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Article

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 properties. 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 $15,787.10 \pm 0.10$ 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, antiacetylcholinesterase, 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 ($IC_{50} = 0.93 \pm 0.01$ mg/mL) and HMGR inhibition (45.41%) using 100 µg/mL. The extract significantly inhibits cell proliferation on the MCF-7 and HepG₂ 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 ($19.11 \pm 0.03\%$), lipids ($32.92 \pm 0.29\%$), and sugars ($40.87 \pm 0.39\%$) 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 (C₆N₆FeK₃), ferric chloride (FeCl₂·6H₂O), trichloroacetic acid, and sodium dihydrogen phosphate (NaH₂PO₄) 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 X₁- Solvent concentration (% v/v), X₂- Sonication temperature (°C), X₃- Sonication time (min), and X₄- 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).

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

where y represents the predicted response which is TPC yield; β_0 is a constant coefficient; β_i , β_{ii} , β_{ij} are regression coefficients for linear, quadratic, and interactive terms, respectively; and X_i and X_j 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).

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).

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 \pm standard deviation from triplicate experiments.

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)):

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

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 distilled 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 $^{\circ}\text{C}$. 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 Shimadzu UV-160A, Kyoto, Japan. A control reaction was carried out using the same procedure but with adding 100 μL of distilled water instead of 100 μL of *Zls* extract at different concentrations. All tests were done in triplicate and the percentage inhibition was calculated as

$$I (\%) = 100 - (V_{\text{sample}} / V_{\text{control}}) \times 100 \quad (3)$$

where I is the percent inhibition of acetylcholinesterase, V_{samples} the initial velocity of the extract containing reaction, and V_{controls} 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 was analyzed 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 HepG₂ and MCF-7 Cells

The cytotoxicity of *Zls* extracts were performed using 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 cultured in DMEM supplemented with 10% FBS, 100 U/mL Pen-Strep, and 2 mM L-glutamine at 37 $^{\circ}\text{C}$ 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)):

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

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:

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

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 $X_2 X_3$, $X_2 X_4$, $X_3 X_4$ 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.

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 *

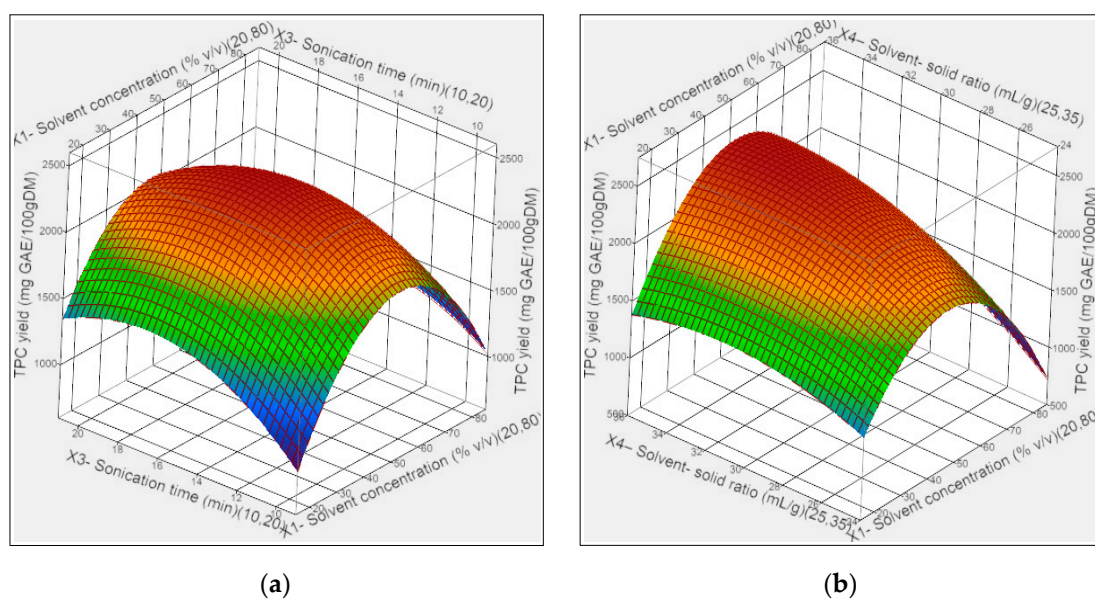
X_4 —Ratio	209.31142	21.14238		525,735.3	98.0118	<0.0001 *
Quadratic						
X_1^2	-664.7757	31.71357	1	2,356,942.5	439.4001	<0.0001 *
X_2^2	-157.6356	31.71357	1	132,528.0	24.7069	0.0003 *
X_3^2	-243.7076	31.71357	1	316,764.8	59.0538	<0.0001 *
X_4^2	-129.108	31.71357		88,900.6	16.5736	0.0016 *
Interaction						
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		

^aCoefficients refer to the general model; ^bDegree of freedom; * significance.

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.



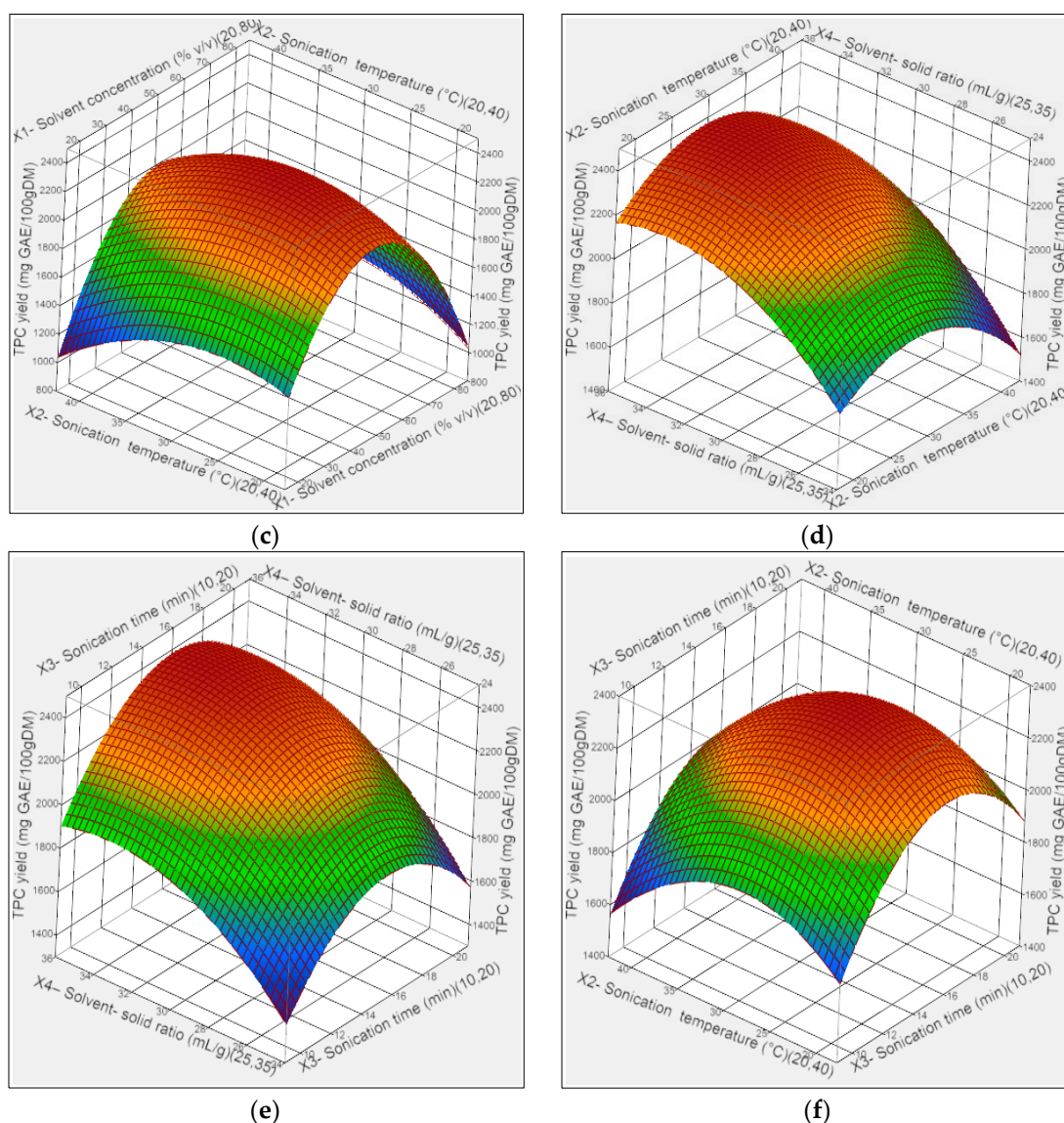


Figure 1. Response surface analysis for the total phenolic yield from *Z.lotus* seeds using UAE with respect to sonication time and ethanol concentration (a); solvent-to-solid ratio and ethanol concentration (b); sonication temperature and ethanol concentration (c); solvent-to-solid ratio and sonication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

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 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 significantly the TPC recovery due to their thermal degradation which confirmed the results of Table 2.

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).

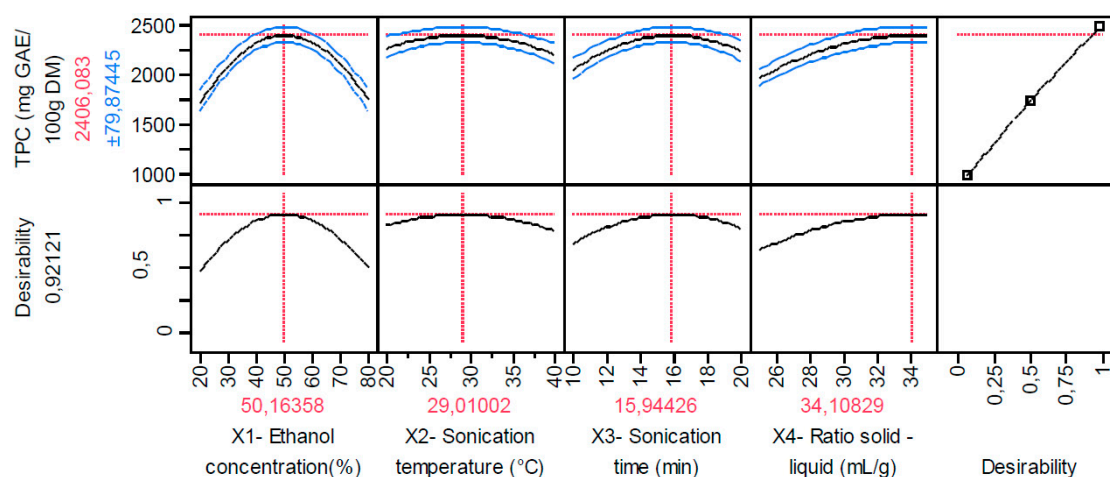


Figure 2. Prediction Profiler of *Z.lotus* seeds extract using UAE.

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, Esmaelian, 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].

Table 3. Biological activities of *Z. lotus* seeds extract using UAE. Results are expressed as means \pm standard deviation.

Factors	Ultrasound Extraction
Sonication time (min)	15.94
Ethanol concentration (%)	50.16
Sonication temperature ($^{\circ}$ C)	29.01
Solvent solid/ratio (mL/g)	34.10
Results	
Recovery of TPC (mg GAE/100 g)	2383.10 \pm 0.87
Recovery of TFC (mg QE/100 g)	486.50 \pm 0.38
Recovery of TTC (mg CE/100 g)	15,787.10 \pm 0.10
DPPH scavenging EC ₅₀ (μ g/mL)	0.39 \pm 0.00
FRAP (mg GAE/100 g)	1670.42 \pm 6.50
AChE assay IC ₅₀ (mg/mL)	0.93 \pm 0.01
HMGR assay (%) (for 100 μ g/mL)	45.41
HepG2 cells IC ₅₀ (mg/mL)	3 \pm 0.50
MCF-7 cells IC ₅₀ (mg/mL)	<0.05 \pm 0.0

Regarding to the TTC yields, the *Zls* extract was for 15,787.10 \pm 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.

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 EC₅₀ value (0.39 μ g/mL) than that of Tunisian *Z. lotus* leaves and fruits extract using methanol (0.10 \pm 0.001 and 0.31 \pm 0.005 mg/mL)[48], Tunisian *Z. lotus* pulp and peel extracts using UAE with an EC₅₀ of 0.28 mg/mL [19], from *Z. mucronata* roots (0.029 \pm 0.05 mg/mL)[49], and from Korean *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 \pm 6.5 mg/100 g) and was found to be higher than those obtained with 471.6 \pm 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 μ g/mL which is in well agreement than our AChE inhibitory from *Zls*. In addition, the standard galantamine showed an IC_{50} of 0.14 μ g/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 μ g/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 μ g/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 HepG₂ 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 IC₅₀ 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 HepG₂ 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 IC₅₀ 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 HepG₂ cells, respectively. Thus, *Zls* extract exhibited no significant activity against HepG₂ 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 HepG₂ and MCF-7 cells [21,74].

In addition, *Z. jujube* fruits extract was found to exhibit activity against MCF-7 cells with an IC₅₀ value of 1.8 mg/mL after 24 h which is higher value than that of *Zls* extract (IC₅₀ less than 0.05 mg/mL after 24 h) [75]. The results of cytotoxic action of *Z. jujuba* extract indicated a reduction in viability and high potent inhibitory effect toward the proliferation of MCF-7 cells, this effect may be due to cell apoptosis [76]. However, the cytotoxicity effect reported from *Zls* extracts against HepG₂ cells was significantly higher than that reported from mung bean sprouts extracts obtained using maceration method with an IC₅₀ of 14.04 ± 1.5 mg/mL. This extract also contained polyphenolic compounds [77]. The effective cytotoxicity toward hepatocellular HepG₂ and breast 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, gallic acid, and (+)-catechin), dimer (procyanidine B2), and polymeric proanthocyanidins in *Zls*, which were applied 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 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 HepG₂ and breast 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 HepG₂ 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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