* using combination of MAE and RSM. This subject deserves further investigation on the seeds of the jujube using the microwave method as we have shown its effectiveness in our previous paper developed from pulp and peel by this one in order to enhance jujube by-products. On the other hand, in order the replace synthetic antioxidant such as butylatedhydroxyanisole (BHA), propyl gallate (PG) and butylatedhydroxytoluene (BHT) that present dangerous toxic effects on human health by natural antioxidant extracted from *Zls* which can be valorized in food industries. In the meantime, this study is driven by the consumer need to nutraceutical food production based on phenolic compounds with a good health promotion, mainly against AD and hypercholesterolemia. Therefore, the current study aimed to investigate the influence of different factors on the extraction efficiency (in terms of recovery and biological activities of total phenolic compounds) by MAE process; In addition, we sought to evaluate the antioxidant, anticholiesterase and anti HMGR activities. Specially, the major phenolic compounds present in the *Zls* extracts were identified using liquid chromatography-high resolution tandem mass spectrometry (LC–MS/MS), and their biological effects were also studied.

Materials and methods

Biological material and chemicals

1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\cdot}$) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Folin–Ciocalteu, gallic acid and all chemicals

used in this study were from Sigma Aldrich co (St. Louis, MO, USA), Biochem-chemopharma (UK) and (Fontenay-sous- Bois, France).

Jujube fruit seeds (*Zizyphus lotus* L.), were harvested in August 2017 from Djelfa a 270 km south of Bouira province, Algeria country (34° 40′ 30″ N, 3° 15′ 30″ E). The representative samples of approximately 5 kg were cleaned from impurities and placed in glass container, kept in equilibrate storage environment (25 °C and 60% relative humidity) for several weeks. Prior to the experiment, the jujube seeds were manually removed from the pulp and allowed to analyze for moisture content, and dried at 40 °C for 24 h (Memmert, Modell 100–800, Schwabach, Germany). The seed samples were ground with an electrical laboratory grinder (WH model 8100 Basic, Beijing, China), a powder obtained was sieved and a fraction size < 250 µm was used and kept in the dark conditions (4 °C) until analyses.

Extraction procedure

Experimental work

The extraction procedure of total bioactive compounds from a powder jujube seeds were carried out two

Table 1 Optimized microwave assisted extraction procedure of phenolic constituents from jujube seeds

Step 1: Vary one factor at a time approach				
Solvant type	Ethanol concentration	Irradiation time	Microwave power	Solvant-to-solid ratio
(Type) [a]TPC yield (mg [b]GAE/100 g)	(% v/v) TPC yield (mg GAE/100 g)	(s) TPC yield (mg GAE/100 g)	(W) TPC yield (mg GAE/100 g)	(mL/g) TPC yield (mg GAE/100 g)
Water: 1996.08 ± 1.35^{d}	20: 2606.41 ± 1.35^{f}	60: 2465.72 ± 1.62^{e}	300: 1914.71 ± 1.35^{d}	20/1: 2254.30 ± 1.35^{c}
50% MeOH: 2254.30 ± 1.35^{b}	30: 3006.25 ± 2.71^{c}	90: 2324.10 ± 0.13^{g}	400: 2255.08 ± 2.71^{c}	25/1: 2347.49 ± 0.13^{b}
50% EtOH: 2819.24 ± 2.34^{a}	40: 4085.13 ± 1.08^{a}	120: 2349.13 ± 1.08^{f}	500: 3051.72 ± 0.13^{a}	30/1: 3053.20 ± 2.71^{a}
50% Acetone: 2159.70 ± 0.13^{c}	50: 2819.32 ± 2.23^{d}	150: 3145.61 ± 0.14^{a}	600: 2582.47 ± 0.54^{b}	35/1: 1809.15 ± 2.84^{d}
	60: 3875.58 ± 4.06^{b}	180: 2930.35 ± 1.35^{b}		
	70: 2676.60 ± 0.94^{e}	210: 2841.94 ± 2.71^{c}		
	80: 2176.68 ± 1.08^{g}	240: 2488.49 ± 0.40^{d}		

Step 2: Central composite design on face					
Run	X_1- Ethanol concentration (% v/v)	X_2- Irradiation time (s)	*X3*- MW power (W)	TPC yield (mg GAE/100 g)	
				Experimental	Predicted
1	−1(20)	−1(90)	−1(400)	2198.75	2381.91
2	1(60)	−1(90)	−1(400)	2363.07	2039.97
3	0(40)	0(150)	−1(400)	1838.81	1236.04
4	−1(20)	1(210)	−1(400)	2370.89	2336.14
5	1(60)	1(210)	−1(400)	3192.49	3969.94
6	0(40)	−1(90)	0(500)	1987.48	2140.64
7	−1(20)	0(150)	0(500)	2887.32	2990.34
8	0(40)	0(150)	0(500)	2871.67	2611.69
9	0(40)	0(150)	0(500)	2425.66	2611.69
10	0(40)	0(150)	0(500)	2668.23	2611.69
11	1(60)	0(150)	0(500)	5633.80	5124.93
12	0(40)	1(210)	0(500)	2699.53	3082.73
13	−1(20)	−1(90)	1(600)	2261.35	2084.18
14	1(60)	−1(90)	1(600)	4084.51	4719.55
15	0(40)	0(150)	1(600)	2230.05	2426.96
16	−1(20)	1(210)	1(600)	2112.68	2038.41
17	1(60)	1(210)	1(600)	7230.05	6649.52

[a]TPC: total phenolic compounds

[b]GAE: gallic acid equivalent

sequential steps, which the first consists in establishing meaningful factors and their levels influencing the extraction efficiency while varying one factor at a time approach (Table 1). However, a second step with an optimization procedure based on response surface design of experiment was performed. The second-order prediction equation for the TPC response was fitted by response surface designs. In these equations, the quadratic terms model the curvature in the true response function. Among of these quadratic models, we have a central composite design which combines a two-level full factorial design, center points, where all the factor values are set to the midrange value and axial points, where one factor is set to a high or low value (star value) and all other factors are set to the midrange value. In this paper, the selections of relative star points give a CCD on Face ($\alpha = 1$). Referring to the results of the one factor at a time approach, the major factors, namely ethanol concentration in water (%), irradiation time (s) and MW power (W) were varied according to CCD layout table (Tables 1 and 2).

A statistical analysis was performed by using JMP (SAS, version 13 Pro). ANOVA allowed determining the regression coefficients of main effects, interactions, response surface effects. The relationship between the experimental levels of the factors studied with the response yield and finally to infer the optimum from the experimental results. Therefore, the three-dimensional surface plots were generated from the equation of the adjusted model following the use of the regression coefficients. In order to validate if the model describe suitably the extraction behavior of the seeds powder, a statistical parameters of the fitted data are defined as the determination coefficient (R^2), adjusted determination coefficient, lack of fit significant and the root mean square error (RMSE). Finally, to ensure the adequacy of the critical predicted solution obtained by CCD models to the experimental condition, additional extraction trials were established and were compared to the optimal conditions.

Table 2 Regression coefficients estimation and their *p*-values for the reduced fitted quadratic model for microwave assisted ethanol extraction processing of phenolic compounds from jujube

Parameters	Coefficients	*P*-value
Model B_0	2611.69	$< 0.0001^*$
Linear		
X_1- Ethanol	1067.29	0.0001^*
X_2- Time	471.05	0.0193^*
X_3- Power	595.46	0.0058^*
Quadratic		
X_1^2	1445.95	0.0010^*
X_3^2	−780.18	0.0291^*
Interaction		
X_1X_2	493.94	0.0257^*
X_1X_3	744.33	0.0030^*

*Significant

Microwave assisted extraction (MAE)

For the extraction procedure, a modified domestic microwave (NN-S674MF, Samsung, Malaysia), cavity size of 22.5 cm × 37.5 cm × 38.6 cm, 2450 kHz, was used. 1.0 ± 0.1 g powder seeds were mixed in 250 mL flask containing a solvent according to one factor at a time and CCD table layouts (Tables 1 and 2). After microwave heating, the volumetric flask was cooled at room temperature. Thereafter, the extract was filtered through Whatman No. 1 paper and kept in shaded flasks at 4 °C for possible analysis. Each trial was carried out in triplicate.

Analytical determinations

Total phenolic content (TPC)

The yield of TPC was determined for *Zls* extracts obtained with microwave method according to the coloric method based on Folin–Ciocalteu reaction used by Hammi et al. [32] with some modifications. The concentration of TPC was calculated using gallic acid as standard with referring to a calibration curve. Results were expressed in mg of gallic acid equivalent per 100 g of dry matter (mg GAE/100 g).

Total flavonoids content

The total flavonoid content was measured spectrophotometrically, at 430 nm, using methanolic aluminium chloride ($AlCl_3$) for 2% as described in Ghafar et al. [33], using quercitin as standard and the concentration of total flavonoids expressed as mg quercitin equivalents per 100 g of dry matter (mg QE/100 g). Samples were measured in triplicate.

Total condensed tannins content

The total condensed tannins content was determined using the method of Hagerman [34]. The absorbance was readied at 500 nm and the results were expressed as mg catechin equivalent per 100 g of dry matter (mg CE/100 g). Samples were measured in triplicate.

Compound Identification by LC–MS/MS

The identification of phenolic compounds from *Zls* was performed by means of high resolution tandem liquid chromatography-mass spectrometry (LC–MS–MS) as described by Guedes et al. [35] with some modifications: liquid chromatography-high resolution tandem mass

spectrometry (LC-HRMS/MS) was carried out using an Elute OLE UHPLC system interfaced with a quadrupole time-of-flight (QqToF) Impact II mass spectrometer equipped with an electrospray source (ESI) (Bruker DaltoniK GmbH, Bremen, German). Chromatography separation was carried out on an Intensity Solo 2 having 1.8 mm C_{18} with 100 × 2.1 mm dimensions column (Bruker Daltonics, Bremen, Germany). The mobile phase was composed of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), the column and the sampler were kept at 35 °C and 10 °C, respectively. The mass analysis was carried out in negative and positive mode using and ESI methodology, being the optimized parameters: −3.5 kV and + 4.0 kV; end plate offset, 500 V, nebulizer gas (N_2) 2.0 bars; dry gas (N_2), 8 $Lmin^{-1}$; dry heater, 200 °C; collision cell energy was set to 5.0 eV. The internal calibration was performed with 250 mL H_2O, 50 mL iPrOH, 750 mL acetic acid, 250 mL formic acid, and 0.5 mL 1 N NaOH solution on High Precision Calibration (HPC) mode. The acquired data were processed by DataAnalysis 4.1 software (Bruker Daltonik GmbH, Bremen, Germany). The compound identification was accomplished by considering the suggestions from the DataAnalysis® program version 4.4 from BRUKER and the Metlin database. The putative structures for the identified compounds were drawn by MassFrag from Bruker (Bremen, Germany) having into consideration the MS/MS fragmentation.

Antioxidant activity by DPPH assay

Radical-scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH·) from *Zls* extracts was measured by the methods of Achat et al. [36].

Antioxidant activity as ferric iron reducing power (FRAP)

The ability of *Zls* extracts to reduce ferric iron (Fe^{3+}) in presence of an antioxidant which gives out electrons, was determined by Hammi et al. [37].

Acetylcholinesterase (AChE) inhibitory activity

AChE inhibition was measured using the method of Falé et al. [38].

HMGR inhibitory activity

The inhibition of the enzymatic activity of 3-hydroxy-3-methylglutaryl reductase (HMGR) was determined according to the method described by Guedes et al. [35].

Results and discussion

Phenolic recoveries and MAE optimized process

The phenolic recovery of jujube seeds sample was optimized in two experimental sessions. Table 1 shows the TPC yield obtained from a single factor experiment of seeds samples with different solvents types, a best selected solvent concentration (ethanol), extraction time and MW power by MAE. It is clear that the yield obtained by ethanol was statistically highly significantly different compared to those obtained by methanol, acetone and water ($p < 0.01$). On the other hand, mass transfer within the sample powder in ethanol was more appropriate with 9, 3, and twofold heighted to the recovery reached by water, acetone and methanol respectively. Similar findings have been observed and reported in our previous work [39]. Therefore, ethanol was used for extracting TPC in next optimization study.

As shown in Table 1, the best result was obtained when ethanol set at 40% (v/v) with a recovery enhanced from 2606.41 ± 1.35 to 4085.13 ± 1.08 mg GAE/100 g. The percentage of ethanol in water was very important in extraction assisted by microwave which resulted in a higher absorption of microwave irradiation power and higher recovery due to the higher of solubility and diffusion coefficient of TPC compounds. The obtained results were in agreement to those demonstrated by several authors using MAE for extraction of phenolic from vegetable matrix [8, 39–41]. For this, 40% was fixed for the next single-factor experiments and 20–60% was selected for the RSM trials.

For the effect of irradiation time on the extraction process, as indicates in Table 1, a TPC recovery has two stages. The recovery increase rapidly and then decreases slowly as extraction progresses. In general, extraction time in MW process is considered a very important parameter that must be taken in consideration because the energy absorbed by matrix causes rapid microwave heat. Our results are in agreement with those of other literature, mentioning that extracts exposed to a longer irradiation times may cause a degradation of TPC following the thermal microwave effect [42]. Moreover, as explained in the previous paragraph, the irradiation time is also influenced by the dielectric properties solvent, like ethanol that can undergo tremendous heating during prolonged exposure to microwave irradiation and so risk losing the desired bioactive components. In the statistical analysis reported in Table 1, 150 s was selected for the next single-factor trials, while the range 90–210 s was selected for the RSM trials.

Moreover, microwave power significantly influenced the TPC yield as shown in Table 1, the yield increased with increasing microwave power from 200 W to 500 W and then decreased for higher powers, the best yield was at 500 W

(3051.72 ± 0.13 mg GAE/100 g) which was selected for the last single-factor trials, while the range of 400–600 W was selected for the RSM study. Several reports have demonstrated the main effect of microwaves, and in many cases the only, is the heating effect [8, 43].

Finally, the TPC yield increased significantly from 2254.30 ± 1.35 to 3053.20 ± 2.71 mg GAE/100 g (Table 1) with the increase of solvent-to-solid ratio up to 30 mL/g. This result could be related to the quantity of solvent in which the vegetable matrix powder is immersed must be sufficient in order to have a good amount of extraction of the TPC, this is generally due to the effect of the microwave heat which accelerates the diffusion of the solvent [29]. The TPC yield in excess to 30 mL/g was non-significant. Thus, this we are referred to the screening study and other works focused on the extraction of herbs by microwaves and 30 mL/g was chosen for the RSM trials.

The mathematical model of TPC yield according to three selected parameters is following Eq. 1:

$$Y_{TPCyield} = a_0 + a_1 Ethanol\% + a_2 time + a_3 MWpower + a_{12} Ethanol * time + a_{13} Ethanol * MWpower + a_{23} time * MWpower + a_1 Ethanol^2 + a_2 time^2 + a_3 MWpower^2 \quad (1)$$

A quadratic model fitted to the TPC recovery from a jujube seeds data involving X_1- ethanol concentration (%), X_2- irradiation time and X_3- MW power is statistically highly significant ($p < 0.01$) with a p-value of 0.0002, while a lack of fit p-value of 0.1344 is non-significant, a strong correlation between the observed and predicted values are observed by the coefficient of determination ($R^2 = 0.92$) and adjusted determination coefficient ($R^2_{Adj} = 0.86$) which were reasonably close to 1, the residuals were distributed randomly and non-patterned therefore the overall model indicating a good deal of reliability of the input response and output factor.

Table 2 provides the ANOVA analysis of all significant coefficients for the reduced model given in Eq. 2.

$$Y_{TPCyield} = 2611.69 + 1067.29 Ethanol\% + 471.05 time + 595.46 MWpower + 493.93 Ethanol * time + 744.33 Ethanol * MWpower + 1445.95 Ethanol^2 - 780.19 MWpower^2 \quad (2)$$

A quadratic model fitted to $Y_{TPC\ yield}$ data showed that linear terms for all independent parameters were statistically significant ($p < 0.05$) and only ethanol concentration and irradiation time ($p = 0.0257$) as well as ethanol concentration and MW power ($p = 0.0030$) showed significant interaction. At same time, the quadratic terms of ethanol concentration and MW power had a significant effect on TPC yield (Table 2).

The desirability function (0.9) was used in the prediction profiler to find optimal factor settings and indicated an extraction efficiency of 6649.52 mg GAE/100 g.

Analysis of response surfaces

The influence of the process parameters and the mutual interactions on the TPC yield is depicted by the three dimensional (3D) response surface graphs as shown in Fig. 1.

The recovery of TPC extracted from *Zls* is increased with increasing of ethanol concentration gradually from 40% to 60% at longer irradiation time (210 s) to reach a value of 8000 mg GAE/100 g (Fig. 1a). Thus, the increase in the time of exposure to microwave irradiations affects positively the efficiency of the extraction efficiency as its linear effect which was very significant. The extraction of TPC from jujubes using microwaves could be enhanced using ethanol in water over a limited range that facilitate the increase of the contact surface between the solvent and vegetable material which results an increase in extraction yields [39, 43]. Additionally, increasing ethanol/water promotes the extraction of more phenolic groups than non-phenolic contents mainly carbohydrates and terpenes. While, from structure plan, some phenolic compounds extracted from plant materials were found to be in complexion forms when are only extracted with organic solvents, for instance, ethanol and methanol as shown by Kamarudin et al. [44]. Furthermore, Fig. 1b showed clearly that increase in ethanol concentration from 20% to 60% causes the increase of TPC in few minutes at a slightly increase of microwave power to 600 W from 1500 to around 3000 mg GAE/100 g. After that, additional microwave power causes negative effect and decreased the yield to 2000 mg GAE/100 g after 600 W, as its significant quadratic negative effect illustrated in Table 3. These results confirm those of single factors experiments illustrating the remarkably significant positive interaction effect between ethanol X_1 and X_3 (Table 3). Higher microwave power decrease the viscosity of extraction solvent and enhances the diffusion rate of soluble phenolic compounds due to higher heat activation in the extraction process. However, increasing in mass transfer phenomena up to a certain power value (in our case > 600 W) can lead to thermal degradation of some bioactive compounds [44, 45]. Moreover, in present work, no significant interaction between irradiation time and MW power was found ($p > 0.05$), but its significant contribution to the extraction efficiency was observed only in their linear terms (Fig. 1c and Table 2).

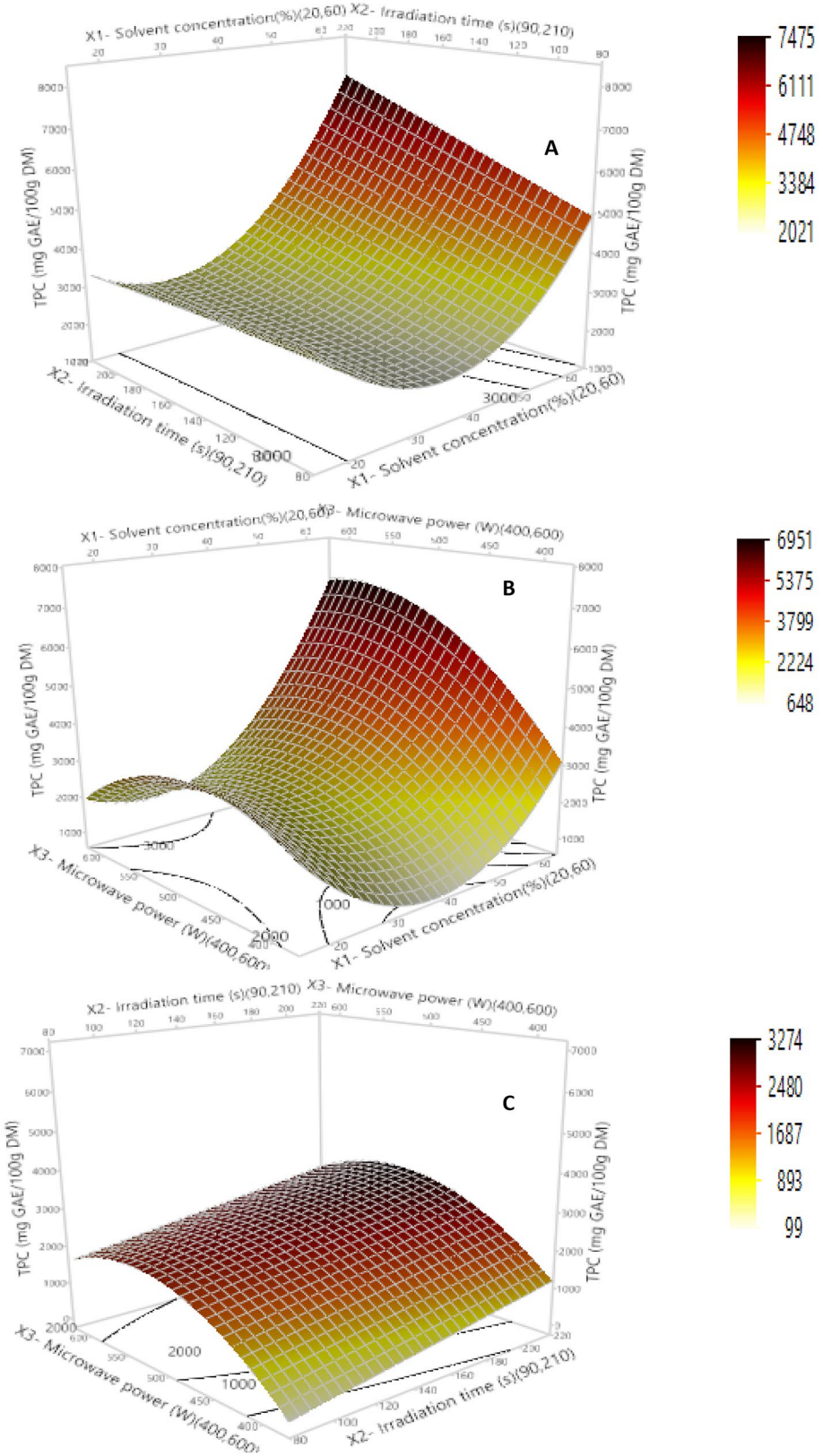


Fig. 1 Response surface plots for the total phenolic recovery from jujube seeds extracted by microwave extraction method with respect to ethanol concentration and irradiation time (**a**); ethanol concentration and microwave power (**b**); microwave power and irradiation time (**c**)

Table 3 Phenolic yields of jujube seeds samples and some biological activities under optimum extraction conditions

Factors	Microwave extraction
Irradiation time (s)	210
Ethanol concentration (%)	60
Microwave power (W)	600
Solvent solid/ratio (mL/ g)	30
Results	
Recovery of total phenolic TPC[a] (mg GAE[b]/100 g)	6709.01 ± 2.20
Recovery of total flavonoids TFC[c](mg QE[d]/100 g)	499.21 ± 0.59
Recovery of condensed tannins TTC[e] (mg CE[f]/100 g)	25,000.74 ± 1.28
DPPH[g] scavenging EC_{50} (mg/mL)	0.000067 ± 0.00
FRAP[h] (mg GAE/100 g)	2039.60 ± 8.43
AChE[i] assay IC_{50} (mg/mL)	0.88 ± 0.02
HMGR[j] assay (%) (for 100 µg/mL)	28.71

[a]TPC: total phenolic compounds
[b]GAE: gallic acid equivalent
[c]TFC: Total Flavonoids Content
[d]TTC: Total condensed Tannins Content
[e]QE: Quercitin equivalent
[f]CE: Catechin equivalent
[g]DPPH: 2, 2-diphenyl-1-picrylhydrazyl
[h]FRAP: ferric iron reducing power
[i]AChE: Acetylcholinesterase
[j]HMGR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase

This study suggested clearly that phenolic compounds extracted from *Zls* using MAE are mainly depended on the ethanol concentration as its linear, quadratic and interaction effects with MW power and time confirming the preliminary experiment results. In meantime, the obtained results are satisfactory by founding ethanol as best solvent giving higher extraction TPC yield which is in agreement with other works demonstrating the use the ethanol as green solvent to enhances the bioactive compounds extracted from plants which is considered to have low toxicity than other organic solvents and permitted to be used in food additives [44].

Recovery in optimal condition

Optimal conditions for yield optimization were 60% ethanol, 210 s and 600 W with a predicted TPC yield of 6649.52 mg GAE/100 g. MAE was carried out at these optimal conditions obtaining a TPC yield of 6709.01 ± 2.20 mg GAE/100 g, close to the value predicted by the model. The recovery of TPC extracted with microwaves through RMS optimization study showed a significantly higher value than those obtained from conventional extraction method (CE) of jujube seeds using ethanol 70% (5100.21 ± 0.20 mg/100 g) [46]. Additionally, TPC value extracted from Spanish jujube fruits using methanol ranged between 1442 and 3432 GAE mg/100 g is significantly lower than obtained results [12]. Similarly, our samples revealed a higher TPC values than those obtained from Chinese jujubes ranged from 454.3 to 1298.9 GAE mg/100 g Zhao et al. [47].

Furthermore, the TFC of *Zls* extracts using MAE (Table 3) was significantly higher than those obtained from other jujubes species (499.21 ± 0.59 mg QE/100 g), in comparison to TFC reported from *Zizyphus jujuba* seeds using ethanol 70% by CE (200.0.1 ± 0.15 mg/100 g) [46]. Similarly, from two different Chinese jujubes that were around 122.1 to 319.5 and 65.1 to 158.6 mg/100 g, respectively [47, 48]. More recently, TFC of *Zj* fruits showed a significantly lower content than ours at different stage of ripening, ranging from 26.7 to 48.5, 19.9 to 34.6 mg QE/100 g in 'Ya Tsao', 'Ta-Jan Tsao' cultivars, respectively [49].

While, TTC of *Zls* was for 25,000.74 ± 1.28 mg CE/100 g which was higher in comparison to polymeric proanthocyanidins extracted from *Zizyphus jujuba* fruits obtained by Wojdyło et al. [12] that showed a value between 939 mg/100 g and 2548 mg/100 g in depends on cultivars. In addition, Gao et al. [11] compared proanthocyanidin extracts from several *Zizyphus* varieties, being the highest content, about 413.7 ± 23.1 mg/100 g, found in *Zizyphus jujuba cv. Zaowangzao* nevertheless significantly lower than our extracts.

The variation in results found for TPC, TFC and TTC in our study using MAE comparatively to those reported

in literature is related to many factors such as plant family, geographical conditions, varieties of plants, extraction method and environmental effects [4, 8, 47, 50]. This study provides a successful RSM optimization study of polyphenols extraction conditions from *Zls* with combination to microwave using method instead of traditional extraction methods which is a crucial step for an industrial process giving a greater recovery of phenolic compounds in less time and solvent consumption due to its heating microwave energy. In addition, it is more important that it contributes to the valorization of natural recourses that is more economic for industry and will be healthier for consumers in general due to its protection of the environment and it uses a green method without toxicity effect. The results presented in this work are in accordance with other research data demonstrating the advantageous of microwaves on the extraction of phytochemicals from plant matrix [26, 29, 51, 52]. However, it will be interesting to know the different phytochemicals present in the *Zls* extracts and evaluate if each of them can be responsible for some biological activities studied in this work.

Identification of bioactive compounds from *Zls* extracts

The content of bioactive compounds extracted from *Zls* obtained by LC–MS/MS analysis in the negative and positive mode (Fig. 2) was demonstrated in a supplementary Table 1. The identification of phytochemicals was carried out using Data Analysis program from Bruker, and pubchem, metlin and others references on the *Zizyphus* genus were used to find the chemical structures. Results indicated the presence of 47 biomolecules based on the exact mass and on the fragmentation patterns. The heatmap was made to compare the intensities of identified compounds and to visualize the higher amount as shown in Fig. 3.

Secondary metabolite

Like all seeds found in vegetable plants, *Zls* extracts have a high content of primary metabolites by comparing the secondary one, but the letter are not negligible given their beneficial effect on human health. The results obtained, during the screening phytochemical by knowing their intensities (Fig. 3), showed that the secondary metabolites are

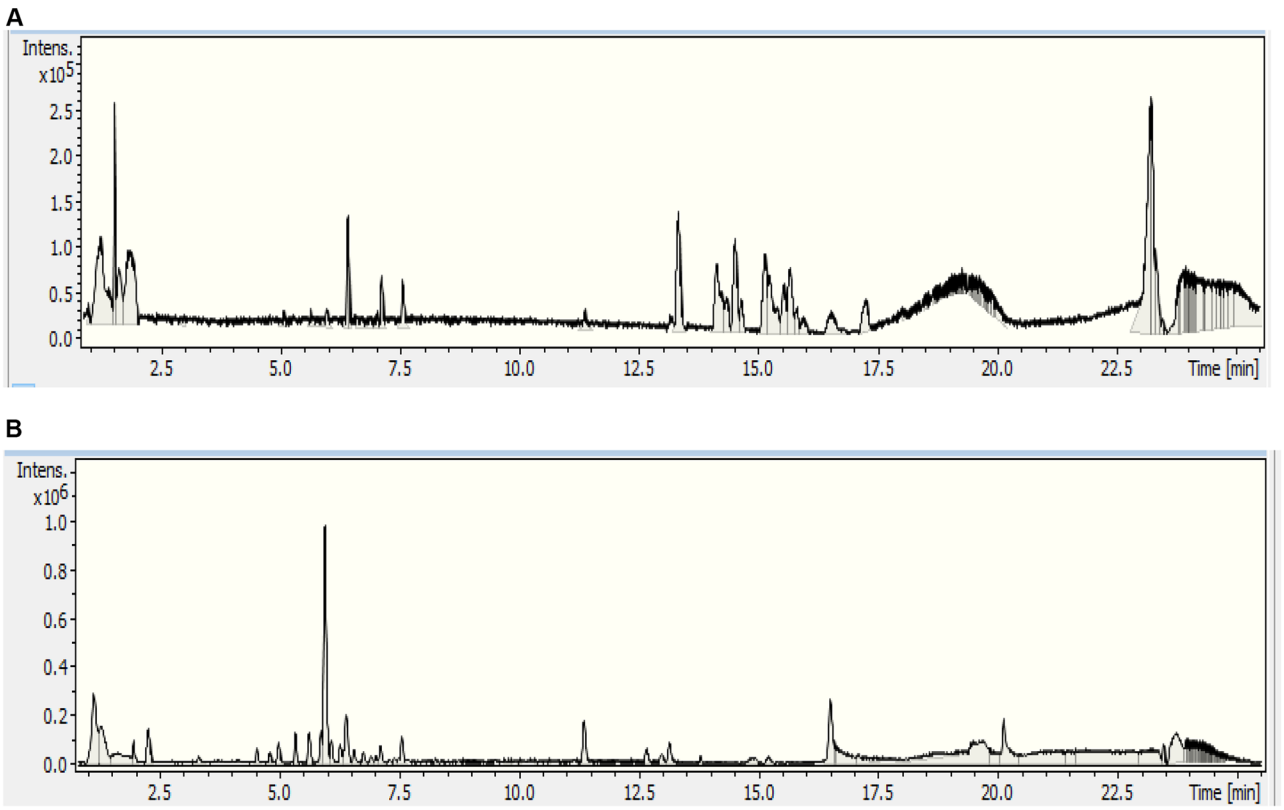


Fig. 2 Total ionic chromatogram and ionic chromatograms extracted from ions identified in *Z. lotus* seeds samples: **a** LC–MS/MS (−) and **b** LC–MS/MS (+)

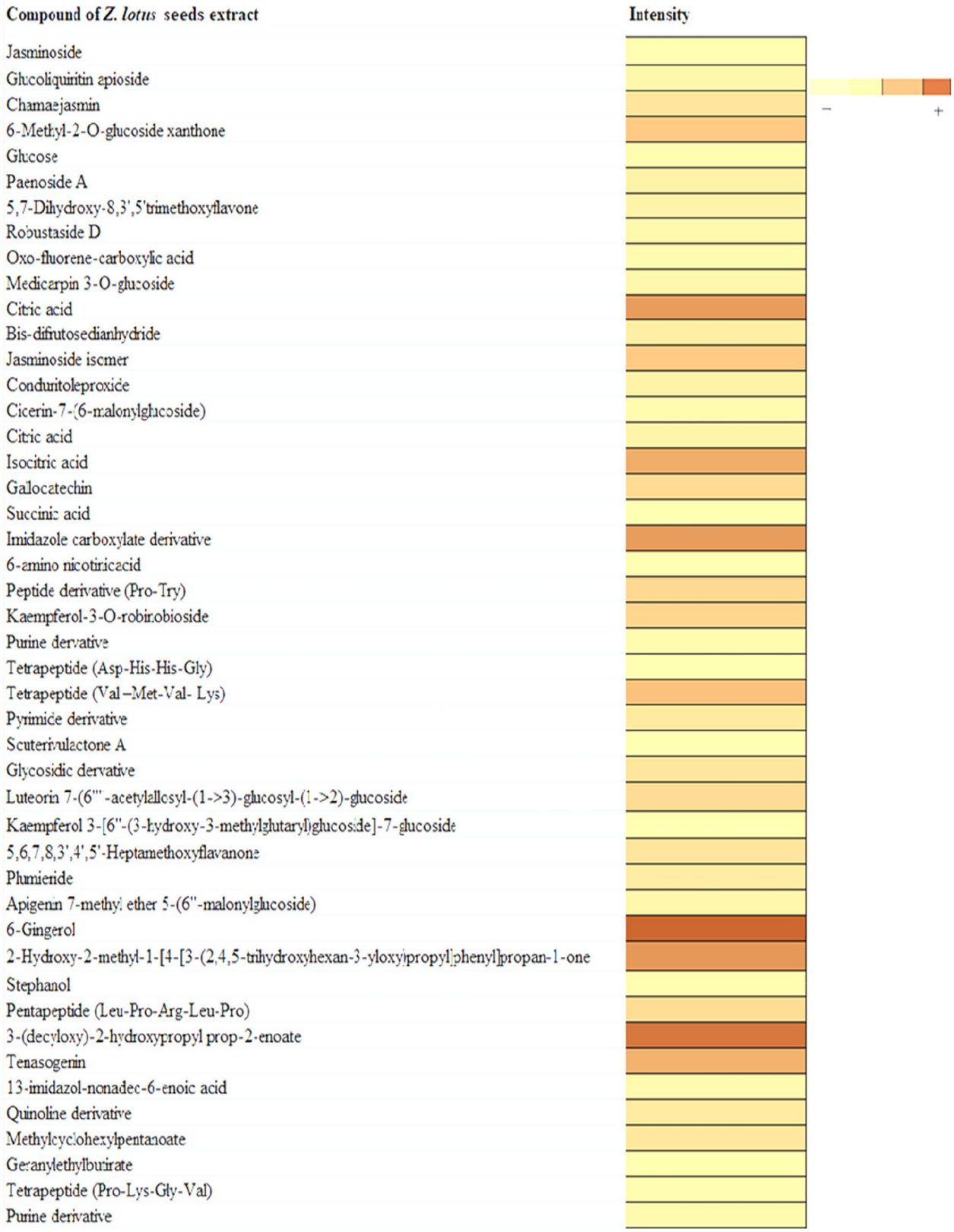


Fig. 3 Heatmap of the chemical profile and of *Z. lotus* seeds samples. Mean values refer to colors from minimum displayed in beige to maximum represented with dark brown

39.62% of all compounds detected in *Zls* samples. It seems that 6-gingerol being the most abundant compound as demonstrated in dark color and found to be around 24.71% of all secondary metabolites. This compound is originated from *Zingiber* and found to exercise a significant biological potential such as anticancer, antioxidant and anti-Alzheimer effects [53, 54]. This compound was previously detected from jujubes [55–58].

Characterization of flavonoids and derivative

Compound with a $[M\text{-}H]^-$ ions at m/z of 701.1780 was detected at 1.44 min and tentatively assigned as paenoside A [59]. This compound was detected from *Delphinium staphisagria* that showed a significant antiproliferative activity against *Trypanosoma cruzi* (epimastigote, amastigote, and trypomastigote forms) [60]. However, compound detected at 1.04 min ($[M+H]^+$) was assigned as biflavonoid at m/z of 543.1253. This compound was assigned as chamaejasmin. This compound was detected from other plants mainly *Stellera chamaejasme* L which showed a very good antioxidant and anticancer effects [61, 62]. This is the first time detecting the presence of this flavonoid in *Zizyphus* genus.

Some flavanones were detected at different retention times 1.03 min and 7.55 min with negative mode at m/z of 711.2074 and 433.1546, respectively. These compounds were tentatively identified as glucoliquiritin apioside and 5,6,7,8,3′,4′,5′-heptamethoxy flavanone, respectively. To the best of our knowledge, this is the first report identifying these compounds in *Zizyphus* genus.

Compound at $R_t = 1.49$ min was observed as isoflavanone at a positive mode with m/z of 435.1288 and was tentatively identified as robustaside D. This compound was detected previously from other plants and was found to exercise antimalarial, antioxidant and antitumor activities against human breast cancer cells from *Peganum harmala L* seeds [63]. However, peaks at 1.60 min, 1.75 min showed a deprotonated molecule with m/z of 431.1348 and 601.1195 with different ion fragments was tentatively identified as medicarpin 3-*O*-glucoside, Cicerin-7-(6-malonylglucoside), respectively. These compounds were deduced for the first time in *Zizyphus* genus [64, 65].

Peaks at R_t of 1.46 min, 7.03 min and 7.58 min with $[M+H]^+$ and $[M\text{-}H]^-$ ions at m/z of 345.0988, 813.2084, 531.1201 were tentatively assigned as 5,7-dihydroxy-8,3′,5′trimethoxy-flavone, luteolin 7-(6′′′′-acetylallosyl-(1- > 3)-glucosyl-(1- > 2)-glucoside and apigenin-7-methyl ether 5-(6′′-malonylglucoside), respectively. These compounds were reported for first time in *Zizyphus* genus. However, two known flavones were characterized in this study. Peak at 5.62 and 7.10 min with m/z of 595.1579($[M+H]^+$), 753.1895 ($[M\text{-}H]^-$) were assigned as kaempferol-3-O-robinobioside, Kaempferol 3-[6′′-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside. Only kaempferol-3-O-robinobioside was previously detected in jujube plant [12].

Furthermore, gallocatechin was the only flavan-3-ols detected at $R_t = 1.99$ min with m/z 307.0791 at positive mode as previously found in Algerian *Z. lotus* and other *Zizyphus* species from leaves and fruits [66].

Figure 3 showed clearly that our samples constitute a significant flavonoids part which are 18% of all metabolites and 50% of all secondary metabolite. *Zls* is one of the used part of jujubes for the treatment of several diseases due to their high content of phenolic compounds especially flavonoids. These were found to be one of the most known substances for their antibacterial, antioxidant and antifungal effect agents [67, 68].

Characterization of terpenes

Peak at 6.49 min showed a deprotonated molecule with m/z of 529.242 and was tentatively assigned as scuterivulactone A. This diterpenoid was never been detected from jujubes and it is known to excerse a good antiinflammatory and anticancer effects from other plant material [69, 70]. Moreover, one iridoid glucoside was detected at 7.57 min with m/z of 469.1315 and tentatively identified as plumieride [71]. Several plumieride derivative were detected from other *Zizyphus* species and found as an efficient antioxidant agent [72, 73]. In addition, two sesquiterpenes were observed at 15.45, 18.19 with m/z of 397.2217, 253.2133 in negative mode. These compounds were assigned as stephanol and geranyl ethyl butirate, respectively [74]. Compound at $R_t = 16.53$ min, which showed an m/z of 449.2965 in positive mode was tentatively identified as tenasogenin. From Fig. 3 we observed clearly that terpenoids are present with high intensities in *Zls* extracts and arround 19% of all secondary metabolites in which tensagenin are 68% of all terpenoids, this is never been detected from other *Zizyphus* species. This compound was very used due to its antidiabetic effect and identified as a pregnane ester from *Marsdenia tenacissima* [75, 76].

Characterization of other compounds

The analysis showed the detection of two secoiridoids with a deprotonated molecular having the same formula ($C_{26}H_{30}O_{13}$) at 1.01 min and 1.71 min with a m/z of 549.1574, these compounds were isotopes and identified tentatively as jasminoside. This compound is detected for a long time from *Jasminum primulinum Hems* [77]. However, compound with $[M\text{-}H]^-$ molecualr ions with m/z 387.1092 at $R_t = 1.09$ min was assigned tentatively as 6-methyl-2-O-glucoside xanthone [78]. For instance, no data detected the

presence of these compounds from *Z. lotus*. Moreover, peak observed at 17.11 with m/z of 319.2797 $(M+H)^+$ was attributed to quinoline derivative. Several works were previously detected quinoline alkaloids in some *Zizyphus* species which is one of most known as antioxidant and anti-aging agent [79, 80].

Primary metabolite

Figure 3 showed that the primary metabolite contain around 56% of all compounds found in our samples. It has been noticed that 3-(decyloxy)-2-hydroxypropyl prop-2-enoate was found as the major abundant compound with 16% of all primary metabolites. This compound was never been reported from *Z. lotus*. Thus, it is used under another structure of 2-propenoic acid from jujubes and showed a significant effect as antioxidant, antiinflammatory and anticancer against breast cell lines [81, 82]. In addition, peptides are present with high intensities in comparison to other primary substances with 13% of *Zls* extracts and 23% of all primary metabolites. *Zls* extracts have been previously found with high protein content (19.11%) which is known by its functional properties (emulsifying activity, foaming capacity, emulsifying stability, water retention and solubility) and nutritional value [83]. Therefore, small peptides are found from *Z. jujuba* seeds as most potentially valuable compound which play an important antioxidant, anticholinesterase roles that can be used as functional food [84, 85] (supplementary Tabble 1).

Characterization of sugars

The examination of chromatograms obtained at negative mode indicated the presence of some sugars at 1.12, 6.92 min with m/z of 179.0529, 539.2965 were clearly identified as glucose and glycosidic derivative [86]. Furthermore, peak observed at 1.67 min with m/z of 325.1090 $(M+H)^+$ was tentatively attributed to bis-D-frutose dianhydride, this compound is considered one of rare class of spirodisaccharides and found often in plants and isolated from microorganisms [87].

Characterization of peptides

The mass spectrometric characterization of compounds observed at 4.98, 6.29 and 16.49 with m/z of 305.1524, 271.1265, 595.4093 $(M+H)^+$ were identified tentatively as dipeptide, pyrimide derivative and pentapeptide. Others small peptides were identified tentatively at retention times of 6.05 min, 6.09 min, 6.66 min and 18.60 min with m/z of 465.1878, 476.3003, 573.2665 and 469.2778 at positive and negative modes were as tetrapeptides with different molecular formula (supplementary Table 1).

Characterization of other compounds

The mass spectrometric characterization of compound at 1.16 min, 1.95 min and 1.98 min with m/z of 191.0168, 191.0169 and 191.0167 were isomers and identified as citric acid and isomers, respectively. In addition, peaks observed at 1.52 min, 2.03 min displayed a deprotonated molecular ions at m/z of 223.0426 and 117.0163 were identified as oxo-fluorene-carboxylic and succinic acids, respectively. These organic acids were detected previously from jujube plant and other herbs and showed a good biological activities [12, 88]. Additionally, two peaks were observed at 5.98 min and 19.36 min with m/z of 376.1267, 355.1531 with $C_{16}H_{19}N_5O_6$ and $C_{17}H_{20}N_6O_3$, respectively at negative mode were assigned as purine derivative.

Compounds observed at 1.72 min, 15.13 min, 16.52 min and 17.23 min in positive mode with m/z of 163.0578, 353.1956, 287.2178, 199.1663 were assigned as conduritol peroxide, 2-hydroxy-2-methyl-1-[4-[3-(2,4,5-trihydroxyhexan-3-yloxy) propyl] phenyl]-propan-1-one, 3-(decyloxy)-2-hydroxypropyl acryate and methylcyclohexyl pentanoate. However, other peaks obtained with $(M+H)^+$ showed the presence of some compounds with retention times of 2.23 min, 4.52 min and 17.06 min with m/z of 254.1582, 139.0492, 363.3061, respectively were identified as imidazole carboxylate derivative, 6-amino nicotinic and 13-imidazol-nonadec-6-enoic acids, respectively [89]. All these compounds were reported for the first time from jujubes and can participate in the studied activities what we have noticed in the Fig. 3.

Effect of MAE on biological activities

Due to the presence of this type of compounds as they are reported to have biological activity, the antioxidant activity and the inhibition of AChE and HMGR were looked for.

Determination of DPPH· and FRAP activities

The antioxidant activity was evaluated with DPPH radical scavenger as shown in Table 3. The *Zls* extracts that exhibited high content of phenolic compounds with a remarkably significant antioxidant effect with EC_{50} value of 0.000067 ± 0.000002 mg/mL. In comparison with other *Zizyphus* species, Choi et al. [90] obtained from Korean *Z. jujuba* seeds (mechu and sanzoin) a lowest DPPH· activity than obtained results (0.3 mg/mL and 0.1 mg/mL respectively). Similarly, Ghazghazi et al. [91] demonstrated DPPH· Activity of 0.31 ± 0.005 mg/mL from *Z. lotus* fruits extracts which is lower than ours from seeds. Our results gave higher antioxidant effects than results obtained by Hammi et al. [32] that reported an EC_{50} of 0.28 mg/mL for Tunisian *Z. lotus*

pulp and peel extracts using UAE. Regarding the iron-reducing power FRAP assay for the *Zls* extracts (Table 3) which is used as one direct method for determination of antioxidant effect from jujubes. The results showed a significant value of 2039.60 ± 8.43 mg GAE/100 g. Our finding are significantly higher than those of Wang et al. [92] (471.6 ± 30.8 mg CE/100 g) obtained from *Z. jujuba cv. Zaowangzao*. The antioxidant capacity of *Z. lotus* extracts have also been evaluated in previous works [4, 31, 32, 93].

Moreover, we can suggest that solvent type has a strong effect on the antioxidant activity which is in agreement to Kim, Son [46]. This activity value is depending on the phenolic compounds contain in the extracts, as well as the extraction method. As demonstrated by Dzoyem, Eloff [16], Wojdyło et al. [12], the presence of flavonoids, such as flavan-3-ols and flavonols in jujube extracts and particularly hydroxyl group position can act as proton donating and contribute to increasing radical scavenging activity. In addition, the presence of others phytochemicals mainly 6-gingerol and other small peptides have probably a good antioxidant potential. Furthermore, it has been reported that the high antioxidant power should be attributed to the effect of microwaves on the structure of the cell of extracts due to the increase of internal pressure and temperature [8, 29, 43].

Determination of AChE inhibitory activity

The inhibition of acetylcholinesterase (AChE) is very used against several neurological disorders such as Alzheimer's disease, senile dementia, ataxia, myasthenia gravis [94] and severe constipation [95]. Several plants have been reported as having AChE inhibitor activity which was attributed to the presence of mainly phenolic compounds and alkaloids and some primary metabolites [16].

The *Zls* extracts were analyzed to determine their ability as acetylcholinesterase inhibitors (Table 3). The study shows that our samples obtained at different concentrations diluted by a factor of 10 exhibited a significant inhibitory activity (0.88 ± 0.02 mg/mL). These results are in accordance to those found by Hernandez et al. [96] from *Hypericum* (Hp_2) extract that showed an IC_{50} value of 0.88 ± 0.08 mg/mL using water for decoction preparation. In the meantime, the same authors demonstrated that other *Hypericum* species ($Hp_,$, $Hp_{3,}$ Hp_4 and Hp_5) obtained in a local supermarket from Portugal showed a lowest AChE activity than *Zls* extracts varying from 0.99 ± 0.12 mg/mL to 1.79 ± 0.34 mg/mL in which some flavonoids were found to be responsible for the AChE activity. In addition, *Globularia alypum* extracted from leaves which show an IC_{50} value of 0.82 ± 0.05 mg/mL [15]. which is in the same range as *Zls* extracts. Moreover, the *Zls* extract had a highest significant AChE inhibitory potential than that reported from *Zizyphus oxyphylla* extracts with an IC_{50} value of 9.58 ± 0.08 mg/mL using *n*- butanol which exhibited a maximum inhibitory effect than other soluble fractions mainly *n*- hexane from the same sample (165.15 ± 0.94 mg/mL) [97]. This indicates that *Zls* extracts obtained using ethanol as extraction solvent exhibited a best inhibition of this enzyme which could be used against Alzheimer's disease or alleviation of severe constipation.

The obtained results showed that MAE extracts contained some level of inhibitory activity against AChE, this may suggest that extraction method were able to extract more active compounds than other extraction methods as seen in previous part with possible AChE inhibitory activity. It could be postulated that *Zls* extracts inhibited AChE activity might be due to phenolic compounds. LC–MS/MS analysis revealed that *Zls* represents a rich sources of phenolic compounds beside of other secondary metabolites and small peptides found with high intensities Fig. 3 which may contribute also to AChE inhibitory activity, and this is precisely where the interest of this study arises in order to have a global idea of the various compounds considered be responsible for this activity, where other future studies requiring to focus on the isolation of certain compounds and to get more from the virtues of this plant by doing an in-depth study on the effectiveness of *Zls* to be exploited as an anti-Alzheimer agent. Several authors described the role of the presence of some bioactive compounds to their AChE inhibitory effects [98–101]. Moreover, the obtained inhibitory effects could be related to the content in different phytochemicals found in the extracts. According to our knowledge, it is the first time that the in vitro inhibition of the acetylcholinesterase enzyme by *Zls* extracts is reported.

Determination of HMGR inhibitory activity

In this study, HMG-CoA reductase activity was measured from *Zls* extracts at optimum extraction conditions that contain highest amount phenolic compounds. The results are demonstrated in Table 3, *Zls* extracts showed an activity of 28.71% as a HMGR inhibitors using 100 µg/mL that is satisfactory as those obtained from *C. olitorius* leaves methanol extracts. Our results are probably due to the components present in *Zls* in high level. The high activity is related to the type of bioactive compounds present in the extracts that can fit the enzyme active site. The modest activity obtained with *Z. lotus* can be explained by the fact that only some bioactive compounds present in *Zls* are responsible for enzyme inhibition and it can only be said that seeds extracts are modest HMGR inhibitors with IC_{50} value similar to those found in literature describing results from polar plant extracts.

The results found in this study is more satisfactory than those obtained from seeds extracts of some plants, mainly *Cannabis sativa* (7.4%), *Cuminum cyminum* (26%), *Nigella sativa* (0%), *Ocimum basilicum* (0%), *Pimpinella anisum* (10.5%) and *Trigonella foenumgraecum* (0%) [102]. While,

it is in the same range than HMGR inhibitors obtained from aerial parts extracts of *Peganum arma* (28.5%) and *Tencrium polium* (28.8%) [102].

However, several *Ziziphus* species that have a high level of secondary substances such as polyphenols, saponin and triterpene that are complexing with cholesterol as binding plasma lipids is due to the sugar chains contained in saponins that attach themselves to triterpene or sterol [103]. These compounds were found to contribute on the inhibition of HMGR and has good hypolipidemic activity [104, 105]. Moreover, *Ziziphus mauritiana* leaves extracts are found to reduce levels of cholesterol, triglyceride and can be used for the treatment of fatty liver and atherosclerosis [106]. More recently, alkaloids from Magnoflorine-containing extracts showed a significant HMGR inhibition [107]. From these reports it seems that especially flavonoids, terpenes and alkaloids are mainly compounds responsible for HMGR inhibitors in our samples as we showed in previous paragraphs.

Several studies found that phenolic compounds are responsible for good HMGR inhibitory which is controlled by the decrease of LDL/HDL ratio as found by Bahramikia, Yazdanparast [108] from hydroalcoholic extracts of *Nasturtium officinale* leaves. Others have reported from that the increase in the biosynthesis and decrease in catabolism of both fatty acids and cholesterol are affected by alcohol extracts which is implicated in the development hyperlipidemia and fatty liver in rats [109]. The good HMGR inhibitory activity is affected also by all factors studied due to the different extraction of bioactive compounds using MAE in our samples especially solvent type and to the best of our knowledge our study is the first improving the HMGR activity from *Z. lotus* species.

Conclusion

In this study, microwaves technology method was used for extraction of phenolic compounds from jujube seeds and evaluation of some biological activities. RSM with CCD was used to optimize the experimental variables. The quadratic polynomial model was obtained with high correlation which can be used to optimize the MAE of polyphenols from jujube seeds. The optimal conditions obtained from optimization study were microwave power at 600 W, irradiation time of 210 s and 60% ethanol (v/v in water). Under these conditions, MAE extract presented high content for TPC (6709.01 ± 2.20 mg GAE/100 g).

Bioactive compounds were profiled through LC–MS/MS analysis, a total of 47 compounds in *Zls* extracts were identified. Among these, 21 have been determined for the first time in jujube genus. Primary and secondary metabolites contain 56 and 39.62% of all phytochemicals, respectively. Jujube samples extracted with MAE for the first time were found to exhibit a significant antioxidant, AChE and HMG-CoA activities in comparison with the previously reported data. Moreover, the *Zls* is a good source of bioactive molecules that are responsible for high antioxidant and enzymatic activities, these results may be useful in the development of new strategies to treat Alzheimer disease and hypercholesterolemia. However, MAE from jujubes encourages the use of of *Zls* as ingredient in industry as food additives. Thus, further comparison with conventional, other innovative techniques and *in vivo* studies are needed to understand not only the advantage of MAE uses but also the activity in biological systems.

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REVIEW ARTICLE

Ziziphus lotus (L.) Lam. plant treatment by ultrasounds and microwaves to improve antioxidants yield and quality: An overview

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Abstract

The purpose of this review is to compile the literature published about different aspects of microwave-assisted extraction (MAE) use and ultrasound-assisted extraction (UAE) applied on jujube worldwide and to compare the results on the antioxidant activity obtained for each extraction method. As a result of the increased consumers demand for natural products, as well as for those of agro-food, nutraceutical, cosmetic industries, and green extraction techniques are nowadays trending to be potential alternatives that can improve antioxidant yield and its quality from an economical and environmental point of view by reducing time, energy, and solvent consumption. Ultrasounds and microwaves are widely used methods in the extraction of active principles due to their cavitation and dipolar rotation effect, respectively. These two techniques provide efficiency of extraction while minimizing the time and preserving the quality of the food matrix, overcoming the disadvantages of conventional techniques characterized by their consumption of large quantities of solvents and providing a sparse quantity of extraction. Jujube, a shrub with a high antioxidant potential, which can be affected by various extraction conditions can be the target of UAE and MAE to increase the antioxidant extraction yield. Exploiting the beneficial properties such as the antioxidant activity can lead to an industrialization process, replacing therefor synthetic antioxidants with natural compounds. These can also help in the development of new nutraceuticals and can be used, for instance, in agro-food industries as preservatives.

Keywords : Microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), antioxidants, *Ziziphus lotus* (L.) Lam plant.

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1 Introduction

Ziziphus genus is a spiny shrub belonging to the family of Rhamnaceas, it is disseminated in tropical regions such as Asia, America, South of Europe, and the northern part of Africa as in Algeria [1]. There are several species of this genus (*Ziziphus vulgaris* Lam, *Ziziphus lotus* Lam, *Ziziphus Spina-christi* (L.) Wild, and *Ziziphus mauritiana* Lam), depending on the soil and climate [2, 3]. The fruits have been edible for millennia [2, 3]. In Algeria, *Z. lotus* (L.) Lam is very abundant [4]. Locally named (Sedra) and the fruit is called 'Nbag' [5]. Several botanists have described the morphological features of the jujube plant (*Ziziphus lotus L.*) which has a perianth pentamer, and the fruit is a drupe the size of a pea or an olive. The leaves are alternate, coriaceous, and accompanied each of two spines straight or crooked. In the most common species, the leaves are small (15 x 10 mm). It is a shrub or a tree frequent in the hot countries, it is cultivated for its fruits. Jujube is located in several regions of Algeria such as in Kabylie region and the southern part (Djelfa, Biskra, and M'sila), as well as in other Mediterranean countries such as Morocco and Tunisia [6].

For decades, researchers and industrial food companies have been increasingly interested in natural antioxidants, due to their properties in food preservation and their significant value for the prevention of diseases related to oxidative stress [7, 8]. The consumer's demand for a natural diet to counteract synthetic antioxidants is the main reason for this search [9-12]. In general, the first process of treatment of several plant materials is the extraction of their crude pigments [13]. Extraction of natural products can be done by various extraction techniques. For several years, conventional extraction methods, including maceration, solvent extraction, Soxhlet extraction, and alembic distillation, all basically utilized in food, medicine, and perfumery [14] have been used. However, many non-conventional methods including ultrasound-assisted extraction (UAE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and enzyme-assisted extraction (EAE) have been proposed due to their enhanced extraction efficiency and environmental friendliness [15-17].

Based on the literature, there has been no review on the extraction techniques of bioactive compounds and antioxidant

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activity of *Z. lotus* (L.) Lam. Therefore, this work aims to provide a comprehensive review of green innovative extraction methods such as UAE and MAE in comparison with traditional methods having as target two important molecule types, polyphenols, and polysaccharides, from different parts of *Z. lotus* (L.) Lam. The effect will be evaluated on health-promoting human food and disease prevention, taking into account their antioxidant activity.

In recent years, the physiological function of foods including fruits, vegetables, and food components such as phytochemicals has received much attention [18, 19]. Possible correlations between the biologically active compounds and human health have generated interest in *in vitro* and *in vivo* studies about these biological activities. The major class of phytochemicals found in plants is related to phenolic compounds which contain a large variety of derivatives including simple flavonoids, tannins, phenols, phenylpropanoids, benzoic acid derivatives, lignans, and lignins [20, 21]. According to Croteau *et al.* [22], the classification of bioactive compounds from plant materials is divided into terpenes, alkaloids, and phenolic compounds. These categories contain a minimum of 8000 types of compounds approximately. Azmir *et al.* [23] suggested that shikimic acid and malonic acid are the pathways of the synthesis of phenolic compounds. While, alkaloids and terpenes come from mevalonic acid and non-mevalonate pathways, respectively. On the other hand, it has been found that polysaccharides represent a vital category as they exhibit numerous pharmacological and biological potential such as antioxidant, anti-inflammatory, and anticancer [24].

Z. lotus (L.) is known for its richness in primary metabolites mainly, protein 19.11%, carbohydrate 40.87%, and lipids 32.92% [25, 26]. For secondary metabolites (Table 1 and Figure 1), *Z. lotus* (L.) demonstrated the presence of many biologically active molecules [1], such as polyphenols (flavonoids and tannins), triterpenes, anthraquinones, alkaloids (cyclopeptides and isoquinolines), and saponosides, everything depends on parts of the vegetable matrix (leaf, root, fruit, and seeds) [27, 28]. The leaves are a source of flavonoids, tannins, alkaloids, and saponins [29-31]. The fruits contain flavonoids, tannins, and saponins [32]. Likewise, the roots are a source of flavonoids, tannins, and alkaloids [33]. Besides containing a higher amount of secondary metabolites, both seed and fruit reveal the presence of important minerals such as magnesium, calcium, and potassium [1]. These compounds are valued for their contribution to a healthy diet and also as ingredients for designing new foods [34, 35]. Among the most isolated compounds from *Z. lotus* (L.), the phenolic acids due to considerable amounts of caffeic acid, gallic acid can be mentioned [34, 35]. Flavonoids like rutin, epicatechin, taxifolin, and catechin can be extracted with organic solvent or mixtures in all parts of the jujube tree. Elsewhere, these compounds may well explain the biological activity, which can be used as control drugs in most pharmaceutical formulations [35]. Table 2 mentioned some isolated compounds from the *Ziziphus* genus and the part of the plant from where they were isolated.

Besides the nutritional composition, jujube has been a dietary food that appears in list A of the medicinal plants of French Pharmacopeia [36]. Several *in vitro* and *in vivo* studies on phytochemical and pharmacological effects have clearly revealed that *Z. lotus* (L.) contains some active molecules responsible for its beneficial effects depending on the part of the plant (root, leaf, seed, pulp, or fruit) mainly as antifungal, antibacterial, antiulcer, anti-inflammatory, antioxidant, and immunostimulant properties [1]. Based on the literature, flavonoid, polysaccharide, protein, and triterpenic acid are the main active molecules responsible for its biological effects. Both flavonoids and polysaccharides are found in both seed and pulp are known for exhibiting antioxidant, antimicrobial, and immunomodulatory properties [1]. However, the triterpenic acids, abundant in leaves, were proposed to be the main active ingredients for the effect on anti-inflammatory and anticancer activities [37]. While, proteins are found in seeds and pulps known for their functional properties such as emulsifying activity, emulsion stability, and water holding capacity [34]. However, the major organoleptic characteristics of plant-derived food (color, taste, ...) are represented in particular by phenolic compounds [38]. Additionally, they are known for their capacity to reduce oxidation reactions by controlling and quenching the reactive oxygen species (ROS) including peroxides, hydroxyl radicals, superoxide, and nitrous oxide that damages food and can be linked to various diseases [39]. The antioxidant activity of *Z. lotus* (L.) extracts is well documented [16, 40-47]. Many methods have been used to evaluate the antioxidant effect of extracts; the most commonly developed method is 2,2-diphenyl-1-picrylhydrazyl (DPPH) which is based on the inhibitory action of vegetable extracts on the free radical activity of ROS. This method is reproducible and time efficient, other methods are also used 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and trolox equivalent antioxidant capacity (TEAC) [48]. According to Bakhtaoui *et al.* [43], *Z. lotus* (L.) fruits showed stronger scavenging free radicals effect when compared to other morphological parts (leaves, root, and stem) [1]. This is influenced by several factors, including their concentration, temperature, type of solvent, ratio, and frequency, as well as the presence of prooxidants and synergists [49]. In parallel to the conventional methods, green extraction processes such as ultrasound and microwave methods on *Ziziphus* species have found to give different antioxidant effects, these differences are well discussed in the next section.

2 Conventional extraction methods of antioxidant from jujube fruit

Several studies have shown the large choice of traditional extraction methods of antioxidants compounds from plant materials, such as maceration, hydro-distillation, and Soxhlet extraction [23]. Generally, this is based on the application of temperature treatment and the use of different solvents depending on the compound to remove or to improve extraction. The most common processes used for the extraction of compounds from plants are either physical or chemical [50]. In addition, the conventional extraction method allows the transfer of heat from the outside to the inside of the sample through the heating medium. Maceration is very used in homemade preparation of

tonic for a long time, which is inexpensive, based on the mixture of solvent with the surface area to get bioactive compounds. Hydro-distillation and Soxhlet extraction techniques are generally used for the extraction of essential oils. They are used for bioactive compounds, thus allowing automatic separation of these antioxidants in root barks from *Z. lotus* (L.) with different solvents. Borgi *et al.* [52] used Soxhlet for extraction of saponin and flavonoid fractions from the leaves and root bark of *Z. lotus* (L.). Borgi *et al.* [4] extracted bioactive compounds from the leaves and root barks of *Z. lotus* (L.) by maceration method. On the other

Table 1: Chemical composition of *Ziziphus lotus* (L.) in different part of jujube

Fraction	Fruits	Pulp & peels	Seeds	Leaves	Root bark	References
Moisture content (%)	-	12.27	6.05	-	9.11	
Carbohydrates (%)	-	65.90	40.87	8720 (mg/100 g)	8.71	
Crude protein (%)	-	3.80	19.11	-	3.18	
Crude fat (%)	-	1.32	-	-	-	
Crudefibe (%)	-	8.41	-	-	47.90	
Ash (%)	-	3.28	1.05	-	2.69	
Pectin (%)	-	3.78	-	-	-	
Vitamin C	5.67	190.65	31.24-170.84	63.40	47.20	
Calorific values Kj/g	-	16.341	-	-	-	1,5, 25, 40, 101
Oleic acid (%)	-	88.12	61.93	-	-	
Elaidic acid (%)	-	7.88	-	-	-	
Linolenic acid (%)	-	-	-	9.15	-	
Saponins (mg/100 g)	-	-	-	340	219	
Polyphenols (mg/100 g)	297–4078.2	325	14.68	664	2009	
Total flavonoids (mg/100 g)	122	173	-	133-199	120	
Total tannins (mg/100 g)	33	929	-	39	156 (Proanthocyanidins)	

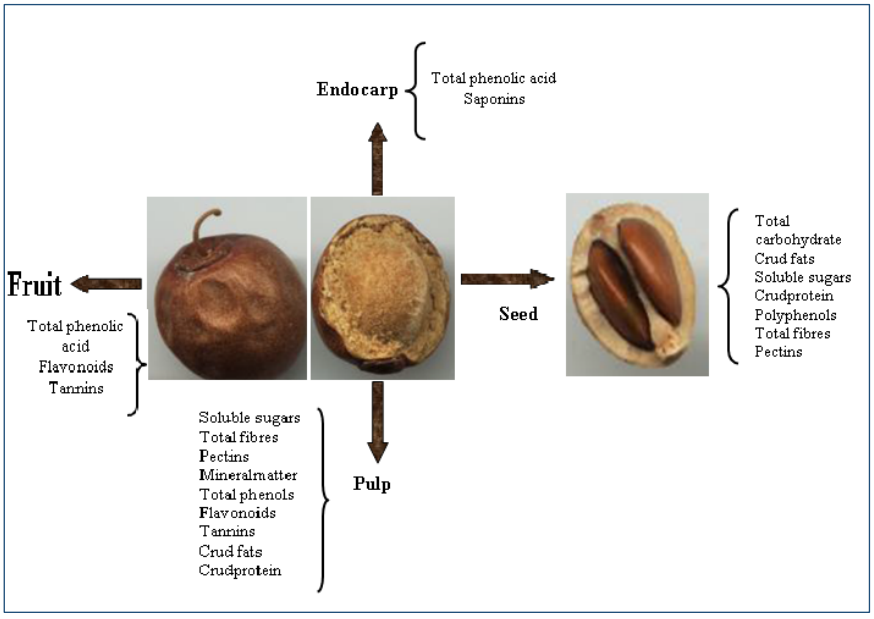


Figure 1: Anatomy of *a Z. lotus* (L.) Lam. fruit

compounds from water due to the flows from the condenser to a separator of the condensed mixture [51].

Many studies have been reporting on the extraction of secondary metabolites from the *Z. lotus* (L.) plant using conventional methods. Indeed, Borgi *et al.* [27] reported the extraction of hand, the effect of *Z. lotus* (L.) root barks extracts' on antiulcerogenic activity using Soxhlet extractor as demonstrated by Wahida *et al.* [53]. Similarly, Naili *et al.* [54] studied the antimicrobial and antioxidant activities of *Z. lotus* (L.) plants growing in the south part of Libya, which showed high content

Table 2: Some classes of polyphenols isolated from *Ziziphus* species

Bioactive compounds	*Ziziphus* species	Fruit	Pulp & peel	Seed	Leave	Stem bark	Branche	References
Phenolic acids								
Gallic acid	*Z. lotus* *Z. jujuba*	+	-	+	-	-	-	102, 103
p-Hydroxybenzoic acid	*Z. jujuba*	-	-	-	+	-	-	29
Syringic acid	*Z. jujuba* *Z. lotus*	+	-	-	+	-	-	29, 102
***p*-coumaric**	*Z. jujuba* *Z. lotus*	+	-	+	-	-	-	29, 102
Ferulic acid	*Z. jujuba* *Z. lotus* *Z. jujuba*	+	-	+	+	-	-	29, 102, 103
Caffeic acid	*Z. jujuba* *Z. jujuba*	+	-	+	+	-	-	29, 103
Acylatedquinic acid derivatives								
Quinic acid	*Z. lotus*	+	+	-	-	-	-	
3-O-caffeoylquinic acid (cholorogenic acid)	*Z. jujuba* *Z. jujuba*	+	+	+	+	-	-	29, 103
Flavonoid aglycones								
Luteolin	*Z. lotus* *Z. jujuba*	+	-	-	-	-	-	102
Quercetin	*Z. jujuba* *Z. lotus* *Z. jujube* *Z. mauritania* *Z. mistol*	+	+	+	+	-	-	29, 102-109
Catechin	*Z. jujuba* *Z. jujuba* *Z. lotus* *Z. jujube* *Z. mauritania* *Z. joazeiro*	+	+	+	-	-	-	29, 102-105, 107, 108
Procyanidin trimer	*Z. jujube*	+	-	-	-	-	-	107
Flavonoid glycosides								
Kaempferol-3-O-glucoside	*Z. lotus* *Z. jujuba*	+	+	-	-	-	-	107
Kaempferol-3-O-robinobioside	*Z. lotus* *Z. jujuba*	+	+	-	-	-	-	107
Vitexin	*Z. jujuba*	-	+	-	-	-	-	109
Quercetin -3-O-glucoside	*Z. mauritania* *Z. lotus*	+	+	-	-	-	-	110
Quercetin-3-O-robinobioside	*Z. jujuba* *Z. mauritania* *Z. jujuba*	+	-	-	-	-	-	107, 110
Quercetin-3-O-rutinoside	*Z. jujuba* *Z. mistol* *Z. mauritania*	+	+	+	+	-	-	29, 103, 104, 106, 110
Quercetin-3-O-rutinoside-7-O-pentoside	*Z. lotus* *Z. jujuba*	+	-	-	-	-	-	107

of polyphenols and alkaloids using different solvents, and considered them a source of phenolic antioxidants and antimicrobials. Benammar *et al.* [55] used the antioxidant effect of *Z. lotus* (L.) root, leaf, stem, fruit pulp, and seed extracted with decoction and the role of different crude extracts plant on human T-lymphocyte proliferation, they have found that the seed extract showed the most potent immunosuppressive effects on T cell proliferation. In addition, Bakhtaoui *et al.* [42] found that the use of bioactive compounds extracted from *Z. lotus* (L.) fruit of Morocco by Soxhlet using methanol enhanced the anti-

helicobacter pylori, gastro-protective, and antioxidant properties. More recently, Marmouzi *et al.* [56] studied the effect of phenolic compounds extracted from *Z. lotus* (L.) fruit and leave by infusion, it showed a very important antioxidant, antidiabetic, and derma protective potential. Furthermore, the identification of these compounds using HPLC-DAD-QTOF-MS showed a high yield in gallic acid with 2715 mg/kg in the leaves and 15000 mg/kg in fruits. In another study conducted by Ghalem *et al.* [44] the antioxidant activity of *Z. lotus* (L.) root from Algeria extract using the Soxhlet method with the use of beta-carotene bleaching test confirmed the antioxidant capacity of these extracts.

Other jujube species components have been extracted using conventional techniques. Indeed, Soxhlet apparatus has been used for extraction of total phenols and flavonoids content from Omani *Z. jujuba* Mill fruits and leaves as well as the antioxidant activity and polarities of jujube crude extracts have been also evaluated [57]. The effect of different extraction solvents using Soxhlet on yield of active metabolites extracted from *Z. jujuba* Mill. leaves was studied by Al-Saeedi *et al.* [58] which confirmed their higher extraction yield and their antimicrobial activities, these results should be considered in pharmacological studies. Furthermore, the phenolic compounds from Apple Kul pulp (*Z. mauritiana* Lam.) were extracted by a Soxhlet extractor using the methanolic as an extraction solvent for 6 hours which was found to be a rich source of polyphenols (52.19 ± 2.38 mg gallic acid equivalents/100 g), tannins (50.20 ± 3.61 mg tannic acid equivalents/100 g), and flavonoids (13.19 ±1.31 mg catechin equivalents/100 g) [59]. Moreover, the *Z. jujuba* Mill. seeds were studied using the conventional method with ethanol/water extracts and analyzed for their bioactive phytochemicals using chromatographic techniques which revealed the presence of many bioactive compounds in which 20 components were identified [60]. On the other hand, Abdula *et al.* [32] used the same method for the extraction of polyphenols from jujube leaves. More recently, Shams *et al.* [61] demonstrated that *Z. jujuba var vulgaris* fruit extracted with maceration method at different extraction conditions give the optimum phytochemical compounds contents using ethanol concentration, pH, extraction time, and extraction temperature of 60%, 3, 180 min, 25°C, respectively. The obtained values were 164.51 mg GAE/g DW, 52.94 mg cy-3-glu 100 g-1 DW, and 137.12 mg LAA 100 g-1 DW for total phenolic, total monomeric anthocyanin, and vitamin C contents, respectively.

Based on what has been cited previously, the conventional extraction methods are characterized by high volumes of solvents and longer extraction time, with a low extraction yield of bioactive compounds. To overcome the limitations of these types of methods, non-conventional extraction methods have been introduced, like microwave and ultrasound -assisted extraction.

3 Ultrasounds and microwaves to enhance bioactive compounds yield and quality

3.1 Application of ultrasound-assisted extraction (UAE) in food research

Ultrasound is a mechanical wave, with frequencies higher than the capacity of the ear to catch [62], which can propagate in material and cause cycles of expansion and compression in the environment. This can create bubbles that surround themselves in a liquid at high speed, called cavitation phenomenon [63]. Ultrasound can also be broadly classified as low-intensity sonication (<1 W/cm^2) and high-intensity sonication (10–1000 W/cm^2) [64]. According to Hielscher *et al.* [65], ultrasound shows a very important expansion in medicine because of its effectiveness. Thus, in medical imaging, ultrasound has been much more interesting compared to other imaging methods [62]. It provides access to quantities such as blood flow mapping as well as their positive impact on human health which is justified in the place of ultrasound in medical diagnostic and therapeutic applications [64].

There are two types of ultrasonic equipment used in laboratories, one is called ultrasound probe; which confirmed a direct contact with the sample to be analyzed, such as the extraction of bioactive compounds from plants in order to accelerate the maceration. Unlike the ultrasonic probe, the second ultrasonic bath is used for homogenization, dispersion, degassing, and cleaning, generally based on the indirect contact and used for enrichment [66, 67].

3.1.1 Cavitation mechanism

Ultrasounds are mainly based on heating. It is the phenomenon of ultrasonic cavitation which is due to the cycles of compression and decompression of water molecules. The mechanical effect of ultrasound at high acoustic pressure forms cavitation bubbles as shown in Figure 2 and allows the acceleration and release of bioactive principles of the plant, via the disruption of cell walls and the intensification of mass transfer [68]. When the medium is introduced under ultrasonic waves, cycles of compressions and rarefactions are formed following the longitudinal displacement of the waves in the particles of the medium. Then, a formation of gas bubbles will take place in these zones of variable pressure while changing their size during the process, this is the cavitation phenomenon, these bubbles will subsequently reach a critical size over a period of a few cycles. Thus, allowing them to collapse violently while releasing large amounts of energy [68]. The size of the cavitation bubble is dependent on the frequency of ultrasound. This cavity can absorb ultrasonic energy more efficiently by expanding rapidly until it can no longer absorb energy when liquid rushes in and the cavity implodes. The cavity containing gas and vapors allows generating enormous local temperatures and pressures creating an environment for a chemical reaction [69].

Due to the beneficial effects of ultrasound in the extraction of bioactive components from plants, it improves the extraction time, by reducing it and giving higher yields [14, 70-71]. Ultrasound is used for plant dehydration [72], drying [65, 73], emulsification, and extraction of bioactive substances [74, 75]. For years, many researchers have demonstrated the importance of ultrasound in the development of agro-food industries [13, 67]. On the economic front, the use of ultrasonic treatment has valuable advantages based on the extraction of materials while preserving the quality of the plant's matrix [70]. In addition, Dalvi-isfahan *et al.* [76] showed that the control of ice nucleation by ultrasound waves is a much better innovative alternative preservation technique in lieu of the freezing foodstuffs, a technique that can alter the nutritional and hygienic quality of the food.

3.1.2 Ultrasound-assisted extraction (UAE) of jujube antioxidants

The application of UAE has been widely used for the extraction of plant materials with high-added value. It seems to be an effective extraction method of antioxidants for jujube fruit. A number of authors have evaluated and optimized ultrasound extraction conditions. Boulanouar *et al.* [45] showed that the extraction efficiency of phenolic compounds from *Z. lotus* hydro-alcoholic extracts under sonication was for 81.44 ± 5.64 mg/g, dry weight which exhibited a good antioxidant effect against ABTS, chelating, DPPH, inhibiting lipoxygenase, reducing superoxide radicals, and ORAC assays with a highest EC_{50} value of 110.64 ± 39.71 µmol TE g^{-1} (d.w) by ORAC assay. Additionally, Hammi *et al.* [43] studied the effect of the independent variables under ultrasound extraction, including ethanol concentration (0-100%), sonication time (5-45 min), ratio of solvent to solid (10-70 mL/g), and sonication temperature varying from ambient temperature to 65°C. The authors reported that the use of ultrasound with high intensity improves significantly the phenolic extraction yield from *Z. lotus* pulp and peel. The results showed that increasing the amplitude and the extraction time leads to a higher extraction yield using a lower temperature. The optimum extraction conditions were found using ethanol concentration of 50%, ratio of solvent to solid of 67 g/mL at 25 min and 63°C. Under these conditions, the extraction yield was for 40.782 mg GAE/g DM with significant antioxidant properties mainly by DPPH (IC_{50} of 0.289 mg/mL) and TAA (IC_{50} of 75.981 mg GAE/g DM) in a shorter extraction time. Moreover, the effects of UAE (20 kHz, 80-95°C, 1-4 h, 20-40 g/mL) on polysaccharide recovery with its antioxidant activities from *Z. lotus* pulp and peel were evaluated by Mkadmini-Hammi *et al* .[77]. The authors reported that the direct UAE process led to the highest yield of polysaccharides (18.88%) and six polysaccharides with an average molecular weight of 2720 kDa were identified (arabinose, rhamnose, glucose, fructose, galactose, and xylose). However, at the optimal conditions of 3h 15min, 91.2°C and water to solid ratio of 39 mL/g, the polysaccharide extract showed a significant DPPH (IC_{50} of 0.518 mg/ml), FRAP (614.39 µmol/L), and anti-lipid peroxidation effects at 50% of 2.417 mg/mL. Similarly, Adeli *et al.* [41] investigated the effect of UAE on yield of water-soluble polysaccharide extracted from *Z. lotus* fruit while obtaining a maximum yield of 13.398 ± 0.019% under optimized conditions as follows: 88.77 W, 29.96 min, 77.73°C and water to raw material ratio 24.44 mL/g with highest antioxidant activities for DPPH (78%) and hydroxyl radical-scavenging (91%).

There are few reports on the extraction of bioactive compounds from the *Z. lotus* plant using UAE. While several reports were found from other *Ziziphus* species demonstrating the good use of innovative extraction techniques as shown in Table 3. Qu *et al.* [78] studied the application of UAE in polysaccharide extraction from *Z. jujuba* Mill. using different solvents and results showed that UAE produces a higher yield of extraction with good antioxidants activity against OH scavenging assay with 68%. Furthermore, UAE enhanced the extraction of polysaccharides from *Z. jujuba cv. Muzao* (ZMP) by UAE using both 29% ethanol and 15% $(NH_4)2SO_4$, the authors used jujube powder with liquid-to-solid ratio (mL/g) of 30 under a power of 70 W for 38 min at 48°C. Following these conditions, the experimental extraction yield of ZMP was 8.18% with a high antioxidant potential compared to DPPH (29.68%) and ABTS radical scavenging (21.45%) at a concentration of 2.5 mg /mL [79]. In another study conducted by Lin *et al.* [80] that utilized UAE for the recovery of polysaccharides from *Z. jujuba* Mill. var. *spinosa* seeds showed a higher yield of polysaccharides (1.05 ± 0.08%) at 52.5 °C, 21.2 min, 134.9W, and ratio of liquid to solid 26.3 mL/g as applied conditions. These results are significantly equated to 0.93 ± 0.14% of 6 hours using the heating water extraction method. The seeds extract scavenged more rates of ABTS (33.41%), superoxide anion (41.72%), and hydroxyl radicals (69.78%), while its chelating capacity of Ferrous ion was up to 42.70%. Similarly, Zemouri-Alioui *et al.* [81] extracted phenolic compounds using UAE on jujube leaves and evaluated their antioxidant activity. RSM study has been used under some extraction conditions including solvent concentration (25-100%), solid/solvent ratio (1/50-1/300), extraction time (1-15 min), and ultrasound intensity (25-100%). The authors demonstrated the positive use of UAE providing 6 g GAE/100g for total phenolic content, under methanol 60%, 75% intensity, time of 10 min and ratio of 1/200. The extract showed a significant correlation with the antioxidant activities against DPPH (3.886 g ascorbic acid equivalents/100g) and FRAPS (2.587 g ascorbic acid equivalents/100g). More recently, in our previous work, we found that phenolic compounds extracted from jujube seeds using UAE under a RSM study have shown a higher yield (2383.10 ± 0.87 mg GAE/100g) at applied conditions of 29.01 °C, 15.94 min, ethanol 50.16%, and liquid to solid ratio of 34.10:1 mL/g. This yield was significantly correlated with the antioxidant activities tested against DPPH (EC_{50} of 0.39 µg/mL) and FRAP (1670.42 ± 6.5 mg/100 g) [82].

The extraction of antioxidants mainly polyphenols and polysaccharides from different parts of the jujube plant by conventional and non-conventional methods have been the subject of several studies. All these results are indicated in Table 3.

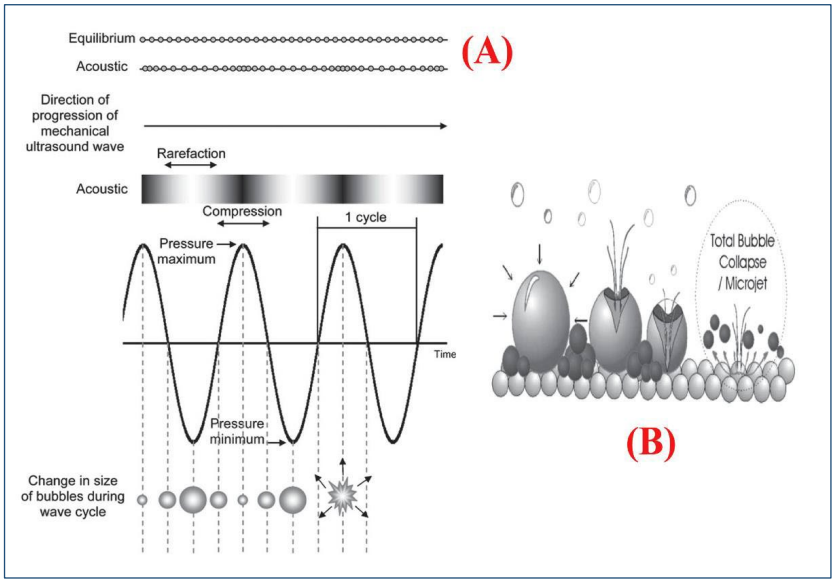


Figure 2: (A) Development and collapse of cavitation bubbles, and (B) schematic depicting classically thought bubble collapse at the solid surface [112]

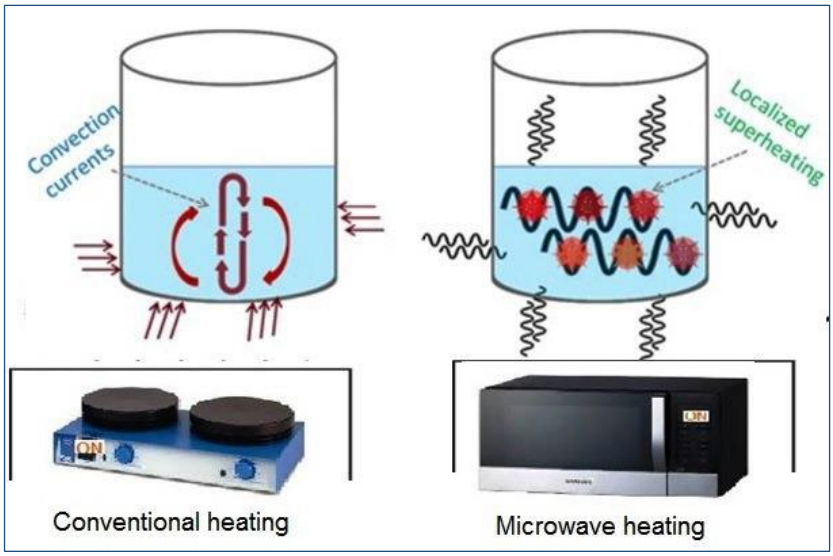


Figure 3: Conventional and microwave heating mechanisms

3.2 Application of microwave assisted extraction (MAE) in food research

The use of microwaves began to appear in the 1950s, the literature reveals that the first microwave oven was introduced in 1955 by Tappan. While during the 1970s and 1980s the widespread use of domestic microwave ovens occurred [83]. Its first application was in chemical synthesis and it was published in 1986 [84], it was used in several domains, such as food processing and drying on industrial process and domestic purposes [85]. Microwaves are a non-ionizing electromagnetic energy [86], with frequencies ranging from 0.3 to 300 GHz. They can be transmitted, absorbed, and reflected, thanks to the laws of optics. Domestic microwave units generally operate at a frequency of 2450 MHz in comparison to industrial applications (915 MHz)[87].

3.2.1 Theory of MAE

Microwave has the capacity to convert a part of plant materials absorbed by electromagnetic energy to heat energy. Microwave heating of plant materials is mainly characterized by the rotation dipole and the ionic conduction [88]. The mechanism of dipole rotation is based on the principle that any molecule under microwave irradiation which generates heat must have a dipole

Table 3: The application of green techniques in the extraction of antioxidants from Zizyphus plant

Extraction method	Category	Vital Products	Matrix	Conditions	Model	Yield	Antioxidant effect	Ref.
UAE	Polysaccharides	Fruit	*Z. jujuba Mill*	15 min 40 °C 80 W	OH scavenging assay.	ND	The extract revealed 68% inhibition. OH scavenging	[78]
	Polyphenols	Fruit	*Z. lotus*	6 min 20 kHz 1g/ 7 mL of a hydro-alcoholic solution (70%)	In vitro ABTS, ORAC, DPPH, chelating, superoxide radicals and inhibiting lipoxygenase were assayed.	81.44 ± 5.64mg/g, dry weight	*Zl* extract exhibited IC_{50} values of 0.049 ± 0.002, 1.406 ± 0.023, 0.042 ± 0.018, 0.138 ± 0.005, 0.001 ± 0.006, 0.129 ± 0.011 mg/ml and 110.64 ± 39.71 µmol TE g^{-1} (d.w.) against ABTS, chelating, DPPH, inhibiting lipoxygenase, reducing, superoxide radicals and ORAC assays, respectively	[45]
	Polyphenols	Pulp and peel	*Z. lotus*	Ethanol 50% 25 min 63°C 67 mL/g	*In vitro* DPPH and TAA assays	40.782 mg gallic acid equivalents/g dry matter	The extract revealed IC_{50} values of 0.289 mg/mL and 75.981 mg GAE/g DM for DPPH and TAA tests, respectively	[43]
	Polysaccharides	Pulp and peel	*Z. lotus*	3h 15min 91.2°C 39 mL/g	DPPH scavenging ability, reducing power and anti-lipid peroxidation assays	18.88%	The *Zl* extract revealed potent IC_{50}values of 0.518 mg/ml), 614.39 µmol/L and 2.417 mg/mL at 50% for DPPH, FRAP and anti-lipid peroxidation tests	[77]
	Polysaccharides	Fruit	*Z. lotus*	88.77 W 29.96 min 77.73°C 24.44 mL/g	DPPH and hydroxyl radical-scavenging activities	13.39±0.019%	The polysaccharide extract revealed an antioxidant effect of 78 and 91% for DPPH and hydroxyl radical-scavenging tests, respectively	[41]
	Polyphenols	Leave	*Z. jujuba Mill*	Methanol 60% 75% intensity 10 min 1/200 mg/mL	FRAP and DPPH assays	6 g GAE/100g	Antioxidant activities against DPPH and FRAP were for 3.886 and 2.587 g ascorbic acid equivalents/100g, respectively	[81]
	Polysaccharides	Fruit	*Z. jujube cv. Muzao*	29% of ethanol 15% $(NH_4)2SO_4$ 30 mL/g 70 W 38 min 48°C	ABTS and DPPH assays	8.18%	The extract revealed to have a moderate antioxidant activity for both DPPH (29.68%) and ABTS (21.45%)	[79]
	Polysaccharides	Seeds	*Z. jujuba Mill* var. spinosa	52.5 °C 21.2 min 134.9W 26.3 mL/g	ABTS, superoxide anion, hydroxyl radicals and chelating capacity of Ferrous ion	1.05 ± 0.08%	The extract showed an ABTS, superoxide anion, hydroxyl radicals and chelating capacity of ferrous ion of 33.41, 41.72, 69.78 and up to 42.70%, respectively	[80]
	Polyphenols	Seeds	*Z. lotus*	Ethanol 50.16% 29.01 °C 34.10:1 mL/g 15.94 min	DPPH and FRAP tests	2383.10 ± 0.87 mg GAE/100g	*Zl* extract exhibited EC_{50} of 0.39 µg/mL for DPPH test and 1670.42 ± 6.5 mg/100 g by FRAP	[82]
MAE	Polysaccharides	Pulp and peel	*Z. lotus*	600 W 40 min 26.69 mL/g	ABTS, DPPH and FRAP tests	13.98 ± 1.55%	The *Zl* extract revealed a good scavenging capacity against ABTS. + (70.45%), DPPH*. (66.02%), and FRAP (A = 0.63)	[16]
	Polysaccharides	Fruit	*Z. jujuba Mill*	4 min 300 W	OH scavenging assay.	ND	The extract revealed 52% inhibition. OH scavenging test	[78]
	Polysaccharides	Peels	*Z. jujuba Mill*	400W 75°C 60 min 30 g water/g powdered jujube	FRAP and DPPH assays	9.02%	Jujube extract showed a scavenger effect against DPPH arround 65 to 75% and FRAP (A= 0.63)	[99]

moment where the molecule is charged (+ or -) like water (H_2O) which according to the polarity of the field, it tries to align with the electromagnetic field by rotary motion (Figure 3). This last causes friction heat [86, 89].

When an electromagnetic field is applied, ionic compounds move at an accelerated rate producing ionic polarization. As the movement of the ions increases, kinetic energy is converted quickly into the thermal energy of the solution [86]. The ability of given material to interact with an electric field by converting energy into heat depends largely on its dielectric properties. Dielectric constant and dielectric loss factor are the parts of dielectric properties. Dielectric constant (ε'), meaning the ability to store electrical energy, while dielectric loss (ε"), describes the material's ability to convert electrical energy into heat [90, 91], according to the following equation:

$$\varepsilon' = \varepsilon'' \tan \delta Eq(1)$$

The dissipation factor (tan δ) is an indicator of the efficiency of the dissipation or absorption of electrical energy in the form of heat by microwave which is described by:

$$p_v = kf\varepsilon' E2 \tan \delta Eq(2)$$

Where:

P = absorbed microwave power (W/cm3)
f = microwave frequency (GHz)
ε"= dielectric loss factor of material
k = a constant
E = electric field intensity for a given volume (volts/cm).

Numerous studies have been published by several authors for microwave applications acquired and tested in the food industry, as shown by Smith *et al.* [93], including drying, moisture determinations, safety guidelines, economy, automation, and robotics Routray *et al.* [86] showed that several factors can affect microwaves extraction of bioactive compounds (gives an example of flavonoids), as suggested by the authors, both polar and non-polar solvents can be used for extraction with respecting substances nature of extraction in each used solvent. On the other hand, the power level, temperature, and time of extraction may affect positively the extraction process and increases the solubility due to the interaction molecules with the opening cell-matrix and the liberation of bioactive compounds. More recently, Chemat *et al.* [13] demonstrated the extraction mechanism of MAE which was supposed to involve several stages that are based on the effect of microwave radiation, which increases the temperature and the pressure of the microwaves during the extraction, these will allow the diffusion of the solvent in the sample matrix and will thus release the active ingredients of the this last. Due to these effects, the advantage of using microwaves is very important because it not only guarantees an efficient extraction allowing the recovery of a maximum of bioactive compounds more quickly compared to the conventional extraction processes but also considered as green technology by reason of the less use of organic solvents [94].

3.2.2 Microwave-assisted extraction (MAE) of jujube antioxidants

The conventional extraction methods, compared to MAE, occurs from the outside to the inside of the substrate, and the heat is not transferred as the same [86]. During this extraction, there is stable conduction due to the concentration of solute in interaction with the solid varies, and this according to the solvent penetrated the matrix, the solubilization of the components and the migration solute from the outside to the solution as well as. The extraction efficiency is not a linear function of extraction time [95].

Several studies demonstrated the efficiency of the MAE process compared to the UAE and the feasibility of using the MAE process at an industrial scale, and it has been very used in green extraction of bioactive compounds from plants and industrial by-products [96]. A number of authors have evaluated and optimized conditions of extraction of bioactive contents using MAE, Dahmoune *et al.* [97] used MAE of *Citrus limon* (L.) Burm. f. peels and compared to UAE and CSE for the recovery of total phenolic compounds. The optimized result for MAE was 48%, 28:1 mL/g, 123s, and 400 W for ethanol as extraction solvent, solvent: solid ratio, irradiation time, and power, respectively. Results show that the maximum predicted TPC recoveries under the optimized conditions for MAE was 15.74 mg GAE/g model. In comparison to UAE and CSE, MAE showed better results in terms of yield and antioxidant activities against DPPH and reducing power. Dahmoune *et al.* [98] used the MAE of total phenolic compounds from the leaves of *Pistacia lentiscus* L. which generates better extraction yield (185.69 ± 18.35 mg GAE/gdw) with higher antioxidant activities in comparison to UAE and CSE with optimal conditions as 46% ethanol, extraction time 60 s, potency density 17.86 W/mL, and liquid/solid ratio 28:1. This is due to the rapid energy-saving heating rates with deep penetration of organic solvent in raw material, leadings to very short extraction times as shown in Figure 3.

More recently, several studies have been carried out for other species of jujube. The influence of microwave heat treatment on jujube plant in terms of storability and quality has been studied. The response surface methodology (RSM) was used to evaluate and optimize MAE in polysaccharide recovery from *Z. jujuba* Mill. peels. For this purpose, jujube fruits were treated by MAE until reaching a temperature level of 45-85°C, microwave powers (250-450 W), extraction time (30-70 min), and solvent to solid ratio (10-70 mL/g). The authors reported that the use of microwave with high intensity improves significantly the yield of polysaccharides of *Z. jujuba* Mill. fruit (9.02% of polysaccharide) at 400 W, 75°C, 60 min, using 30 g water/g powdered jujube with a good antioxidant effect against DPPH (around 65 to 75%) and FRAP (A= 0.63) [99].

For instance, there is no report about the use of MAE to enhance the extraction of phenolic compounds from the *Z. lotus* (L.) species. However, our previous work focused on polysaccharide extract from *Z. lotus* (L.) pulp and peel using MAE under RSM

study. The effect of the independent variables, including microwave power (200-600 W), irradiation time (20-40 min), and a liquid/solid ratio (20-40 mL/g) have shown that MAE improved significantly the polysaccharide extraction yield from *Z. lotus* (L.) pulp and peel at 600 W, 40 min, and 26.69 mL/g [16]. Under these conditions, the *Z. lotus* extract exhibited a good antioxidant capacity against $ABTS^{+}$ (70.45%), $DPPH^{\cdot}$ (66%), and FRAP (A of 0.63) [16].

3.3 Comparison between conventional methods with the green extraction processes

There are several conventional extraction techniques such as maceration, water distillation, steam distillation, combined water and steam distillation, and enfleurage. Comparing these techniques to newer extraction methods, they consist of low-cost solvent extraction that uses heat and/or agitation. Generally, these conventional techniques make it possible to increase the solubility of the target compounds while improving the mass transfer during the extraction stage, several factors are important in the solvent system, which concern volatility, selectivity, density, toxicity, reactivity, miscibility with aqueous media, viscosity, and purity. On the other hand, these conventional methods result in low selectivity and high consumption of organic solvents, in addition to contamination and loss of analytes [40]. However, green technologies such as ultrasound and microwaves are increasingly studied, the latter combines both ecological and economic aspects in order to contribute to the process of sustainable development of the world population by seeking new (modern) means. Extract valuable compounds from plants, herbs, algae, and other organizations (e.g., bioactive compounds such as essential oil, antioxidants, oil, and dyes). According to several researchers, this is the best strategy for 2030 allowing the greatest reduction in waste while valuing the by-products rejected by reusing them in order to reduce energy, especially since the majority of the extractions used consume enormous quantities of energy and solvents with a high environmental impact. So-called green techniques are a better alternative to conventional methods for good respect for the environment and effective sustainability [13, 66]. Furthermore, Chemat *et al.* [67] has developed some principles of innovative extraction, in particular the reduction of large volumes of solvents, energy consumption, extraction time, production of by-products instead of waste with high added value and sourcing reasoned the recovery of a safe natural extract. In addition, on an industrial scale, these principles of green extraction methods have been able to improve the sustainability of production compared to conventional methods.

4 Conclusions

This brief review paper described the uses of emerging technologies mainly ultrasounds and microwaves to enhance antioxidant extraction yields and quality from *Z. lotus* (L.) Lam plant. In order to substitute synthetic antioxidants and palliate the inconvenience of conventional techniques which consume large quantities of solvent and time versus a lower yield, the UEA and MAE extraction technologies could be used. This would present a significant economic gain for the industry. The interest of this review was to show the benefits of antioxidants in protecting humans against free radicals and the benefits of using new environmentally friendly extraction technologies. Furthermore, the valorization of this local plant which could be used as an inhibitor agent of oxidation phenomena, the possibility of exploitation of these antioxidants at an industrial scale, and its commercialization is indicated in this mini-review. Finally, in the next few years, these green methods including UAE and MAE could provide an innovative approach to increase the production of specific compounds extracted from the *Ziziphus* plant for use as nutraceuticals or as ingredients in the field of modern food engineering.

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ORIGINAL PAPER

LC–ESI–MS/MS analysis, biological effects of phenolic compounds extracted by microwave method from Algerian *Zizyphus lotus* fruits

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Abstract
Jujube fruit is a considerable source of antioxidants that could be used as ingredients against several diseases. The optimization of microwave-assisted extraction (MAE) of bioactive compounds from *Z. lotus* pulp and peel (*Zlp*) was achieved using response surface methodology. The effect of the extraction parameters (microwave power "MW", extraction time, ethanol/water solvent and liquid–solid ratio) on the total phenolic compounds (TPC) was well described by second-degree regression model. The liquid chromatography-high resolution tandem mass spectrometry (LC–MS–MS) was done. Best results were obtained at 600 W MW power, 180 s irradiation time, 51% ethanol concentration and 47:1 mL/g solvent-to solid ratio obtaining 7473.38 ± 740.55 mg GAE/100 g of TPC, 1019.96 ± 75.03 mg CE/100 g of TFC, 14,253.11 ± 2453.86 mg CE/100 g of TTC. LC–MS–MS determined that from all phytochemicals, 44.51% of the extract was flavonoids which represent 80.75% of all secondary metabolites from which 5-hydroxy-7-*O*-nerylflavanone is found to be the most abundant one. The extracts revealed the presence of 34 bioactive compounds, from which 10 phenolic compounds have never been previously identified from *Zizyphus* plant. The *Zlp* extract exhibited a greatest antioxidant effects by PAOT, DPPH, and FRAP activity; as well as, a lowest cytotoxic effects against both HepG2 and MCF-7 cells. In a concentration of 1 mg/mL, *Zlp* produced a strong AChE inhibition. This research revealed that MAE is more rapid and an effective method to extract TPC recovery which can be used in food matrixes and/or in nutraceutical formulations for its good biological effect.

Keywords *Zizyphus lotus* · Bioactive compounds · Microwave assisted extraction · RSM optimization · Biological activities

Introduction

Reactive oxygen species (ROS) play an important role in progression of degenerative diseases such as cancer, Alzheimer, inflammation, autoimmune, and cardiovascular diseases [1, 2]. This seems to be attributed to the strong oxidative stress, caused by unbalance between antioxidants and oxidants, including ROS [3, 4]. The antioxidant nutrients are considered a sophisticated system developed by our organism against oxidative stress, thus reducing the risk of chronic

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diseases [5]. In most food industries, synthetic antioxidants have been used as a solution against ROS that causes lipid peroxidation resulting in product alterations (color and flavor) [6]. Propyl gallate (PG), butylatedhydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and butylatedhydroxyanisole (BHA) are the main food antioxidants (Biglari etc.). However, the uses of these synthetic antioxidants have been substituted by natural antioxidants due to their toxic and tumorigenic effects [7]. Beside, medicinal plants are well known as veritable source of antioxidants thanks to phenolic compounds that preserve the quality of products and inhibit oxidative mechanism. More recently, an increasing demand on the uses of safe antioxidants obtained from plant and food by-products as additives and health-promoting ingredients in food, pharmaceuticals, and cosmetics have been mentioned [3, 8, 9].

Zizyphus is a fruit tree, which belongs to the family of Rhamnaceas. This tree is originated from tropical regions such as Asia and America, in Europe and also in all the North of Africa as Algeria. Furthermore, the forms of this tree vary with the soil and climate. Since millennia, the consumption of its fruits is very vast [4]. The edible fruit of jujube (*Zizyphus lotus* L.) is present in abundance in the Mediterranean region (Morocco, Algeria and Tunisia) with various traditional therapeutic use [10]. It is known in some regions of Algeria as '*Sedra*'. In others, It's called '*Nbag*' [4, 11]. More recently, the use of certain traditional medicines as drugs has increased very significantly and considered as a natural alternative to synthetic drugs. Aromatic plant products have played an important part in human culture and folklore medicine [12]. In addition, several scientific studies have shown the state of many biological properties from this plant and its constituents through in vitro and in vivo studies, jujube has been a dietary food, it appears in the list A of the medicinal plants of French pharmacopeia [13]. *Zizyphus lotus* L. fruits (*ZLp*) is a famous Algerian species used in several traditional treatments for its antioxidant, anti-inflammatory, anti-diabetic and hypoglycemic activities. The different part from this medicinal plant offers a variety of specific therapeutic properties (root, leaf, stem bark and fruit) due to its diversity of bioactive compounds present in the plant, especially polyphenols that are known by neutralization of free radicals with a significant antioxidant capacity [4, 14, 15]. Hence it is more interesting to see the compounds responsible for this biological activity extracted from jujubes.

Bioactive compounds extraction from *ZLp* has been investigated in the last decades focusing mainly on conventional solvent extraction (CSE). Alternatively, modern techniques such as microwave-assisted extraction (MAE), the use of supercritical fluid (SFE), accelerated solvent extraction (ASE) and ultrasound assisted extraction (UAE) have been widely used to promote extraction in terms of reducing energy and time with obtaining a higher bioactivity which is not the case for traditional techniques that are found to consume energy, time, solvent and participate to the degradation of bioactive compounds [3, 4, 16–18]. Among these techniques, UAE seems an efficient technique in term of profitability and economic in relation to the shortened time and to the power reduced of the extraction, thanks to its physiochemical effect of absorption assigned to the cavitation phenomenon induced with the ultrasound waves in the extraction medium [19]. On the other hand, MAE is a new method based on the heating of the sample matrix which can accelerate the vaporization of solvent, raising the intracellular pressure and causing the rupture of the cell walls to release the bioactive compounds [20]. The use of microwaves for extracting plant constituents has been found to improve three times the yield of total phenols extracted compared to other extraction methods. This green technology known for its extraction efficiency is still in their infancy [17, 21, 22]. As a result, alongside the majority of previous research focused on various bioactive compounds found in this plant, mainly polyphenols, polysaccharides, proteins and lipids. The ecofriendly-extraction of polyphenols from jujube by green technology based on MAE could be one of the solutions deemed to be a promoter for the valorization of vegetable plants for the protection of the environment and the consumer [22]. This green extraction methods applied to plants will meet the challenges of the twenty-first century on recovering bioactive compounds without spoiling the environment through the use of organic chemical solvents.

To the best of our knowledge, no study has been reported on the effect of microwaves and ultrasound in the extraction of phenolic compounds from *Zlp* comparatively to the conventional organic solvent process. This present work is motivated by the increased need of the agro-food industries to develop the production of new functional and nutraceutical ingredients by optimizing the extraction conditions, as well as the increased consumers demand for natural food products with important nutritional values. In addition, there is no research available in the literature concerning the identification of phenolic profile from *Zlp*. Therefore, the present work deals with (i) the determination of optimum MAE by RSM using as factors: irradiation time, ethanol concentration, solvent-solid ratio in terms of the TPC recovery, (ii) identification of the compounds present in *Zlp* by liquid chromatography high resolution mass spectrometry (LC-HRMS/MS) and (iii) evaluation of some biological activities of the optimum extract.

Materials and methods

Raw material

The fruits material of *Zlp* were collected from Djelfa, Algeria (34° 40′ 30″ N, 3° 15′ 30″ E). The pulp and peels were

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dried at 40 °C for 24 h in a ventilated oven (Memmert, Modell 100–800, Schwabach, Germany) and ground by an electrical grinder (WH model 8100 Basic, Beijing, China) and the powder was passed through a standard sieve, and only the fraction with particle size < 125 µm was used. The powder was stored in closed airtight bottles and kept in the dark conditions (4 °C) until analysis.

Experimental work

Before MAE optimization, a preliminary study based on single-factor experiments was performed to evaluate the influences of process parameters (Table 1). When one variable was not studied, it was kept constant. The experimental level was defined according to the preliminary experiment results. Each factor was evaluated at different levels within the following intervals: 300–700 W, 30–300 s and 10–70% for microwave power, extraction time and ethanol concentration respectively. However, pH and particle size were fixed in all experiments at 4 and 125 µm respectively.

Based on the experimental results of the preliminary single-factor study, major significant influence factors were selected. Then, a RSM based on a central composite design (CCD) for MAE was conducted to optimize the extraction processes (Tables 2). Regression analysis of the data to fit a second-order polynomial equation was carried out according to the following general equation (Eq. 1) which was, then, used to predict the optimum conditions of the extraction process.

$$Y = B_0 + \sum_{i=1}^{k} BiXi + \sum_{i=1}^{k} BiiX^2 + \sum_{i>1}^{k} BiiXiXj + E, \quad (1)$$

where Y represents the response function (in this case, the TPC); B_0 is a constant coefficient; Bi, Bii, and Bij represent linear, quadratic, or interaction regression coefficients,

Table 1 Central composite design with the observed responses for phenolic compounds yield from *Zlp* using microwave-assisted extraction (MAE)

Run	X_1— Irradiation time (s)	X_2— Microwave power (W)	X_3— Solvent-to solid ratio (mL/g)	X_4— Ethanol concentration (% v/v)	TPC yield (mg GAE/100 g DM)	
					Experimental	Predicted
1	180 (+1)	300 (− 1)	50 (+1)	30 (− 1)	7094.41	7054.59
2	120 (− 1)	700 (+1)	20 (− 1)	30 (− 1)	5816.37	5914.58
3	150 (0)	700 (+1)	35 (0)	50 (0)	8215.96	7654.08
4	120 (− 1)	300 (− 1)	20 (− 1)	70 (+1)	3896.71	3918.11
5	120 (− 1)	700 (+1)	50 (+1)	30 (− 1)	7198.74	7410.62
6	120 (− 1)	700 (+1)	50 (+1)	70 (+1)	7355.24	7498.36
7	180 (+1)	700 (+1)	50 (+1)	70 (+1)	7094.41	7059.09
8	120 (− 1)	700 (+1)	20 (− 1)	70 (+1)	4267.08	4158.29
9	120 (− 1)	300 (− 1)	50 (+1)	70 (+1)	6729.26	6636.12
10	120 (− 1)	300 (− 1)	20 (− 1)	30 (− 1)	5252.99	5139.71
11	120 (− 1)	300 (− 1)	50 (+1)	30 (− 1)	6168.49	6013.69
12	150 (0)	500 (0)	50 (+1)	50 (0)	7381.32	7294.14
13	180 (+1)	300 (− 1)	50 (+1)	70 (+1)	7003.12	6992.36
14	120 (− 1)	500 (0)	35 (0)	50 (0)	6846.63	6842.05
15	150 (0)	500 (0)	20 (− 1)	50 (0)	4689.61	5021.49
16	180 (+1)	500 (0)	35 (0)	50 (0)	6727.96	6977.24
17	180 (+1)	700 (+1)	20 (− 1)	30 (− 1)	5884.19	5828.72
18	150 (0)	500 (0)	35 (0)	50 (0)	6965.31	7114.52
19	150 (0)	500 (0)	35 (0)	50 (0)	6919.66	7114.52
20	150 (0)	500 (0)	35 (0)	70 (+1)	6079.81	6281.56
21	150 (0)	500 (0)	35 (0)	30 (− 1)	7147.88	7190.83
22	150 (0)	300 (− 1)	35 (0)	50 (0)	6426.70	7233.28
23	180 (+1)	700 (+1)	20 (− 1)	70 (+1)	3145.53	3387.77
24	150 (0)	500 (0)	35 (0)	50 (0)	8192.69	7114.52
25	180 (+1)	300 (− 1)	20 (− 1)	70 (+1)	4303.59	3943.11
26	180 (+1)	300 (− 1)	20 (− 1)	30 (− 1)	5905.05	5849.37
27	180 (+1)	700 (+1)	50 (+1)	30 (− 1)	7589.98	7656.01

TPC total phenolic content, *GAE* gallic acid equivalent

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Table 2 Estimated regression coefficients for the quadratic polynomial model and the analysis of variance (ANOVA) for the experimental results of TPC from *Zlp*

Parameter	DF	Estimated coefficients	Sum of squares	F-value	Prob > F
		7114.5237	41,407,285	12.9234	< 0.0001*
X_1	1	67.59694	82,248	0.3594	0.5600
X_2	1	210.39819	796,813	3.4817	0.0867
X_3	1	1136.3241	23,242,185	101.5561	< 0.0001*
X_4	1	− 454.6311	3,720,410	16.2562	0.0017*
Quadratic					
X_1^2	1	− 204.8755	107,933	0.4716	0.5053
X_2^2	1	329.16208	278,608	1.2174	0.2915
X_3^2	1	− 956.7013	2,353,570	10.2839	0.0075*
X_4^2	1	− 378.3236	368,045	1.6082	0.2288
Interaction					
X_1X_2	1	− 198.8785	632,842	2.7652	0.1222
X_1X_3	1	82.811685	109,724	0.4794	0.5019
X_1X_4	1	− 171.1659	468,764	2.0483	0.1779
X_2X_3	1	155.51643	386,966	1.6908	0.2179
X_2X_4	1	−133.6724	285,893	1.2492	0.2856
X_3X_4	1	461.0678	3,400,436	14.8581	0.0023*
Lack of fit	10		1,703,270.2	0.3266	0.9082
Pure error	2		1,043,056.2		
Total error	12		2,746,326.3		
R^2				0.937801	
r^2 adjusted				0.865235	
CV%		7.58			
Corr.Total	26		44,153,611		

X_1: Irradiation time

X_2: Microwave power

X_3: Solvent-to-solid ratio

X_4: Ethanol concentration

*means that the concerned factor is significant

respectively. *Xi* and *Xj* represent the coded independent variables.

In order to define the interaction of the independent variables and their effects, the 3D response surface plots have been used from the mathematical equation of fitted the polynomial model. The factor levels were coded as − 1 (low), 0 (central point or middle) and 1 (high), respectively. Analysis of variance was performed for the response variable (TPC recovery) using the full model where *p*-values (distributed into linear and interaction factors) indicated whether the terms were significant or not. To control the appropriateness of the proposed model from RSM and giving the MAE optimal conditions, a comparison with the experimental results was established. The efficiency of the MAE method (optimum conditions) was compared with UAE and CSE based in respect to TPC recovery (response) and biological activities.

Microwave-assisted extraction (MAE)

The TPC was extracted from *Zlp* powders using a modified microwave oven apparatus (2450 kHz, Samsung Model, Malaysia). This microwave was equipped with a numerical digital control system for power adaptable (from 100 to 900 W) and irradiation time. One gram of plant material powder was extracted into 500 mL flask containing aqueous ethanol solvent. Different conditions of extraction were occurred by varying the four variables or parameters studied as ethanol concentration (30–70%), irradiation time (120–180 s), microwave power (300–700 W) and solid-to-solvent ratio (1:20–1:50 g/mL) (Table 1). After irradiation, the flask was taken out and cooled in an ice water bath (4 °C). The mixtures were filtered by Whatman No.1 paper and collected in a volumetric flask than centrifuged at 1000×*g* for 5 min. The extract was stored at 4 °C until use. Each trial was carried out in triplicate.

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UAE and CSE methods

The extraction of TPC from *Zlp* was carried out in an ultrasonic bath UAE (J P.SELECTA 195 W 50/60HZ, Spain) according to the method optimized by Hammi et al. [23] and by conventional solvent extraction by the method described by Dahmoune et al. [8].

Analytical determinations

Total phenolic content (TPC)

Total phenolic content in *Zlp* was determined according to the Folin–Ciocalteu method of Hammi et al. [23]. A calibration curve was established to calculate the TPC concentration using gallic acid as standard and the results were expressed as mg of gallic acid equivalent per 100 g of dry matter (mg GAE/100 g DM).

Total flavonoids content (TFC)

The total flavonoid content was determined according to the Ghafar et al. [24] method. The TFC results were expressed as mg quercetin equivalents per 100 g of dry matter (mg QE/100 g DM). Samples were measured in triplicate.

Total condensed tannins content (TTC)

The total condensed tannins content was determined using the method of Berkani et al. [3], based on the condensation of phenolic compounds with vanillin in acid medium. The TTC was expressed as mg catechin equivalent per 100 g of dry matter (mg CE/100 g DM). Samples were measured in triplicate.

Compound identification by liquid chromatography–high resolution tandem mass spectrometry (LC–HRMS/MS)

The identification of phenolic compounds from *Zlp* was performed by means of liquid chromatography-high resolution tandem mass spectrometry (LC–MS–MS), as described by Guedes et al. [25].

Biological activities

Total antioxidant power by PAOT technology The total antioxidant activity of *Zlp* was determined according to Kaci et al. [26] by using PAOT (Pouvoir AntiOxydant Total) Liquid® technology (WO 2020/109736A1). Results were expressed as PAOT Score per gram of extract (PAOT Score/g) [27], according to the following formula:

$$\text{Antioxidant activity} = \left(\frac{EP_{product10} - EP_{control0}}{EP_{control0}}\right) \times 100, \quad (2)$$

where $EP_{control0}$ is the electrochemical potential at time 0. $EP_{product10}$ is the electrochemical potential obtained after 10 min registration in presence of tested antioxidants or studied supplements samples.

Scavenging activity against DPPH· radical The free radical-scavenging ability of *Zlp* was expressed as the percentage of inhibition of DPPH· radical. The free radical-scavenging activity was measured according to the colorimetric method used by Achat et al. [28] where the % scavenging activity was determined according to Eq. 3.

$$\%\ \text{Scavenging activity} = \left(\frac{A_{control} - A_{extract}}{A_{control}}\right) \times 100, \quad (3)$$

where $A_{control}$ is the absorbance of DPPH and solvent. $A_{extract}$ is the absorbance of DPPH and sample extract. Trolox and BHA were used as positive controls

The effective sample concentration required to inhibit 50% of the DPPH radical (EC_{50} value) is obtained by linear regression analysis of the dose–response curve plot between% inhibition and concentrations.

Ferric-reducing antioxidant power (FRAP) activity The ability of the extracts tested to reduce ferric iron (Fe^{3+}) present in potassium ferricyanide to ferrous iron (Fe^{2+}) in presence of an antioxidant that has the power to give out electrons, was determined by Hammi et al. [29]. A calibration curve was plotted using gallic acid as standard and the results are expressed in terms of antioxidants having an iron reduction capacity equivalent to that of equivalents in gallic acid of 1 mg per 100 g of dry matter (mg GAE/100 g DM).

Anticholinesterase activity Anticholinesterase activity (AChE) was determined using AChE assay, an enzyme localized in the neurosynaptic gaps and neuromuscular junctions, used in the treatment of Alzheimer disease according to Berkani et al. (2021). All tests were done in triplicate and the percentage inhibition was calculated as:

$$I\,(\%) = 100 - \left(\frac{Vsample}{Vcontrol}\right) \times 100, \quad (4)$$

where I is the percent inhibition of acetylcholinesterase. Vsample: the initial velocity of the jujube extract containing reaction and Vcontrol: the initial velocity of the control reaction.

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Anti-proliferative activities on HepG2 and MCF-7 cells The MTT viability test against the human tumor cell lines HepG2 (ATCC#HB-8065), from human hepatocellular liver carcinoma cell lines, and MCF-7 HTB-22), from breast cancer were used for evaluating *Zlp* cytotoxicity according to the method described by Berkani et al. (2020b). At different concentrations of extract, the assays were done in 8 × 12 replicates and the cell viability percentage was calculated by the following equation:

$$Viability\,(\%) = \left[\frac{(Abs\,595 - Abs\,630\,of\,experimental\,wells)}{(Abs\,595 - Abs\,630\,of\,control\,wells)}\right] \times 100 \quad (5)$$

Statistical analysis

Each extraction trial and all the analyses were carried out in triplicate and all the data in this paper have been reported as means ± S.D. Influence of each factor on the TPC yields in the single-factor experiment for the MAE was statistically assessed by ANOVA and Tukey's post hoc test with a 95% confidence level. Data obtained from the CCD trials for the MAE were statistically analyzed using ANOVA for the response variable in order to test the model significance and suitability. The $p < 0.05$ and $p < 0.01$ were taken as significant and highly significant level, respectively. The JMP (Version 10.0, SAS) software was used for the analysis of all experimental results and the construction of CCD. Component Analysis (PCA) was applied to show the possible correlation between phenolic compounds extracted by different methods and antioxidant activity.

Results and discussion

Optimization of MAE conditions

Modeling and fitting the model using response surface methodology

Based on the preliminary study (data not shown), the effects of extraction parameters such as nature of solvent, microwave power, extraction time, concentration of solvent and solvent-to-solid ratio were systematically were chosen. Then, the extraction efficiency of the microwave process was estimated by measuring bioactive compounds. The experimental design matrix and the response *Zlp* extracts obtained in the trials of the CCD from 27 experiments are presented in Table 1. The regression coefficients were determined using least squares as a technique of individual linear, quadratic and model interaction [30]. As shown in the Table 2, for the TPC response, ethanol concentration and solvent-to-solid ratio have significant linear effects ($p < 0.05$), while no significant effect of irradiation time (X_1) was found. However, TPC shows significant quadratic effects for the solvent-to-solid ratio (X_3^2) with a negative effect. On the other hand, the irradiation power (X_2^2) showed no significant effect on TPC yields. Regarding the effect of interaction between the variables, a significant effect was observed between the solvent-to-solid ratio (X_3) with ethanol concentration (X_4) (Table 2). Three second order polynomial models were fitted to generated data to describe the significance and adequacy of the model and the empirical relationship between the TPC and operational conditions. The below predictive equations in term of significant independent variables were obtained (Eq. 4). Table 2 reports the statistical analysis of the regression model.

$$Y(TPC) = 7114.52 + 1136.32X_3 - 454.63X_4 - 956.7013X_3^2 + 461X_3X_4 \quad (6)$$

Very high F-value and a very low p-value ($p < 0.0001$), indicating that these models were highly significant and it can be used to optimize the extraction variables (Table 2). In addition, the determination coefficients for TPC yields was for $R^2 = 0.93$ and Adj. $R^2 = 0.86$, were reasonably close to 1, with low value of CV (< 10%) (Table 2). This indicates a high degree of correlation between the observed and predicted values, which implied that only 3, 6 and 1% respectively of these variations could not be justified by the models. Also, other parameters were insignificants as the lack of fit ($p > 0.05$) for the TPC response which provide also the validity of the experimental model. The coefficient of variation (CV) was within the acceptable range (˂ 10%) [31, 32]. This indicates that variation in the mean value is small and satisfactorily develops an adequate response model.

Analysis of response surfaces

The response surfaces of regression models were plotted by their three-dimensional profile in order to study the effects of interaction between independent variables on the TPC extraction yields (Fig. 1A–F). According to Hayat et al. [33], maintaining two independent variables at their zero levels and using the z-axis against two independent variables, a response can be drawn. The surface response (3D) is considered as the diagram permitting to represent the regression equation.

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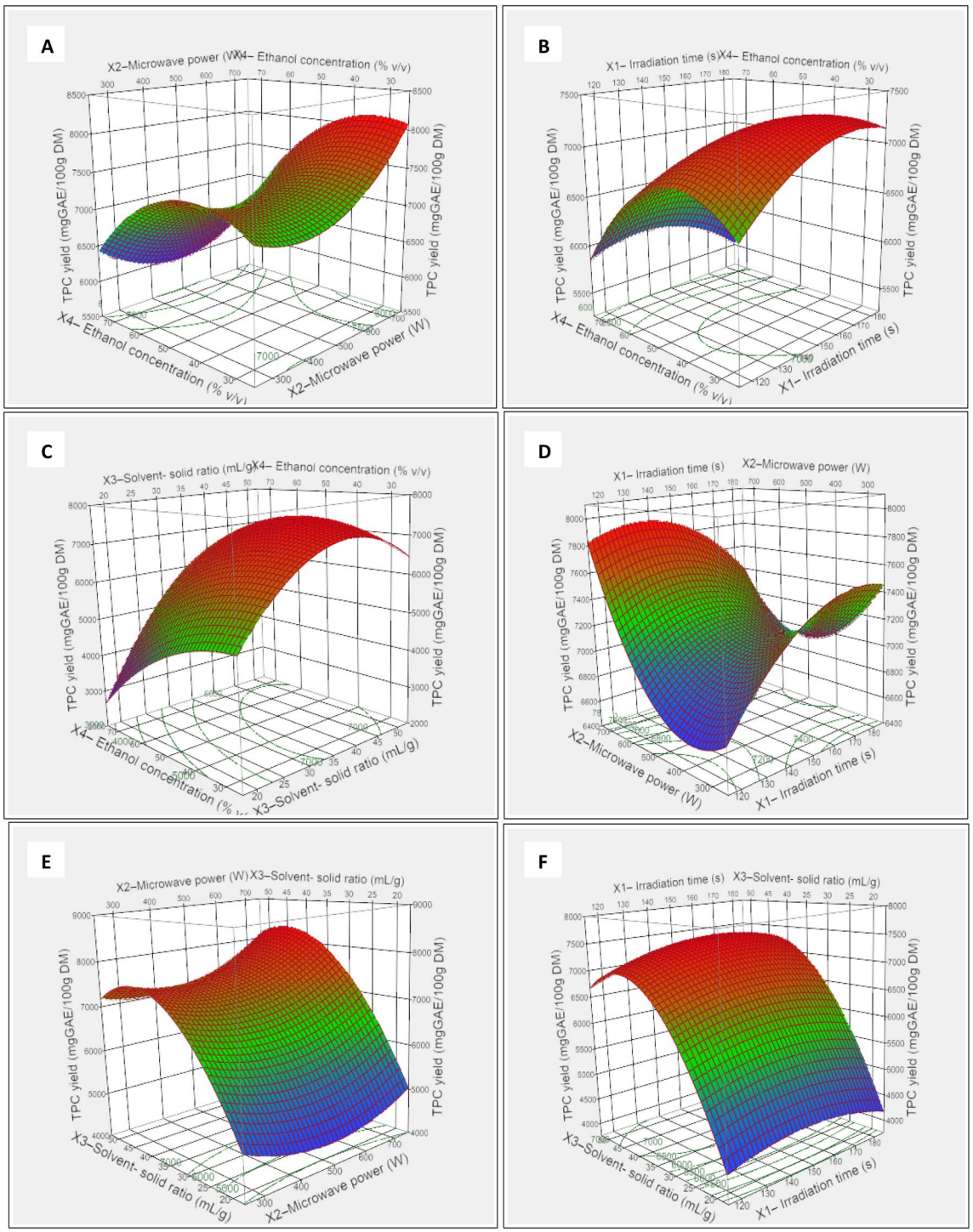


Fig. 1 Response surface analysis for the TPC yields from *Zlp* with MAE with respect to ethanol concentration and solvent-to-solid ratio (**A**); ethanol concentration and irradiation time (**B**); ethanol concentration and microwave power (**C**); /irradiation time and microwave power (**D**); microwave power and solvent-to-solid ratio (**E**); irradiation time and solvent-to-solid ratio (**F**)

Response surface analysis of TPC yield

The effect of ethanol concentration on each of the factors (solvent-to-solid ratio, microwave power and irradiation time) was shown in Fig. 1. Figure 1A shows that the TPC could be greater than 6000 mg GAE/100 g for a low solvent-to-solid ratio with ethanol concentration between 30 and 40%. At 50% ethanol concentration, the TPC yield

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increased up to 7000 mg GAE/100 g with the significant increase in extraction time up to 180 s, whereas, above this extraction time with additional ethanol concentration, the extraction yield decrease gradually (Fig. 1B). As shown in the Fig. 1B, the TPC content increases considerably and could reach more than 8000 mg GAE/100 g at higher power with an increase in ethanol concentration up to 50%. But TPC decreases progressively when the microwave power decreased (500 W) and ethanol concentration increases over 50%. Indeed, as shown in Table 2, the yield of TPC depends mainly on ethanol concentration as its linear and interaction effect (positive effect) with solvent-to-solid ratio were highly significant ($p < 0.01$). Figure 1 (D, E) describe the interactions between the irradiation power and each of the other two factors (irradiation time and solvent-to-solid ratio) on the TPC recovery. Figure 1D shows that the amount of TPC increased with increasing microwave power and irradiation time during the initial extraction followed by a decrease at mean values. The graphs suggested that the irradiation power has an interaction effect with the extraction time and linear effect ($p < 0.01$) on the TPC yield (Table 2). Our results are in agreement with those found by different authors who underlined that all factors influencing the output of phenolic compounds extraction from medicinal plant tissues [34, 35]. Figure 1E clearly shows that with a significant increase in the solvent-to-solid ratio from 30 to 47 mL/g, the extraction yields increased approximately to 7000 mg GAE/100 g with a slight decrease when power increased. This trend could be explained by the total absence of inertia effect between the extraction power and the solvent-to-solid ratio, but we can say that the yield of TPC from *Zlp* depends mainly on the solvent-to-solid ratio, since its quadratic and linear effects were very significant ($p < 0.01$). Figure 1F depicts the effect of the solvent- solid ratio with the irradiation time, the TPC content is maximized (6500 mg GAE/100 g) at a high solvent-to-solid ratio with an extraction time of less than 150 s, but it decreases considerably when the solid to liquid ratio begins to decrease.

Validation and verification of predictive model

The MAE optimal conditions obtained for TPC recoveries were: 51% ethanol, 600 W microwave power, 180 s irradiation time and 47 mL/g solvent-to-solid ratio. To ensure the equation of the model and the adequacy of the chosen experimental model compared to the predicted model, the optimal response values were tested at the selected optimal values. The experimental values of extraction yield for the TPC was very close to the predicted values. According to Zhang et al. [30] the adequacy of the regression response of the model compared to that of the experimental model is necessarily due to a very strong correlation between the actual results and the expected results to express the desired optimization, so we can say that the model has been validated.

Comparison between MAE, UAE and CSE

The efficiency of TPC yields using MAE was compared with other methods such as UAE and CSE (Table 3). The results indicate that MAE showed a significantly higher TPC extraction capacity (7473.38 ± 740.55 mg GAE/100 g) ($p < 0.05$) compared to UAE (6841.41 ± 4.61 mg GAE/100 g) and CSE (4818.72 ± 7.02 mg GAE/100 g). This may be due to the long extraction time and the amplitude of the UAE which had a negative influence on the recovery of the phenols. In the work of Al-Saeedi et al. [12] who reported a TPC of 64.89 ± 0.44 mg GAE/100 g from the fruit of Oman *Zizyphus jujuba* (*Zj*) which is significantly lower than our results using the methanol as extraction solvent. In addition, Spanish jujube fruit has a content of 1442 to 3432 mg GAE/100 g according to Wojdyło et al. [36]. Similarly, Cosmulescu et al. [37] have shown that the TPC content was between 475.3 and 1634.4 mg GAE/100 g from the fruit of *Zj* by a conventional extraction method, these quantities are lower than our results found from *Zlp*.

However, the TFC extracts obtained by MAE, UAE (1019.96 ± 75.03; 1047.46 ± 1.89 mg QE/100 g respectively) were significantly higher ($p < 0.05$) than those obtained by CSE (934.41 ± 6.05 mg QE/100 g). We suggest there, that the recovery of flavonoids was influenced negatively by longer extraction time. Our results are superior to those obtained by Al-Saeedi et al. [12] of Oman variety fruit of *Zj* 27.43 ± 0.18 mg QE/100 g using chloroform as extraction solvent, likewise for those obtained by Koley et al. [38] who reported TFC content from 8.36 to 21.97 mg QE/100 g from *Zj*. The flavonoid extract of *Zj* showed a significantly lower content than ours, ranging from 19.9 to 48.5 mg QE/100 g [37]. Moreover, the TTC extracts by MAE were very significantly higher ($p < 0.01$) ($14,253.11 \pm 2453.86$ mg CE/100 g) than those obtained by UAE and CSE respectively ($10,339.50 \pm 0.1$; 4629.68 ± 0.08 mg CE/100 g). Gao et al. [39] Compared proanthocyanidin extracts from several *Zizyphus* varieties, among those containing the highest content about 413.7 ± 23.1 mg/100 g DM from *Zj cv. Zaowangzao* which is significantly lower than our extracts obtained from *Zlp*.

It is more interesting to mention that all the factors studied have a significant influence on the jujube yield and therefore on the antioxidant activity which was improved by MAE with the evaluation of each parameter and the interaction effects that may exist. In which this work has combined the three responses studied to have similar optimal conditions which will allow future readers to deduce that the methodology used for MAE from *Zlp* extracts made it possible not only to improve the TPC

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Table 3 Comparison of extraction yield of bioactive compounds from *Zlp* by microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), and conventional solvent extraction (CSE)

	MAE	UAE	CSE
Extraction method/Factors			
Irradiation time (min)	3	25	120
Ethanol concentration (%)	51	50	50
Microwave power (w)	600	–	–
Temperature (°C)	–	63	60
Solvent solid/Ratio (mL/g)	47	67	50
Results of bioactive compounds			
Recovery of total phenolic TPC (mg GAE/100 g)	7473 ± 740^{a}	6841.41 ± 4.61^{b}	4818.72 ± 7.02^{c}
Recovery of total flavonoids TFC[a] (mg QE[b]/100 g)	1019.96 ± 75.03^{a}	1047.46 ± 1.89^{a}	934.41 ± 6.05^{b}
Recovery of condensed tannins TTC[c] (mg EC[d]/100 g)	$14{,}253.11 \pm 2453.86^{a}$	$10{,}339.50 \pm 0.1^{b}$	4629.68 ± 0.08^{c}
Results of biological activities			
PAOT Score/g	339 ± 0.30^{a}	156.5 ± 0.02^{b}	113 ± 0.01^{c}
DPPH scavenging EC_{50} (µg/mL)	0.24 ± 0.01^{a}	0.55 ± 0.03^{b}	1.05 ± 0.12^{c}
FRAP activity (mg $_{GAE}$/100 g)	3305.18 ± 9.75^{a}	1284.53 ± 10.99^{b}	1284.53 ± 10.99^{b}
AChE[i] assay % (10 mg/mL)	25.00 ± 3.81^{a}	19.66 ± 0.16^{b}	–
HepG2 cells IC_{50} (mg/mL)	$< 0.02 \pm 0.77^{a}$	$< 2.04 \pm 1.81^{b}$	–
MCF-7 cells IC_{50} (mg/mL)	$< 1.36 \pm 5.48^{a}$	$< 3.99 \pm 3.36^{b}$	–

Results are expressed as means standard deviation (±)

[a] *TFC* total Flavonoids content

[b] *QE* quercitin equivalent

[c] *TTC* total condensed Tannins content

[d] *CE* catechin equivalent

yields but also knowing that of flavonoids and tannins for their possible individual or collective use. In addition, the polyphenols are varied because of their capacity to react with each other and may be with other substances. Thus, there has been an hydrolysis or a polymerization of these molecules in smaller or larger having a strong antioxidant activity [40]. According to some authors Zhang et al. [30], MAE gives a very good yield in a minimum of irradiation time, herein its advantageous compared with other methods. Moreover, the increase of the interaction of the cells of the matrix leads to the transfer of the tissues so facilitates the interaction of electromagnetic fields. The energy transfer from the inside to the outside world will allow the promotion of the solubility of the solvent.

Identification of phenolic compounds from *Zlp* extract

In order to identify the bioactive compounds from our samples, LC–HRMS/MS was carried out, LC–HRMS in the negative and positive mode was performed and the compounds are indicated in Table 4. Compounds were tentatively identified under the analytical conditions by Data Analysis program from Bruker, and the chemical structures were suggested by pubchem, metlin and published reference on the same plant species or genus. In order to visualize the relationship between the compounds by knowing the intensities and antioxidant activities, the heatmap represented in Fig. 3 was used. This visual representation allows confirming which of compounds are more active in terms of antioxidant effect. Table 4 indicates the presence of 34 compounds based on their occurrence in plants from literature and by taking into account the exact mass and the fragmentation pattern and availability of reference standards and to the best of our knowledge have not been previously reported in *Zlp*.

Identification of secondary metabolites

The following three major peaks obtained at 1.96, 2.32 min detected with a deprotonated molecule at m/z 549.1590 and 169.0126 were assigned as caffeic derivative glycosylated and gallic acids. These compounds were analogous to those previously identified in *Zizyphus* species [41, 42].

Flavan-3-ols and proanthocyanidin were characterized in this study. Compounds observed at R_t of 1.21 and 1.74 min with m/z 525.1609 ($[M+H]^+$), 455.0950 ($[M-H]^-$) with different ion fragments were proposed to be barbatoflavan and epigallocatechin 3-*O*-vanillate,

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Table 4 Identification proposal of the phenolic compounds present in the *Zlp* by LC–MS/MS in ESI negative and positive mode

R_t (min)	Intensity	$[M—H]^-/[M+H]^+$	Fragments ion (m/z); intensity (%)	Error (mg/kg)	Molecular formula	Tentative identification	References
1.05^-	29,390	387.1089	341.1061 (42.9); 119.0321 (40.9); 101.0219 (23.2); 161.0427 (13.5); 149.0425 (11.2); 83.01 (3.2)	2.5	$C_{21}H_{16}N_4O_4$	Indole derivative	125554521 (pubchem)
1.11^-	17,000	179.0535	59.0105 (100); 71.0110 (90.1); 96.9570 (39.9)	14.6	$C_6H_{12}O_6$	Glucose	5793 (pubchem)
1.21^+	52,178	525.1609	354.1041 (100); 273.0782 (98); 245.0469 (37.7); 145.0473 (17.6);	− 1.2	$C_{24}H_{28}O_{13}$	Barbatoflavan	47366 (metlin)
1.49^-	7764	475.1235	133.0110 (100); 115.0009 (28.8); 71.0098 (19.9); 56.9939 (1.7)	2.3	$C_{23}H_{24}O_{11}$	Luteolin 5,3′-dimethyl ether 7-glucoside	49394 (metlin)
1.58^-	14,314	431.1347	89.0208 (100); 119.0311 (4.1); 341.1040 (8.1)	0.1	$C_{22}H_{24}O_9$	Isosakuranetin-7-*O*-rhamnoside	52823 (metlin)
1.74^-	9174	455.0950	112.9824 (100); 68.9919 (51.8)	7.4	$C_{23}H_{20}O_{10}$	Epigallocatechin 3-*O*-vanillate	47348 (metlin)
1.96^-	6618	549.1590	179.0529 (84.4); 383.1130 (31.8); 323.0925 (18.7); 161.0429 (19.4); 341.1037 (11.9)	19.8	$C_{26}H_{30}O_{13}$	Caffeic acid derivative glycosylated	985394 (metlin)
1.97^+	11,640	139.0486	121.0380 (100); 93.0434 (72.5)	19.4	$C_6H_6N_2O_2$	4-Methylpyrimidine-2-carboxylic acid	723920 (metlin)
1.98^-	36,208	191.0170	87.0054 (100); 85.0257 (47.9); 111.0066 (37.2); 57.0306 (17.7)	8.52	$C_6H_8O_7$	Citric acid	Berkani et al. [4]
2.01^-	2632	117.0158	73.0260 (100); 45.9963 (96.0); 68.9922 (94.7)	20.80	$C_4H_6O_4$	Succinic acid	Berkani et al. [4]
2.32^-	290	169.0126	NF	3.25	$C_7H_6O_5$	Gallic acid	Benabderrahim et al. [41] Zhao et al. [42]
3.74^+	4362	195.1203	45.0326 (100); 109.9576 (24.9); 135.0422 (13.1); 107.0483 (9.4)	19	$C_8H_{12}N_4O$	Pirimidine derivative	926880 (metlin)
5.67^+	148,518	393.2040	NF	5.17	$C_{25}H_{28}O_4$	5-Hydroxy-7-*O*-nerylflavanone	52674 (metlin)
6.11^+	5386	465.1873	407.1842 (16.4)	5.1	$C_{18}H_{24}N_8O_7$	Tetrapeptide (Asp-Gly-His-His)	121608 (metlin)
6.12^+	9780	476.3004	45.0328 (100); 133.0834 (26.5)	3.8	$C_{21}H_{41}N_5O_5S$	Tetrapeptide (Leu-Cys-Lys-Leu)	176048 (metlin)
6.43^+	60,668	407.2194	254.9096 (0.3); 365.1489 (0.3); 372.3353 (0.3)	6	$C_{26}H_{30}O_4$	5-Methoxy-7-prenyloxy-8-C-prenylflavanone	52663 (metlin)
6.50^+	13,542	611.1521	303.0459 (100); 304.0494 (20.4); 465.0962 (11.8)	14.0	$C_{27}H_{30}O_{16}$	Quercetin-3-*O*-robinobioside	Wojdylo et al. [36]

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Table 4 (continued)

R_t (min)	Intensity	$[M—H]^-/[M+H]^+$	Fragments ion (m/z); intensity (%)	Error (mg/kg)	Molecular formula	Tentative identification	References
6.57^+	11,738	301.1146	106.0636 (100); 132.0428 (50.4); 225.0994 (61.0); 197.1041 (35.5)	− 1.1	$C_{11}H_{16}N_4O_6$	5-Oxoprolylaspar-aginyl glycine	487255 (metlin)
6.59^+	5296	467.2203	331.0835 (1.2); 290.5074 (0.9)	− 4.6	$C_{20}H_{30}N_6O_7$	Tetrapeptide (Val-Pro-His-Asp)	244401 (metlin)
6.84^+	6326	619.3095	103.0375 (100); 191.0891 (30.7); 235.1127 (18.2)	16.6	$C_{28}H_{42}N_8O_8$	Pentapetide (Pro-Tyr-Pro-Arg-Ser)	264985 (metlin)
7.25^+	7332	417.2332	45.0328 (100); 89.0586 (42.4); 133.0838 (25.3); 177.1098 (12.9); 144.0754 (12.6)	5.4	$C_{17}H_{30}N_4O_7$	Tetrapeptide (Leu-Val-Asp-Ala)	182319 (metlin)
7.33^+	11,710	317.1460	106.0638 (100); 132.0424 (18.2)	− 5.6	$C_{12}H_{20}N_4O_6$	Tripeptide (Ser-Pro-Asn)	19282 (metlin)
8.06^+	3365	301.1136	106.0638 (100)	− 2.2	$C_{10}H_{20}O_{10}$	Dioside	70700334 (pubchem)
8.41^+	13,106	505.2559	419.2203 (2.3); 113.0570 (2.3); 124.0784 (1.5)	− 3	$C_{19}H_{36}N_8O_6S$	Tetrapeptide (Ala-Met-Gln-Arg)	107754 (metlin)
8.48^+	4558	433.1185	61.0274 (100);132.0427 (50.3); 208.0571 (18.6); 269.0881 (17); 311.0968 (13.1); 327.0893 (12.2)	− 12.87	$C_{21}H_{20}O_{10}$	Apigenin-7-*O*-glucoside	Khan et al. [49]
9.54^+	25,954	225.0990	132.0428 (100); 104.0485 (17.7)	− 4.5	$C_8H_{16}O_7$	Glycoside	102183608 (pubchem)
10.15^+	8300	255.1559	209.1506 (100); 123.1152 (47.8); 191.1413 (44.7); 109.0998 (28.9); 167.1052 (22.8)	2	$C_{14}H_{22}O_4$	9-(3-Hydroxy-5-oxocyclopent-1-en-1-yl)nonanoic acid	689561 (metlin)
10.91^+	4476	301.0728	182.0871 (57.3)	− 7.09	$C_{16}H_{12}O_6$	6-Methylluteolin	49158 (metlin)
10.99^+	3366	255.0957	181.0607 (26.5); 204.9606 (23.3)	2.1	$C_{11}H_{14}N_2O_5$	Glycine derivative	500366 (metlin)
11.17^+	7960	255.1568	209.1511 (100); 123.1152 (36.5); 191.1404 (27.9); 109.1010 (17.9); 167.1043 (21.3)	− 1.6	$C_{10}H_{18}N_6O_2$	Glycine derivative	347687 (metlin)
14.99^+	6378	291.2494	45.0327 (100); 161.0930 (15.9); 151.0927 (10)	31.7	$C_{13}H_{10}N_4O_3$	Guanidine derivative	104888008 (pubchem)
15.10^-	11,328	353.1957	96.9565 (61.5); 122.9722 (2.9)	2.0	$C_{19}H_{32}O_7$	Blumenol C-glucoside	95146 (metlin)
16.50^+	49,376	595.4105	309.1994 (100); 310.2025 (21.6); 221.1492 (1.1)	− 6.6	$C_{30}H_{58}O_{11}$	Stearyllisomaltse	9894835 (pubchem)
16.53^+	98,018	287.2188	69.0689 (100); 111.1153 (54.1); 199.1664 (4.3)	13.9	$C_{16}H_{30}O_4$	3,6-Dihydroxy-4-ethylidene-6-ethyldecanoic acid ethyl ester	10803152 (pubchem)

Peaks with a minus/plus superscript were analyzed in negative/positive mode. Compounds are indicated by retention time (R_t) order and numbered according to the appearance in each chromatogram, negative $[M-H]^-$ or positive mode $[M+H]^+$

NF not fragmented

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respectively [43, 44]. However, compounds with retention times of 5.67, 6.43 min showed a MS^2 fragmentation characteristic to flavanones with m/z of 393.2040, 407.2194 at positive mode were tentatively identified as 5-hydroxy-7-*O*-nerylflavanone and 5-methoxy-7-prenyloxy-8-C-prenylflavanone, respectively. In addition, a flavonoid with methoxy groups was at $R_t = 10.91$ with a [M + H] + m/z of 301.0728 which was assigned as 6-methylluteolin [45]. The flavonoid aglycones were in accordance to those identified in previous reports from other plant materials that showed a significant antioxidant properties [46]. To the best of author's knowledge, these compounds are reported from *Zizyphus* genus for the first time.

Four compounds were characterized as flavanones glycosides in this study. Peaks observed at 1.49, 1.58 min, with $[M\text{-}H]^-$ ion at m/z of 475.1235 and 431.1347, were tentatively identified as luteolin 5,3′-dimethyl ether-7-glucoside and isosakuranetin-7-O-rhamnoside, respectively [47, 48]. Our study is the first detecting the presence of these compounds from *Zizyphus* genus. However, two known flavones, including quercetin-3-*O*-robinobioside ($R_t = 6.50$ min), apigenin-7-*O*-glucoside ($R_t = 8.48$ min) were found with positive mode in the samples. These compounds were in accordance with previous funding from jujube samples [15, 49].

Figure 3 shows clearly that both glycoside and aglycone flavonoids are 44.51% of all phytochemicals and 80.75% of all secondary metabolites detected in our samples. 5-hydroxy-7-*O*-nerylflavanone is found to be the most abundant flavonoid from our samples. We can say that the antioxidant activities of *Z. lotus* extracts will be mainly due to flavonoids group by knowing the intensities of each compound present in extracts as we mentioned them at different color in heatmap. Alkaloid group was characterized in this study and observed at 1.05 min with m/z of 387.1089. This compound was assigned as indole derivative. This last is detected also from other plants and it is known as one of antimalarial, antioxidant and anti-inflammatory agents [50]. In addition, peak observed at $R_t = 15.10$ min with a $[M\text{-}H]^-$ ion at m/z 353.1957 was attributed to blumenol C-glucoside. This compound showed a good pharmacological effect in several plant material like major class of terpenes, thus it is demonstrated by Amat-ur-Rasool et al. [51] from Indian *Bombax ceiba* that is a good antioxidant. Others suggested that it is one of inhibitors on pro-inflammatory cytokines [52].

Identification of primary metabolites

Peaks observed at 1.11, 8.06, 9.54 and 16.50 min with positive and negative modes at m/z of 179.0535, 301.1136, 225.0990 and 595.4105 were attributed to glucose, dioside glycoside and stearyllisomaltse, respectively. These compounds are not present with high intensities in our sample and some of them are in accordance to other data detecting sugars from *Zizyphus mauritana* fruits [53].

Recently, several studies have given a considerable interest to the bioactive peptides derived from plant materials which have a large biological effect on human health. Otherwise, different parts of jujubes such as fruits, roots, seeds and leaves have been used against the treatment of several diseases [54, 55]. However, several small peptides are detected in *Zlp* extracts. Peaks observed at R_t of 3.74, 14.99 min with m/z of 195.1203, 291.2494 were tentatively assigned as pyrimidine and guanidine derivative. In addition, three glycine derivatives were tentatively identified at 6.57, 10.99 and 11.17 in positive mode with m/z of 301.1146, 255.0957 and 255.1568, respectively. Furthermore, five different tetrapeptides were identified tentatively in positive mode at different retention times 6.11, 6.12, 6.59, 7.25 and 8.41 min. These compounds were assigned with different molecular formula $C_{18}H_{24}N_8O_7$, $C_{21}H_{41}N_5O_5S$, $C_{20}H_{30}N_6O_7$, $C_{17}H_{30}N_4O_7$ and $C_{19}H_{36}N_8O_6S$, respectively. However, one pentapeptide was detected at 6.84 min with m/z of 619.3095, and one other tripeptide was observed at 7.33 min with m/z of 317.1460. These compounds were in accordance to those identified in previous reports from *Zizyphus* species and others data isolated bioactive compounds from plants [56, 57]. Figure 3 showed the presence of 14.04% peptides from total compounds present in our *Zlp* extracts and we mentioned 31.30% from total primary metabolites. Several studies have been characterized the antioxidant potential of peptides from several plants as demonstrated from *Zj* fruits [58]. Thus, *Zlp* have a non-negligible amount of peptides which may participate in the studied antioxidant activities.

The examination of chromatograms obtained at negative and positive modes indicated the presence of some organic acids at 1.97, 1.98, 2.01, 10.15 and 16.53 min with m/z of 139.0486, 191.0170, 117.0158, 255.1559 and 287.2188, respectively were assigned tentatively as 4-methylpyrimidine-2-carboxylic, citric, succinic, 9-(3-hydroxy-5-oxocyclopent-1-en-1-yl)-nonionic and 3,6-dihydroxy-4-ethylidene-6-ethyldecanoic ethyl ester acids. In addition to other substances, these acids have found to exert antioxidant and anti-Alzheimer effects from several plants [59, 60].

Antioxidant activities

In order to evaluate the antioxidant activities of *Zlp* extract obtained by the three methods studied, several tests were used such as PAOT, $DPPH^{\cdot}$ and FRAP assays (Table 3).

The greater antioxidant capacity of the phenolic extracts was attributed to a significantly lower EC_{50} ($p < 0.01$). The MAE extract showed the higher antioxidant (0.24 ± 0.01 µg/mL) than UAE extract (0.55 ± 0.03 µg/mL) and CSE (1.05 ± 0.12 µg/mL). Our results are in the same range to our previous works on *Zl* seeds by UAE (0.39 µg/mL) [3] and

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lower antioxidant capacity than that of MAE obtained from jujube seeds (0.067 µg/mL) [4]. These results are in accordance with Ghazghazi et al. [61] and works who have used MAE for *Zj* from Tunisia and Oman. Similarly our results obtained by UAE and CSE are greater than those of Hammi et al. [23] reported an EC_{50} of 0.2895 mg/ml for Tunisian variety extract but close to those of MAE extract. Moreover, the iron-reducing power of the *Zlp* extracts revealed a highly significant effect by the MAE extract (3305.18 ± 9.75 mg GAE/100 g), compared with the UAE and CSE extracts (1284.53 ± 10.99 and 672.86 ± 4.87 mg GAE/100 g, respectively). Our results are much higher than those of Wang et al. [62] (471.6 ± 30.8 mg CE/100 g DM) obtained from *Zj cv. Zaowangzao*. In the meantime, the PAOT technology evaluating the antioxidant effect which is used for the first time in jujube plant was well marked by the extracts from MAE (339 ± 0.30 PAOT Score/g) than the other two methods (Table 3). Thus, we confirmed the highest correlation between phenolic compounds and antioxidant effect where MAE showed the highest level. Our results are in accordance to [63] demonstrating the higher correlation between bioactive compounds and both PAOT and DPPH activities in wine samples. In addition to the remarkably antioxidant compounds present in *Zlp* which are mainly responsible for antioxidant effect (Pearson test n = 0.94) in comparison to some standard antioxidant showed in that work such as trolox, myrecitin and epicatechin (544.16 ± 16.81, 677.78 ± 7.41 and 730.2 ± 93.73 mg PAOT/L, respectively). As well as, by comparing to other source of antioxidants such as Arkovital® Pur'Energie and synthetic supplement showing a PAOT Score of 0.034 ± 0.0009 and 0.045 ± 0.0037 mg GAE, respectively [64]. These results are very lower to that found in jujube extracts by comparing to equivalent of gallic acid where the highest PAOT Score was obtained by MAE (0.012 ± 0.0004 GAE/g) followed by UAE and CSE (0.004 ± 0.0000 and 0.002 ± 0.0004 GAE/g of extract, respectively). This means that MAE method is more efficient for the recovery of phenolic compounds than the herein tested UAE and CSE methods. The present study confirmed the results obtained by the other antioxidant activities that microwave extract presented a significant and high antioxidant and chelating activities than UAE and CSE extracts.

The antioxidant capacity by PAOT, DPPH scavenging and FRAP assays of the extracts was evaluated by PCA analysis in order to demonstrate the relationship between these last and phenolic antioxidants. PCA was applied to the jujube extracts (MAE, UAE and CSE) for phenolic compound (TPC, TFC and TTC) and antioxidant activity, where the two chosen factors justified 100% of total variance. The resulting plots allowed selecting the better extraction method of *Zlp* antioxidant compounds, and clearly divided the samples into three groups, depending on the extraction method as shown in Fig. 2. The first group which explains 90.18% of the total variance showed a negative correlation with PC1,

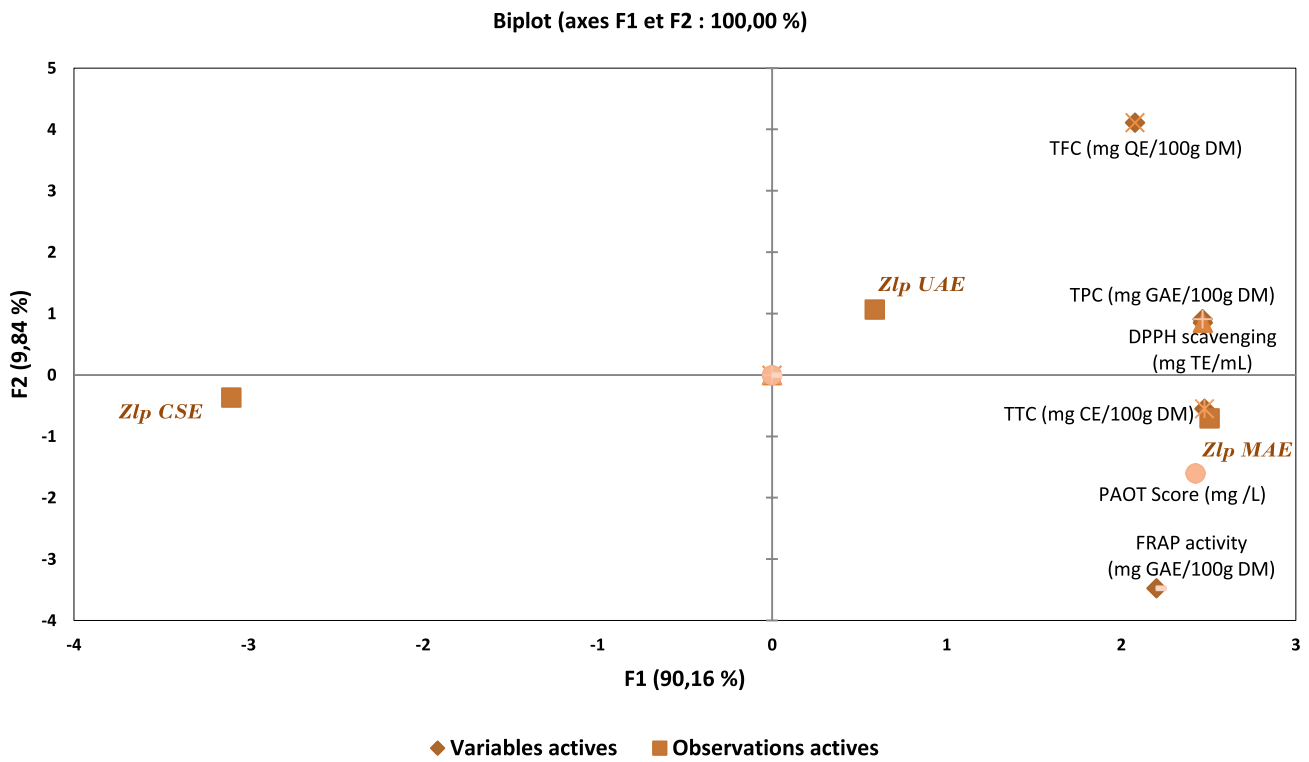


Fig. 2 Principal component analysis of TPC for *Zlp* with MAE, UAE and CSE

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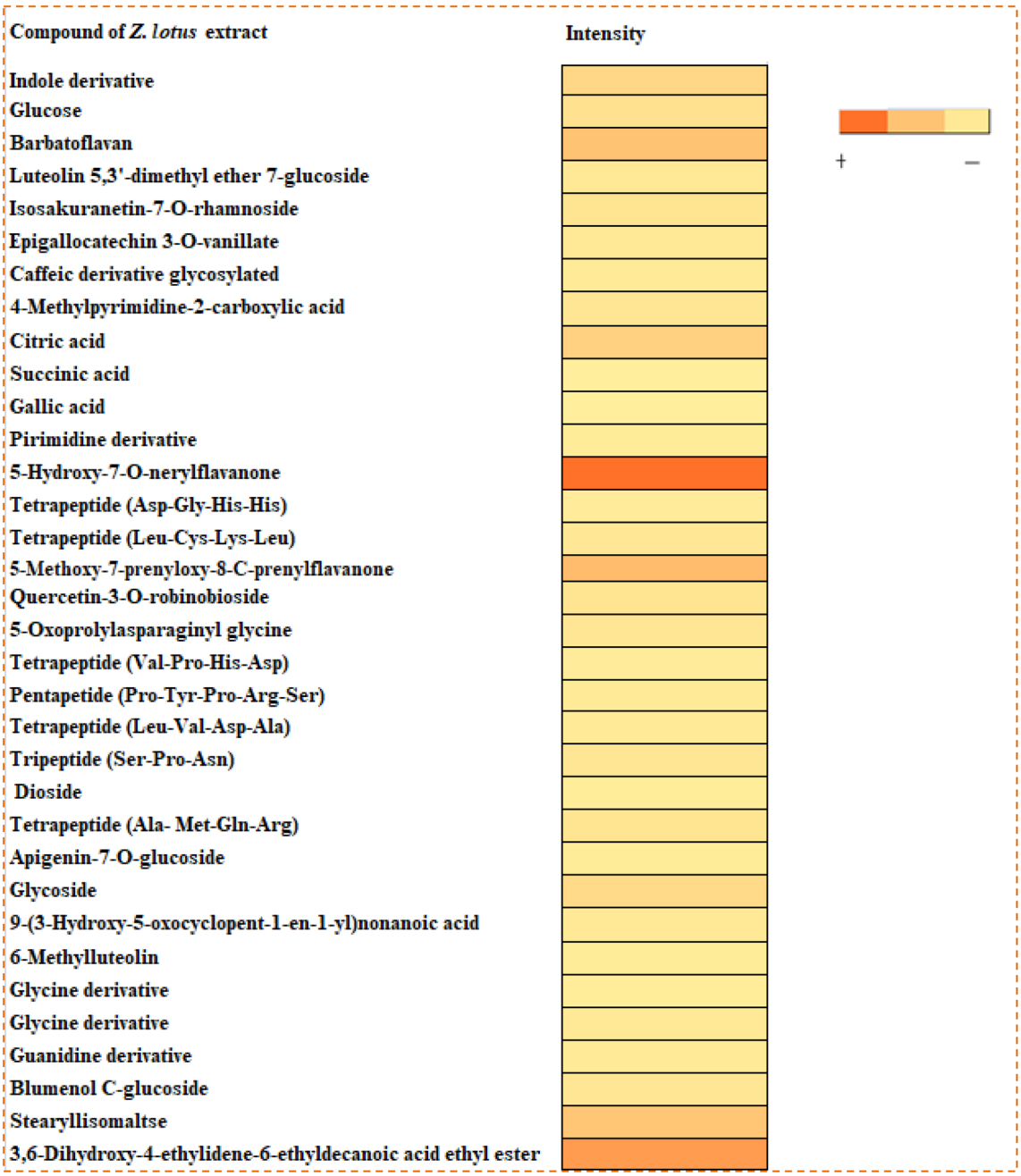


Fig. 3 Heatmap of the chemical profile and of *Zlp* extracts. Mean values refer to colors from minimum displayed in light orange to maximum represented with dark orange (Color figure online)

thus confirming that MAE was the best extraction method for phenolic compounds with potent antioxidant activity. The highest correlation was found between antioxidant activity (PAOT, FRAP) and TTC, as well as between DPPH and TPC. Thus, our results suggest that phenolic compounds might have a good influence on the antioxidant capacity of the *Zlp*. In addition to UAE which showed a positive correlation with PC2. The best correlation between the UAE and both TPC and TFC were observed in this group with a remarkably higher correlation with DPPH assay. While, the third group (PC3) explains only better 9.84% of the experimental variability, which could essentially be associated to the CSE method and TFC.

The mechanism responsible may be due to the bioactive compounds present in jujube extract, such as phenolic and organic acids, flavonoids aglycones and glycosides, peptides and some of sugars as we have seen by LC–MS/MS analysis. Figure 3 demonstrated that most of these phytochemicals

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with high intensities are polyphenols (55%) known which could provide hydrogen and electron to the radicals [65]. Their activities could be attributed to the presence of a high number of hydroxyl groups which are present in their aromatic rings [66]. As well as, the increasing of these activities due to the content of other compounds is associated to the strong reducing of the oxidized state which causes an increase in antioxidant capacity. Nevertheless, *Zlp* extract showed another combination of phenolic compounds with other primary metabolites, mainly peptides and derivative that are complexing together in plant extracts and increase the antioxidant effect [58]. Furthermore, from a heatmap we clearly notice that combination between glycoside and aglycone flavonoids (80.75%) are the most determinant of the antioxidant activity in jujube extracts where 5-hydroxy-7-*O*-nerylflavanone was the most abundant. It should be emphasized that the proposed approach is limited for the identification of all the compounds contained in this extract mentioned for the first time from pulp and peel. This will open up to other more innovative studies requiring the purification of this extract and the isolation of this majority compound in order to benefit more from their use as a nutraceutical agent.

Anticholinesterase activity

The AChE inhibition assay of *Zlp* for both MAE and UAE is represented in Table 3. MAE exhibited a significant effect on AChE inhibitory activity ($25.00 \pm 3.81\%$) when compared to UAE ($19.66 \pm 0.16\%$), this was in accordance with the antioxidant activities showing the higher positive effect by MAE than other techniques. These results are in agreement with our previous works on jujube seeds demonstrating a lowest IC_{50} value by MAE than by UAE (0.88 ± 0.02 and 0.93 ± 0.01 mg/mL, respectively) where we have seen an AChE inhibition of 50% in seeds which is twice lower than that of pulps, while a same tendency has been observed on the method of choice (MAE > UAE) [3, 4]. In comparison to data in literature, these results are mainly due to the concentrations of phenolic compounds present in jujube extracts which gave a proportional AChE inhibition of many plant matrix [67–69]. These results suggested that *Zlp* is a good AChE inhibitor and could be exploited against treatment of several desease mainly Alzheimer's disease and alleviation of severe constipation [4].

Antioproliferative activity

The antiproliferative activity of *Zlp* extracts was evaluated by measuring the cytotoxic effect on $HepG_2$ (human hepatocellular liver carcinoma cell) and MCF-7 (breast cell) lines as represented in Table 3. The recovery of jujube phenolic extracts that killed 50% of the cells (IC_{50}) was determined from dose–response curves. Similar observations than antioxidant assay were observed for this test. The antiproliferative effect on both $HepG_2$ and MCF-7 cell lines as observed to be maximum in extract obtained by MAE with lower IC_{50} value ($< 0.02 \pm 0.77$ and $< 1.36 \pm 5.48$ mg/mL, respectively) in comparison to that noticed by UAE extracts ($< 2.04 \pm 1.81$ and $< 3.99 \pm 3.36$ mg/mL, respectively), while demonstrating that our extracts exhibited minimum toxicity. A value of 0.1 mg/mL is the limit of toxicity to human cell lines [70]. Thus, we can notice that the jujube extracts excerce a significant higher effect against both MCF-7 and $HepG_2$ cells. Our results are in accordance to that obtained in our previous work on jujube seeds [3], which are mainly due to the nature of bioactive compounds present in jujube extracts including mainly flavan-3-ols such as monomer [as (−)-epicatechin, gallocatechin gallate, (+)-catechin] and other procyanidins, the extraction methods [3, 15]. Such finding demonstrated that *Zlp* is one of the most active antitumor agents that caused the severe cytotoxicity in the studied lines.

Conclusion

In this study, the microwave extraction process was applied according to a RSM modeling optimization to predict and estimate the behavior of some bioactive compounds namely total polyphenols, flavonoids and condensed tannins extracted from *Zlp,* as well as, the parameters influencing the biological activities (including $DPPH^{\cdot}$, FRAP, PAOT, AChE, MCF-7 and HPG_2 testes).

- The mathematical models generated by the RSM design proved to be appropriate for predicting the responses chosen in each experimental study. The optimal conditions obtained from optimization study were microwave power at 600 W, irradiation time of 180 s and 47 mL/g solvent-to-solid ratio and 51% ethanol (v/v in water). These are very important factors which afect both the yield and biological activities of jujube extracts.
- The comparison between the three extraction methods revealed the efficiency of MAE technique in terms of TPC, TFC and TTC than UAE and CSE ones. Under these conditions, MAE extract presented high content for TPC (7473.38 ± 740.55 mg GAE/100 g) with an extraction of 10 times lower approximately than that of UAE and CSE.
- Indeed, MAE showed the highest correlation values between antioxidant capacity and phenolic compounds, as demonstrated by $DPPH^{\cdot}$, FRAP and PAOT tests. This method presented a highest AChE effect and antiproliferative effects against MCF-7 and HPG_2 on jujube extracts.

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- LC–MS/MS analysis identified 34 phytochemicals including primary and secondary metabolites. To the best of our knowledge, 10 phenolic compounds have not been reported before in *Zizyphus* species. Moreover, the *Zlp* are rich in phytochemicals that are responsible for high antioxidant activity.
- From an industrial point of view, the phenolic extracts obtained by MAE can be used by exploiting their biological properties (food additives, nutraceuticals, etc.) in order to replace the conventional extraction methods as CSE.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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