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Présentée par
ABBOU Amina

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Recherche de nouvelles molécules d'origine végétale à intérêt industriel et thérapeutique

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ABBOU Amina

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Communications

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Abbreviation list

AE: aceton extract

APTT: activated partial thromboplastin time

EAI: emulsifying activity index

EE: ethanol extract

FA: foaming activity

HC: heat coagulability

MGC: minimum gelling capacity

OHC: oil holding capacity

PE: propanol extract

PHPC: *Pinus halepensis* seeds protein concentrate

PS: protein solubility

PT: prothrombin time

SH: surface hydrophobicity

WHC: water holding capacity

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General introduction

General introduction

Plants have always been alienated from man, first to feed himself and also to relieve his ills. In Africa and around the world, more than half of the population is using traditional medicine and herbal remedies for its primary health care because of the high cost of drugs and their unavailability on the market, as well as the disappointment with the prescribed drugs. In the 19th century, phytotherapy tooks a decisive turn with the birth of extractive chemistry which gave this discipline a much stronger scientific character. In the same time, the birth and rapid development of synthetic chemistry have competed with the natural medicines **(Barros, Ferreira, Queiros, Ferreira, & Baptista, 2007).** Subsequently, as several synthetic additives have been limited in several countries, due to their long-term adverse toxicological effects, including carcinogenicity **(Council, 1996)**. The current trend among consumers to look for a more natural food has prompted industries to research, develop and apply new natural herbal products. This, either to improve the product through their functional properties or for preservation through their antioxidant and antimicrobial properties and incorporate them into their products as alternatives to synthetic preservatives **(Burt, 2004).**

The Mediterranean region possesses a rich and little valued flora, even though it constitutes the majority of energy resources. The Aleppo pine (*Pinus halepensis* Mill.) is certainly the main resinous species in the Mediterranean region; it constitutes very extensive stands. In Algeria, the Aleppo pine covers an area of 850,000 ha and extends essentially in the northern part of the country **(Kadik, 1987**). It has been an integral part of the Mediterranean landscape for at least 3 million years. The Aleppo pine's range is explained by its great resistance to drought and its great sensitivity to low temperatures. This area covers 3.5 million hectares **(Quézel, 2000a)**. In view of the size of the area occupied by this tree and the often underestimated potential it represents for the wood industry, studies to improve knowledge of this species and promote its management are numerous and essential. Especially, since these trees produce oil seeds. These seeds are the lightest among the Mediterranean pines because it is estimated that one kilogram of seeds contains 45 000 and 65,000 kilograms of very good quality from a nutritional and organoleptic point of view, traditionally used by our ancestors for food flavoring, cooking and medicinal virtues thanks to their ease of use, effectiveness and undeniable advantages **(Chambel, Climent, Pichot, & Ducci, 2013**). They have been well known since ancient times and widely used in traditional medicine to treat many diseases. They mainly contain a fixed oil, proteins, sugars and polyphenols **(El Omari, et al., 2020).**

Unfortunately their traditional uses are increasingly marginalized and to date no industry has valued them in either the food or pharmaceutical industry (except in Tunisia where they produce a dessert cream based on its seeds) and these seeds are not studied enough, the little work that has been done in this direction has focused on their lipid fraction and volatile compounds.

Because of the great availability of this well supplied tree of these oil seeds which seem very interesting and the lack of valorization and use despite their remarkable taste, it seemed very important to us to find ways of valorization in the pharmaceutical industry (by using its polysaccharide fraction which has never been solicited) and in the food industry (by using the protein fraction for their functional properties and by designing a vegetable milk based on these seeds) to confirm the functionality of this food.

The subject of this thesis is therefore part of the search for new ways to valorize these seeds, the manuscript is divided into 4 chapters as follows:

- Chapter 1:

- ➢ Bibliographical synthesis on the seeds of Aleppo pine;
- ➢ The possible ways of valorization of the seeds of Aleppo pine (we explained why we chose to valorize their polysaccharide and protein fraction and why this matrix was very interesting for the production of vegetable milk).

- Chapter 2:

Study of the effect of precipitation solvent on the antioxidant, in vitro anti-inflammatory and anticoagulant activity of Aleppo pine seed polysaccharides.

- Chapter 3:

Optimization of functional properties (solubility, foaming and emulsifying activity) as a function of pH, NaCl and phosphate buffer.

- Chapter 4:

Study of the manufacturing process of a vegetable milk based on Aleppo pine seeds, where we studied the effect of each unit operation on the chemical and physical quality of the product.

Chapter I:

Literature review

Chapter I: Literature review

I. Pinus halepensis Mill. seeds

I.1 The genus Pinus:

The genus *Pinus* (Pine) belongs to the Pinaceae family, native and widespread on the European, African, Asian and American continents **(Djerrad, Kadik, & Djouahri, 2015)**. It is divided into three subgenera which are *Pinus, Ducampopinus* and *Cembrapinus*. These subgenera are divided into sections **(GUIT, NEDJIMI, GUIBAL, & CHAKALI, 2015)** and includes more than 110 plant species. It is the largest genus of this family, to which it is named. Pines are conifers and softwoods (they secrete resin). They are evergreen trees (or shrubs in their dwarf forms) of varying sizes. Their bark, when old, is cracked or forms more or less scaly patches. The buds are large, often resinous, with many scales. Their leaves are flattened green needles with a serrated outline. Depending on the species, they are grouped by 2, 3 or 5. Pines are monoecious trees: the male kittens are on long branches near the base of the year's growth, cylindrical, numerous, yellow or red in colour; the female kittens are lateral or terminal. The female cones evolve in 2 to 3 years to become the fruits of the plant: in the first year they are pollinated but not fertilized and grow in the second year. Once mature, the female cones may release their seeds immediately or after several years **(Farjon, 2005)**. Pinaceae are coniferous trees from 2 to 100 m high, their leaves are green needles, prickly or not, more or less long, attached alone to the twigs, or joined in pairs or in rosettes **(Feikh, 2014)**.

I.2 *Pinus halepensis* Mill

Pinus halepensis Mill (Aleppo pine) is the scientific name given by Philip Miller in 1768 to the Aleppo pine **(Nahal, 1962)**. It is a long evergreen perennial tree that reaches heights up to 20 m **(Mauri, Di Leo, De Rigo, & Caudullo, 2016)**. It belongs to the Pinaceae family and Pinoideae subfamily that comprises 250 species **(Amri, et al., 2013)**. Ecological and geographical studies showed that *P. halepensis* Mill., grows at lower altitudes in arid and semiarid regions favoring a Mediterranean climate independent of the soil type **(Mauri, et al., 2016)**. It is a thermophilic species that can survive and grow in various environments. This species grows in two periods of season (Autumn and Spring) **(Fotelli, et al., 2019)**. Its ecological amplitude is very vast, occupying nearly 6.8 million hectares throughout the Mediterranean basin **(Newton, Allnutt, Dvorak, Del Castillo, & Ennos, 2002)**.

I.2.1 Systematic

According to **Quézel (2000b)**, the Aleppo Pine is the characteristic essence of the semi-arid Mediterranean bioclimatic stage, it belongs to: (Table I)

Table I : Systematic of *Pinus halepensis*

Classification	
Reign	Plantae
Junction	Phanerogams
Under junction	Gymnosperms
Order	Pinales
Family	Pinaceae
Genus	*Pinus*
Species	*halepensis*

Scientific name: *Pinus halepensis.*

Common name: Aleppo pine

French name: pin d'Alep

Arabic name: Sanaoubar al-halabi.

I.2.2 Distribution air

The Aleppo Pine (*Pinus halepensis* Mill.) has been an integral part of the Mediterranean landscape **(figure 1)** for at least 3 million years. It is the species most widely used in reforestation for soil protection. It is drought resistant and not very tolerant to other factors such as low fertility soils and arid climates. The Aleppo Pine's range and its limits can be explained by its great resistance to drought and its high sensitivity to low temperatures **(BARBERO, 1992**).

I.2.2.1 In the Mediterranean basin

Pinus halepensis is in a spontaneous state around the Mediterranean basin and occupies more than 3.5 million hectares **(Quézel, 2000b)**. It is very widespread in North Africa, especially in Algeria and Tunisia (its forests occupy more than 2.5 million hectares) **(Sghaier & Ammari, 2012a)** and in Spain where it finds its optimum growth and development (it makes up 15% of the wooded area) **(BARBERO, 1992)**. In Italy, it covers 20,000 ha and remains close to the

coast. It is little represented in Greece and Turkey by relatively large settlements in Jordan **(BADOT & GARREC, 1993) (Table II).**

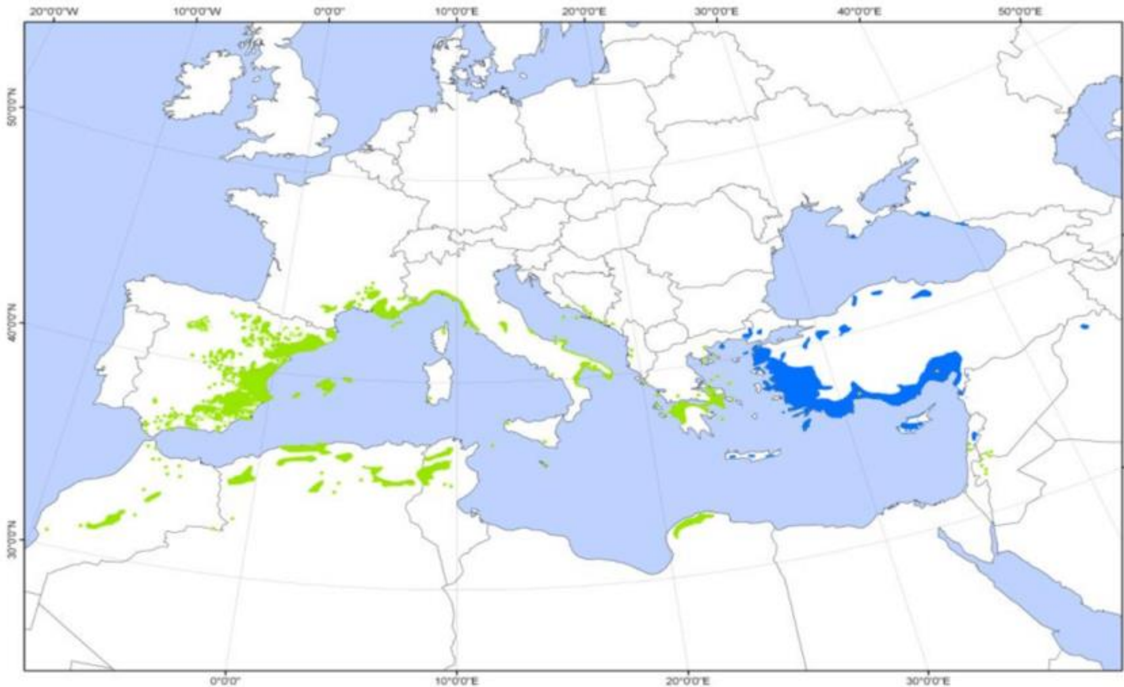

Figure 1 : Distribution air of Aleppo Pine in the Mediterraneen region (green)

Table II: Distribution air of Aleppo pine in the Mediterraneen region

Country	**Area (hectares)**	**Sources**
Algeria	800 000	**(Tatar, 2012)**
Morocco	65 000	**(Assmaa, Said, & Najib, 2011a)**
Tunisia	297 000	**(Sghaier & Ammari, 2012b)**
France	202 000	**(Ripert, et al., 2001)**
Spain	1 046 978	**(Assmaa, Said, & Najib, 2011b)**

Italy	20 000	**(Seigue, 1985)**

I.2.2.2 In Algeria

In Algeria, it is spread over 800,000 hectares, occupying 35% of the forested area. According to **Paul (1951)**, the Aleppo pine has vast stands in Oran (Sidi-Bel-Abbès, Saïda, Tlemcen, Tiaret), in Algiers (Media, Boghar, Monts des Bibans), on the Saharan Atlas (Mount Ouled Nail) and in southern of Canstantine (Aurès, Tébessa region) **(Figure 2).**

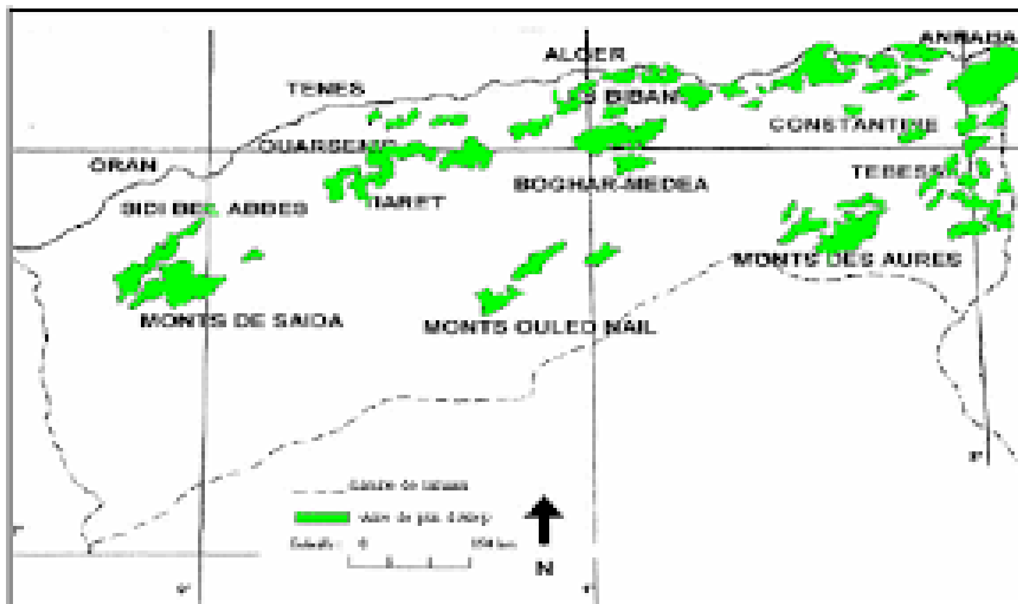

Figure 2: Distribution of Aleppo pine in Algeria Paul (1951).

I.2.3 Botanical characteristics of Aleppo pine

It is a second-growth coniferous forest tree that can sometimes reach 30 meters in height **(Figure 3.D)** is often leaning and not very straight with a crushed, irregular and clear crown, but its branches are fairly spread out **(Becker, Picard, & Timbal, 1982).**

Bark: Rich in tannins, initially smooth and silvery in color, then becomes cracked with grey-brownish scales **(Kadik, 1987).**

Branches: Are light green, then light grey, quite fine. It is polycyclic because this tree often makes a second growth in the same year. The buds are non-resinous, ovoid, sharp, brown with free scales fringed with white **(Kadik, 1987).**

Leaves or needles: are 6 to 10 cm long **(Figure 3.A)** with a width of 1 mm, are thin, soft, smooth and sharp, grouped in twos in brushes at the end of the twigs **(Nahal, 1962).**

Cones: Are large with a size of 6 to 12 cm (**Figure 3.C**) with a 1 to 2 cm thick peduncle, often isolated and reflected. They are purple then glossy brown with flattened patches, persistent several years on the tree **(Nasri, Khaldi, & Triki, 2004**).

Wood: Used in boxes, for the manufacture of paper pulp and poles, if its shape allows it. It is a perfect wood for the construction of piles or boats **(Burnel & Pélissier, 2009).**

Seeds: The Aleppo pine tree also produces an edible seed, small 5 to 7 mm long-winged, gray-brown on one side and gray speckled with black on the other **(Kadik, 1987).**

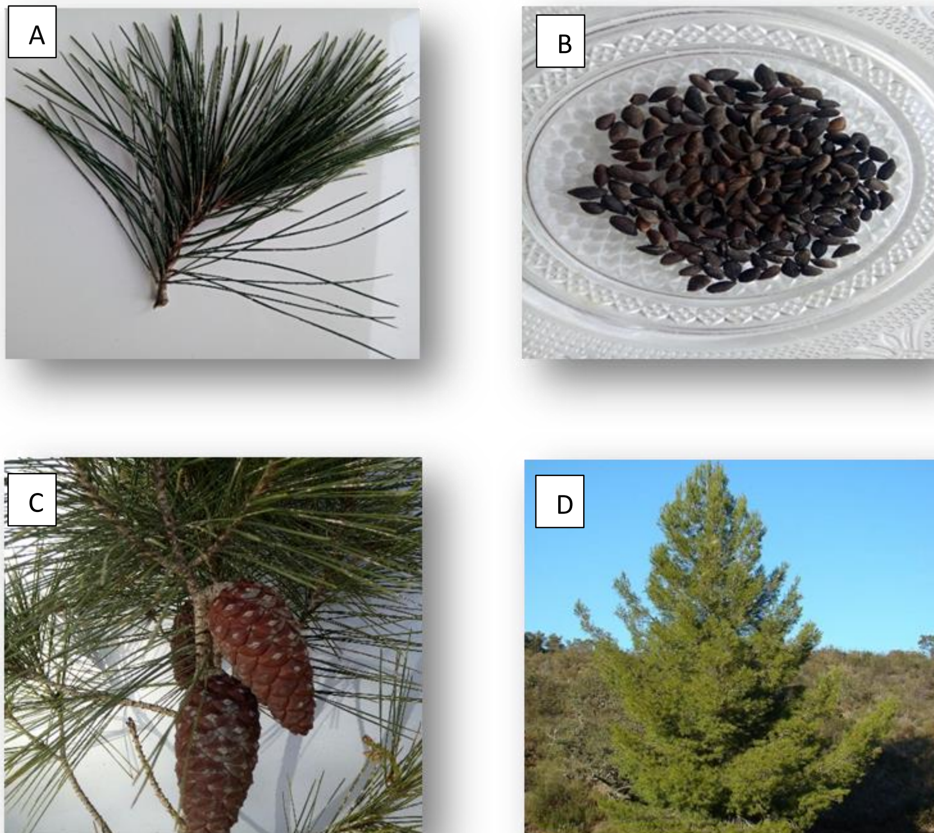

Figure 3: original photographs of (a) the needles, (b) the seeds, (c) the cones and (d) the tree of *Pinus halepensis*.

I.2.4 Climatic requirements

The Aleppo pine is found in the Mediterranean bioclimatic stages: upper arid, semi-arid, sub-arid and humid. The *P. halepensis* Mill., (Aleppo pine) is the most pine species that mainly grows at lower altitudes in arid and semiarid areas with a Mediterranean climate, independently of the soil type, although it is also found at higher altitudes in Morocco (more than 2000 m) **(Mauri, et al., 2016)**. It is a heliophilic and xerophilic species **(Ayari, Salah, & Daniel, 2014)** and is a thermophilous resistant species that can survive and grow in various environments. It uses several adaptation methods to overcome drought measures such as reducing stomatal conductance under water shortage. This species grows in climatic conditions arranged between −2 and 10 °C and annual precipitation between 350- and 700-mm. Spring and Autumn (the temperature and water are available) are the period during which Aleppo Pine grows importantly **(Fotelli, et al., 2019).**

1.2.5 Traditional uses of Aleppo pine

The use of Aleppo pine as a chemotherapeutic agent for infectious diseases in the Mediterranean area in the 12th century was described in the books of Maimonides **(Casal & Casal, 2004)**. Medical treatments of the Byzantine and Ottoman period in Middle Eastern countries also used Aleppo pine to treat haemorrhages (internal), as haemostatic (in external wounds), in the prevention and treatment of diabetes according to the Greco-Arab and Islamic model, respiratory tract diseases (catarrh and colds), dental problems (toothache) and injuries. The method of use was fumigation (inhalation, or exposure of the affected body part) **(Lardos, 2006)**. A survey of the scientific and ethno-pharmacological literature published since 2000 revealed the use of Aleppo pine as a medicinal plant in many regions around the Mediterranean **(González-Tejero, et al., 2008)**

Until today, Aleppo pine needles and seeds are used in the eastern region of the Mediterranean to cure diabetes, using a standard decoction of 50 g of seeds or needles taken internally, 150 cc twice a day; and for sexual weakness, use 10-15 g of the seeds consumed daily **(Azaizeh, Saad, Khalil, & Said, 1970; Daoud, 2008; Said, Khalil, Fulder, & Azaizeh, 2002).** In the region of Calabria, Italy, the decoction of buds and needles of Aleppo pine is used to treat bronchitis and joint inflammation by the bath **(Passalacqua, Guarrera, & De Fine, 2007)**.

Experimental studies have revealed that the essential oils of *Pinus halepensis* cones have a remarkable activity for wound healing **(Süntar, Tumen, Ustün, Keleş, & Akkol, 2012)**. The antibacterial activity of Aleppo pine essential oil was tested against strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli and Bacillus cereus*, the results revealed that this essential oil had a moderate activity against all bacterial strains, with the exception of *Pseudomonas aeruginosa* and *Escherichia co*li which were found to be highly resistant **(Abi-Ayad, Abi-Ayad, Lazzouni, Rebiahi, & Ziani_Cherif, 2011).** Raw sap is used in Lebanon for cold and coughs, externally for wounds and venereal diseases. The astringent pulverized bark is applied to wounds; the resin is used as an antiseptic **(Schiller, 2014).**

Wood:

Aleppo pine is considered the most widely used species for reforestation in Algeria. Aleppo pine wood can be used, after removal of the resin, for the manufacture of paper pulp **(Nahal, 1962)**. The Aleppo pine has a white wood, heartwood and light fawn color (light red) and of poor quality in antiquity, the Greeks worshipped it and it was sought after for shipbuilding **(Dupérat, 2002)**. Currently, it is used for making crates and frames, it is also a good firewood. It was used by sawyers to make pallets and packaging (where fruit production is important) and in particular squares, pieces for assembling crates using largely peeled wood. Aleppo pine wood, even if it is not of exceptional quality, is nevertheless suitable for more noble and therefore more remunerative jobs than those it is currently used for. In particular, light packaging could be a driving sector for large volumes of sawn timber **(Bedel, 1986).**

Resin:

Aleppo Pine gives about 3 kg of resin (the gem) per tree per year. The pure gem contains 20 to 24% turpentine and 75 to 80% cellophane. This gem also has very interesting medicinal uses **(Kadik, 1987)**. According to **Barceloux (2008)**, Turpentine oil extracted from pine resin has been used since ancient times as a herbal treatment for various diseases. Hippocrates (370-460 BC) mentioned the value of turpentine oil as an emmenagogue and inhibitor of nasal discharge. The Greek physician Dioscorides used turpentine oil as an aphrodisiac. The Romans used this oil for a wide variety of internal and external illnesses, including stroke, lethargy, depression and pleurisy **(Boulaacheb, 2009).**

The different chemicals that make up pine resin and essential oil are agents that can cure various diseases and ailments due to their biological activity. According to "Plants for a

Future" "turpentine is antiseptic, diuretic, rubefacient and vermifuge. It is a valuable remedy used internally in the treatment of kidney and bladder diseases and is used both internally and as a steam and friction bath in the treatment of rheumatic diseases. It is also very beneficial for the respiratory system and is therefore useful in the treatment of mucous membrane diseases and respiratory ailments such as coughs, colds and tuberculosis. Externally, it is a treatment for various skin conditions, wounds, sores, burns, boils, etc. used in the form of plasters, poultices, herbal steam baths and inhalers". Aleppo pine resin is used for the treatment of muscular pain, as a disinfectant of the respiratory and urinary tracts and as an antifungal agent in North Africa. Aleppo pine resin is used for the fermentation of wine and in the manufacture of pills and suppositories **(Boulaacheb, 2009)**

I.3 Aleppo pine seeds

I.3.1 Seed structure

Aleppo pine seeds are 6-7 mm long and weigh on average 13.54 ± 0.07 mg, covered with a dark mottled seed coat which contributes significantly to the total weight of the seeds and can reach a quarter of their total weight; the diameter of the seeds (without the seed coat) is 1.84 ± 0.02 mm, the thickness of the nutrient tissue is 0.47 ± 0.01 mm and the diameter of the embryo is 0.82 ± 0.02 mm **(Figure 4, Figure 5)) (Salvatore, et al., 2010)**

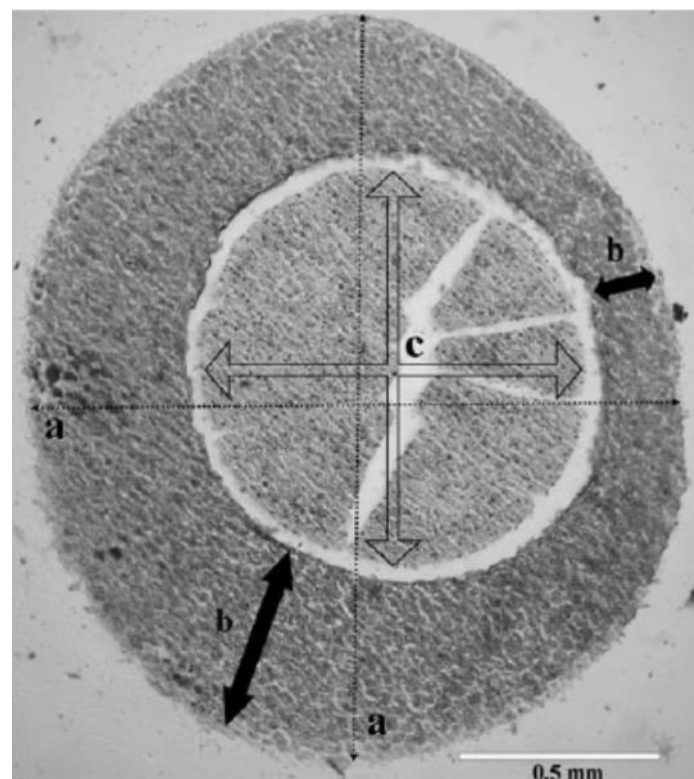

Figure 4: Optical microscope view of the cross section of an Aleppo pine seed, (a): diameters of the seed, (b) :thickness of the nutritive tissue, (c) diameter of embryo (Salvatore, et al., 2010)

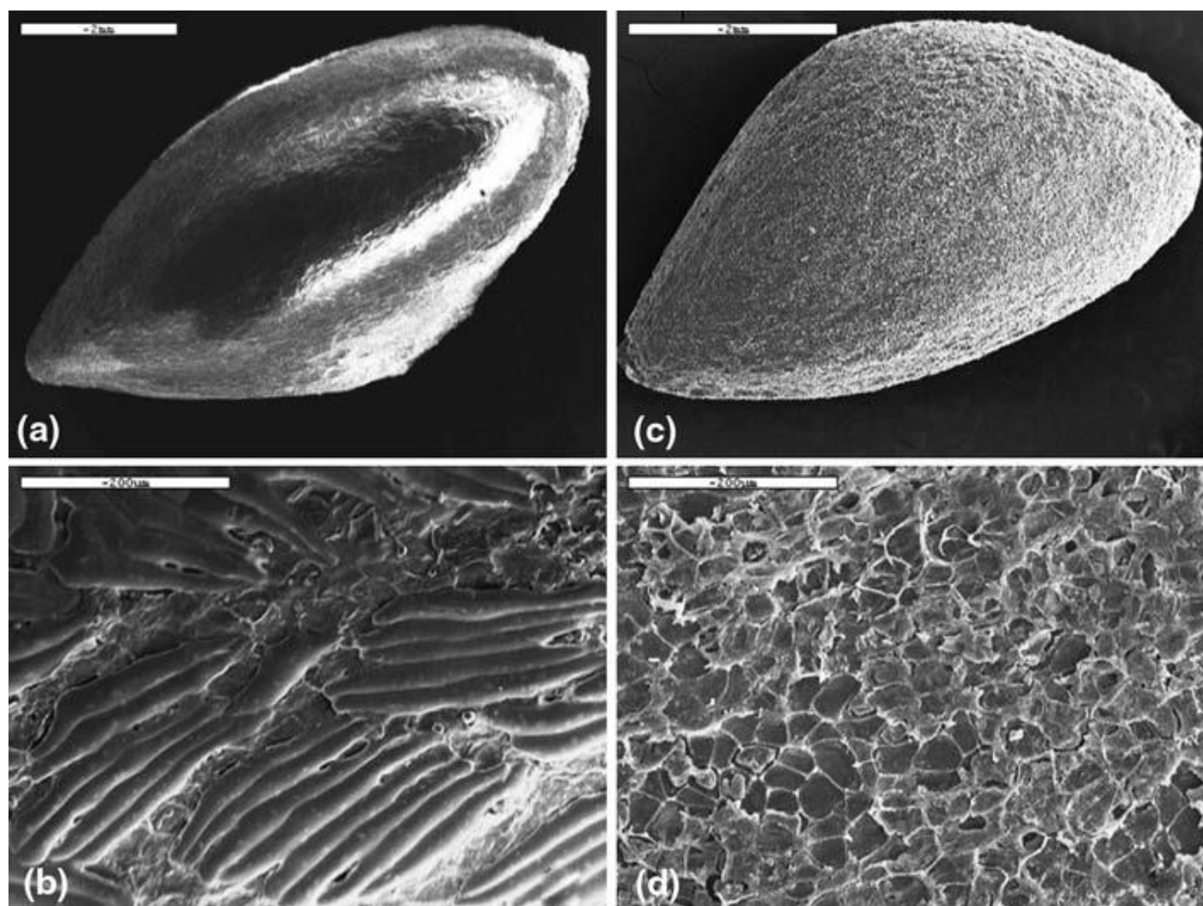

Figure 5 : Scanning electron microscope view of the two origins and details of Aleppo pine seeds (a) The majority of seeds appeared enveloped; (b) a detailed view of the seeds showing the wax globules and epidermal cells; (c) the majority of MAR seeds appear to have a rough surface; (d) a detailed view of the MAR seeds shows the uncovered integument (Salvatore, et al., 2010).

I.3.2 Previous studies on Aleppo pine seeds

Pine seeds have been the subject of several phytochemical studies in order to identify its active ingredients. Numerous studies have highlighted the richness of the extracts of these seeds in several heterogeneous constituents. Most of these studies focused on their composition and physicochemical characteristics, but especially on their lipid fraction. **(S Cheikh-Rouhou, et al., 2006).**

S Cheikh-Rouhou, et al. (2006) studied the proximal composition of Aleppo pine seeds grown in Bizerta (Tunisia) and the fatty acid composition and thermal and physical properties of the lipid fraction. The analysis showed the following composition (on a dry weight basis): 22.7% protein, 43.3% oil, 8.3% ash and 25.7% total carbohydrates. Potassium, magnesium

and calcium were the predominant mineral elements present in the seeds and together reached approximately 1%. Oleic and linoleic elements were the major unsaturated fatty acids (27.3% and 48.8%, respectively), while the major saturated fatty acids were palmitic (8.75%), myristic, rnyristoleic, palmitoleic, margaroleic, stearic. Linolenic, arachidic, eicosenoic, eicosadienoic, eicosatrienoic, behenic and lignoceric acids were also detected. Thermal profile of *Pinus halepensis* Mill. seed oil, determined by its DSC melting curve, showed that the entire liquefaction of Aleppo pine seed oil occurs at -8°C. The physico-chemical properties of the oil are as follows: saponification index 190, peroxide value 3.18, iodine value 117 and a low acidity of 0.61%. The results suggest that the production of oil from the seeds of *Pinus halepensis* could have a potential use in the food, pharmaceutical, cosmetic and other non-food industries.

Another study of **Salma Cheikh-Rouhou, Besbes, Blecker, Deroanne, and Attia (2008)** was based on the oxidative stability and certain quality parameters of two oils from black cumin (*Nigella sativa* L.) seeds of Tunisian and Iranian origin and an Aleppo pine oil of Tunisian origin which were evaluated at 60°C and 100°C. Absorbance at 232 and 270 nm, peroxide value, acidity, viscosity and colour of Iranian black cumin oil were slightly modified compared to those of Tunisian black cumin oil and pine oil. Absorbance at 232 and 270 nm, acidity and viscosity increase rapidly after reaching the induction period for the three oils studied. The results obtained showed that the order of oxidative stability is as follows : Iranian black cumin oil > Tunisian black cumin oil > Pin. Iranian black cumin oil resisted heat treatment for a long period (50 - 55 days and 45 hours, at 60 and 100°C, respectively). This may indicate that Iranian black cumin oil could withstand the heat treatments that are applied in culinary and other industrial applications.

Salma Cheikh-Rouhou, Besbes, Lognay, et al. (2008) also analyzed and compared the sterol composition of oils extracted from nigella seeds and Aleppo pine seeds. The highest level of total unsaponifiable matter was found in Aleppo pine seed oil (17.23 g/kg oil versus 15.58 g/kg oil). β-sitosterol was the major sterol in both oils, accounting for 44% and 54% of the total sterols in Nigella and Pinus seed oils respectively. The major sterol was stigmasterol in nigella seed oil (16.57-20.92% of total sterols) and campesterol in *Pinus* seed oil (11.42% of total sterols). TMS 484, D7-stigmasterol, D7-avenasterol and cholesterol were detected at lower levels in both oils. The high level of b-sitosterol in pine seed oil may make it the most suitable and effective in reducing blood cholesterol and preventing coronary heart disease.

Dhibi, Mechri, et al. (2012) carried out a discrimination study based on antioxidant compounds, antioxidant capacity and fatty acid profile of *P.halepensis* cones and seeds. The total amount of phenols was about 72 times higher in the cone extract than in the seed extract. Anthocyanin and carotenoids were respectively 10 and 12 times higher in the cone extract. Cone and seed extracts differed significantly in terms of free radical scavenging activity on the 2,2-diphenyl-1-picrylhydrazyl (DPPH-) radical (86.65 vs. 16.97% respectively) at a concentration of 1mg/mL. The cone extract had greater antioxidant power on the radical cation 2,2-azino-bis 3-ethylbenzothialozine-6-sulfonic acid) (ABTS-+) than the seed extract (IC50 of 0.368 vs. 2.345 mg mL-1). The profile of cone oils revealed richness in saturated fatty acids (41.5%) and fatty acids high in trans fatty acid isomers, with trans-linoleic acid predominating (4.74%). However, polyunsaturated fatty acids in seed oil accounted for more than 64% of total fatty acids. Cones had significant antioxidant activities and high levels of bioactive compounds. The cone is therefore a potential source of natural antioxidants that may offer several health benefits. However, the lipid extract from the seeds appears to have greater nutritional value as a polyunsaturated oil than the cone oil, which is rich in saturated and trans fatty acids.

Dhibi, Flamini, Issaoui, and Hammami (2012) evaluated the heating conditions of *Pinus halepensis* seed oil to assess the effect of thermo-oxidation on the bioactive compounds contained in the samples. The following parameters were monitored: ultraviolet absorption at 232 and 270 nm, fatty acid composition, oxidation stability, antiradical activity and aromatic profiles. Under the thermo-oxidation process, trans fatty acids (TFA) increased and conjugated linoleic acids of the acid isomers appeared. The antiradical activity decreased significantly from 73.3% to 52.5% after 120 min of heating. Oxidative stability decreased. The volatility profile was strongly influenced by heating with the formation of new volatile compounds, such as the two (E,Z)-2,4-decadienal and (E,E)-2,4-decadienal. α-pinene isomers, and the main terpene compound was very vulnerable to heating conditions. Despite the particularly pleasant aroma and nutritional value of *P. halepensis* seed oil, it should not be used for cooking. Indeed, under frying conditions, its antioxidant properties and oxidative stability are lost and potentially toxic substances of compounds such as trans fatty acids and unsaturated aldehydes are formed.

Another study of **Dhibi, et al. (2014)** evaluated the cis, trans and linoleic acid conjugated fatty acids in fresh Aleppo pine oil heated to frying temperature (180°C) and correlated with

antioxidant characteristics. The results showed that fresh Aleppo pine oil had a low oleic/linoleic ratio (0.4) and the total trans fatty acid content in the fresh oil reached 1%. The main trans fatty acid was 18:2 n-6 in both fresh and hot oil. Individual trans fatty acids increased with significant differences with heating time. Linoleic acid conjugated fatty acids occurred after 4 h and increased significantly, accounting for 10% of the total trans fatty acids after 10 h. Total trans fatty acids were negatively correlated with α-tocopherol, γ tocopherol and carotenoids and positively correlated with DPPH. The stability index of the oil showed a significant negative correlation with trans fatty acids. A principal component analysis (PCA) showed a clear discrimination between fresh and heated oil. Temperature, heating time, degree of unsaturation and antioxidants are combined factors that significantly affect the isomerization rate and nutritional quality of Aleppo pine seed oil.

The study of **Nabil Kadri, et al. (2015)** focused on the physicochemical characteristics of the seeds of certain Pinus species (*Pinus halepensis Mill., Pinus pinea L., Pinus pinaster and Pinus canariensis)* grown in northern Algeria. Results showed that the seeds are composed of 19.8 to 36.7% oil, 14.25 to 26.62% protein, 7.8 to 8.6% moisture. Phosphorus, potassium and magnesium was the predominant element present in the seeds. Physicochemical properties of pine seed oil indicate the acidity (4.9-68.9), iodine (93.3-160.4) and saponification (65.9-117.9) indices. Analysis of the oil showed that the main unsaturated fatty acids for the four species were linoleic acid (30-59%) and oleic acid (17.4-34.6%), while the main saturated fatty acid was palmitic acid (5-29%).Gas chromatographic analysis and mass spectrometry of the volatile oils of *P. halepensis* Mill, *P. pinaster and P. canariensis* indicated that the main volatile compound was limonene, with relative percentages of 3.1, 7.5 and 10.8, respectively. In addition, this study showed that Aleppo pine seeds are the richest in lipids (36.7%), proteins (26.62%) and total sugars (5.55%) (**Table III**).

In another study of **Kadri, Khettal, Yahiaoui-Zaidi, Barragan-Montero, and Montero (2013)** The lipid fraction of *Pinus halepensis* Mill. seeds was extracted and separated by column chromatography. Different classes of glycol-ipids (GL) and phospholipids (PL) were then separated and identified by liquid chromatography-mass spectrometry (LC/MS). A relatively high level of GL was found compared to the PL content. Four classes of glycolipids were detected: Esterified steroglucosides (ESG), monogalactosyldiacylglycerols (MGDG), cerebrosides (Cer) and digalactosyldiacylglycerols (DGDG). Six classes of phospholipids have also been identified: Phosphatidic acid (PA), phosphatidylethanolamine (PE),

phosphatidyl inositol (PI), phosphatidylserine (PS), phosphatidyl glycerol (PG) and diphosphatidyl glycerol (DPG). The method for the quantitative determination of the sugars that make up the glycolipid classes is described. The maximum ratio of sugars was observed in digalactosyldiacylglycerols (DGDG). The main unsaturated fatty acids for both glycolipid classes are oleic and linoleic acid, while the main saturated fatty acids are arachidic, palmatic and stearic acids in varying proportions.

And then, **Kadri, et al. (2014)** studied the lipid fraction of *Pinus halepensis* Mill. seeds for possible antiangiogenic activity. The neutral lipid, glycolipid and phospholipid fractions of Aleppo pine were complexed with β-cyclodextrin and analyzed by proton nuclear magnetic resonance (H1 NMR). Their cytotoxic activity was evaluated on three cell lines (human basal epithelial cell adenocarcinoma (A549), human colon adenocarcinoma (HCT15) and human myeloma (HL60)) by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium (MTT). Anti-angiogenic activity was evaluated in vitro on endothelial cell tubule formation and in vivo on the chorioallantoic membrane of the White Leghorn chick embryo (WLC). These results imply that neutral lipids, glycolipids and phospholipids from Pinus halepensis Mill. seeds are non-toxic and have antiangiogenic activity at concentrations of 1 mg/ml and 10 mg/ml, which may be useful in preventing angiogenesis-related diseases and subsequent cancer.

Rigane, et al. (2019) have applied a discriminatory analysis considering as variable dependency the oil yield, the phenolic content and the fatty acid profile according to the place of cultivation of *Pinus halepensis* Mill. The antioxidant activity of the oils collected from the Kasserine centre and the Foussana countryside were also studied. The oil of *Pinus halepensis* Mill. collected in Foussena had the highest value of oleic acid (62.45%), while the oil collected in Kasserine was characterized by a high percentage of palmitic acid (5.73%) which freezes this oil at low temperatures. On the other hand, phenolic compounds showed significant correlations with antioxidant activities. In addition, the anti-metastatic activity of fatty acids in *Pinus halepensis* Mill. oil was studied on the U87-MG cell lines. The fatty acid extract showed inhibitory activities on the proliferation and cell cycle progression of U87-MG cell lines. These results give credibility to the therapeutic potential of this plant against cancer. Given the potential use, knowledge of the composition of Pinus halepensis Mill. oil is of major importance. The diversity of applications of *Pinus halepensis* Mill. oil gives this plant great industrial importance.

Table III: Chemical composition *P. halepensis* seeds (Nabil Kadri, et al., 2015).

Composition	Percent %
Proteins	22.7
oil	43.3
Ashes	8.3
Total carbohydrates	5.7
Potassium, Magnesium & calcium	1
Total polyphenols	3.71
Flavonoids	0.80
Unsaturated fatty acids :	
Oleic acid	27.3%
Linoleic acid	48.8%
Saturated fatty acids :	
Palmitic Acid	8.75%

The seeds of *Pinus halepensis* Mill are therefore very rich in primary metabolites (Sugars, proteins, protein reserves) and secondary (Total phenols and flavonoids) as they have a high concentration of trace elements (Phosphorus, potassium, magnesium, zinc, iron, copper and manganese). They have very interesting oil from a nutritional and therapeutic point of view but should not be used for cooking. However, no studies have been carried out on their protein and carbohydrate fraction and no agri-food value has been published.

II. Valorization pathways for Pinus halepensis Mill. Seeds

II.1 Polysaccharides

II.1.1 General on polysaccharides

Polysaccharides are polymers of natural origin consisting of several bones linked together by osidic bonds. They are covalent chains of 20 to several thousand monosaccharide units **(Dhahri, 2017)**. There are in nature many varieties of polysaccharides that may be of plant origin such as starch, cellulose, pectins, or alginate or agar which are derived from algae, animal such as heparin or microbial (dextrans, xanthans) **(Joseph, Aravind, George, Varghese, & Sreelekha, 2013)**.

Polysaccharides are stable, non-toxic, biodegradable, biocompatible and water-soluble. They exist in many forms and have properties that make them extremely suitable for biomedical, pharmaceutical and cosmetic applications. A wide range of polysaccharides are already used in medical and food applications such as cellulose, heparins, starch and pectin **(Persina, et al., 2011)**.

II.1.2 Classification and nomenclature of polysaccharides

Polysaccharides can be classified according to their structure, chemical composition, solubility, source and applications. According to their chemical composition, they are classified into two types: Homopolysaccharides (homoglycans) which are made up of a single type of monosaccharides such as cellulose and glycogen which are made up only of glucose and heteropolysaccharides (heteroglycans) which are made up of different monosaccharides such as heparin which is made up of α -l-idopyranosyluronic acid 2-sulphate and 2-deoxy-2-sulfoamino-α-d-glucopyranose 6-sulphate **(Xiao, et al., 2011)**. Depending on the nature of the glycan-bound molecules, polysaccharides can also be glycoproteins or glycolipids **(Liun, Willfo, & ChunlinXun, 2015)**.

Polysaccharide is the name given to a macromolecule composed of a large number of monosaccharides, the residues are linked together by glycosidic bonds. They can be linear,

branched or repeating. Their nomenclature follows the general principles of stable organic products and the nomenclature for carbohydrates **(IUPAC-IUBMB, 1997).**

II.1.3 The sources of polysaccharides

- **Animal sources**

Animal polysaccharides belong to the glycosaminoglycan (GAG) family and are derived from proteoglycans (**Liun, et al., 2015**). These polymers are either involved in the structure of connective tissues (dermatan sulfates and chondroitins), or in cellular communication via their functional properties (heparins and heparan sulfate) (**BOUAL 2013/2014**).

These polymers consist of neutral residues (glycosamine) and glucuronic acid plus at least sulfate. Heparin is a polymer strongly bound to proteins, it is a molecule with immediate anticoagulant activity used in the curative and preventive treatment of thromboembolic diseases (**BOUAL 2013/2014**). Chitosan is found in the exoskeletons of arthropods **(Francis Suh.J.-K & Matthew, 2000).**

- **Vegetable sources**

The study of plant polysaccharides requires first of all an awareness of the very great structural variability partly linked to their numerous biological functions (**Benaoun, 2017**). The walls of plant cells are essentially composed of polysaccharides, which explains the majority of these molecules in the biomass. There are two groups of plant polysaccharides according to their function: reserve polysaccharides and structural polysaccharides. Algae are the largest source of plant polysaccharides, known as algal polysaccharides.

- **Bacterial sources**

Among the most studied bacterial polysaccharides are dextran, produced by *Leuconostoc mesenteroides* and glucosine produced by *Agrobacterium tumefaciens* **(Singh, Kaur, & Kennedy, 2015**).

Microorganisms produce a wide variety of exo-polysaccharides with unique physical and chemical characteristics. These exo-polysaccharides can be homopolysaccharides or heteropolysaccharides and because of their unique structure, they have potential applications in the food and pharmaceutical industries **(LILLY, WILSON H. A, & G., 1958).**

II.1.4 Biological activities of polysaccharides

The structural diversity of polysaccharides, whatever their origin (animal, vegetable or microbial) gives its macromolecules numerous biological activities. Thanks to their interactive and regulatory properties, polysaccharides participate in the control of cellular activity (proliferation, differentiation, adhesion and migration) but also in the activity of many enzymes **(Colliec-Jouault, et al., 2004).**

II.1.4.1 Antioxidant activity

Several studies have proven the very strong antioxidant activity of polysaccharides, according to **Badrinathan, Shiju, Sharon Christa, Arya, and Pragasam (2012),** Sulphated polysaccharides have shown good radical scavenging activity at low concentrations. According to **Y. J. Li, et al. (2015)** the strongest antioxidant activity expressed for the protection of the human body from damage caused by ROS, is noted for fucoidane followed by alginate and laminarane.

II.1.4.2 Anti-diabetic activity

The incidence of diabetes has risen dramatically, becoming the third most serious chronic disease after cancer and cardiovascular disease **(Zhu, et al., 2014). Zhou, Yan, Bai, Li, and Huang (2015)** studied the hypoglycemic activity and potential mechanisms of polysaccharides from *Misgurnus anguillicaudatus* in diabetic mice. Oral administration of these polysaccharides significantly decreased levels of glucose, triglycerides, total cholesterol and low-density lipoprotein-cholesterol (LDL-cholesterol) in the blood and increased levels of high-density lipoprotein-cholesterol (HDL-cholesterol) and insulin in diabetic mice, along with an increase in body weight and insulin content of the pancreas. These polysaccharides also have potent anti-inflammatory and antioxidant activities, as evidenced by the decrease in tumor necrosis factor-α, (TNF-α) and interleukin-6, it also significantly improves blood markers of liver failure and renal function in diabetic mice.

II.1.4.3 Hepato-protective activity

Quan, et al. (May 2013) tested the protective effect of *Boschniakia rossica* (Orobanchaceae) polysaccharides against hepato toxicity induced by carbon tetrachloride. Carbon tetrachloride resulted in increased serum tumour necrosis factor (TNF-α) and hepatic nitric oxide levels and inducible positive regulation of nitric oxide synthetase and cyclooxygenase-2 proteins in liver tissue. Pretreatment of mice with polysaccharides isolated from *B. rossica* reverses these

parameters. A hepatoprotective effect of *B. rossica* polysaccharides is reported by histopathological examination of liver sections. The results indicate that *B. rossica* polysaccharides play a protective role in the acute liver injury induced by carbon tetrachloride. A hepatoprotective effect *of B. rossica* polysaccharides may be due to high antioxidant defense potentials, which suppress inflammatory responses and apoptosis of liver tissues.

II.1.4.4 Anticancer activity

Cancer is described as a heterogeneous set of cells that develop in tumour microenvironments with a complex ecology. Conventional cancer treatments, such as surgery, chemotherapy and radiotherapy, show limitations due to poor prognosis and severe side effects. Nowadays, increasing attention is focused on natural products in an attempt to find new and effective anti-cancer agents with less toxic effects **(Bao, Yuan, Wang, Liu, & Lan, 2013)**

Numerous studies have suggested that polysaccharides may inhibit tumour growth by common mechanisms such as prevention of oncogenesis through oral consumption of active preparations; direct anticancer activity, such as induction of tumour cell apoptosis; immunopotentiation activity associated with chemotherapy; and inhibition of tumour metastasis **(Zong, Cao, & Wang, 2012).**

II.1.4.5 Anticoagulant activity

According to **Souza, et al. (2015)** Sulfated polysaccharides isolated from marine algae and invertebrates are a complex group of macromolecules that are widely studied as anticoagulants and anti-thrombotics without the undesirable risk of bleeding.

According to **Mulloy a.B, Moura˜o.P.A.S, and Gray.E. (2000)** Four distinct classes of sulfated polysaccharides (heparin, dermatan sulfate, fucosylated chondroitin sulfate and fucoidane from algae) all have anticoagulant activity due to their interaction with enzymes and inhibitors of the coagulation system.

II.1.4.6 Anti-inflammatory activity

Polysaccharides have proven their anti-inflammatory power for a very long time **(Lindsay, et al., 2006)**. Polysaccharides can selectively adhere to pathogens and block the adhesion of microbial pathogens to target cells, thus exerting the anti-inflammatory function and anti-infectious effect **(J.-H. Lee, et al., 2006)**. For example, a zwitterionic polysaccharide

produced by certain bacteria has a clear preventive and therapeutic effect against abscesses. The mechanism of action of the polysaccharide is to activate T cells through MHC II, so it can have an anti-inflammatory effect. So far, there are oligosaccharides and polysaccharides that are present as oral and topical anti-inflammatory agents **(Kasper, Onderdonk, Crabb, & Bartlett, 1979)**.

II.1.4.7 Other activities

As the understanding of the structure and function of polysaccharides after administration deepened in the 1950s, scientists discovered that polysaccharides of different structures had many properties and bioactivities **(L. Shi, 2016)**. In addition to the activities mentioned, new polysaccharide activities are always present on an ongoing basis. For example, **Yu, et al. (2004)** have isolated and studied two new pure polysaccharides from the *Nerium indicum* flower family for their neuroprotective effects on neurons against Serum-deprivation and beta-amyloid (Aβ) peptide. Pretreatment of the polysaccharides significantly reduced the number of apoptotic neurons revealed by DAPI staining when the neurons were exposed to a serum-free medium. In addition, the polysaccharides could also decrease the caspase-3 activity triggered by Aβ peptides. Western blot analysis indicated that the polysaccharides stimulated the phosphorylation of PDK-1 (Serine 241) and Akt (Threonine 308). The polysaccharides provide a lead for the future development of the neuroprotective agent against neuronal death in neurodegenerative diseases and the neuroprotective agent **(L. Shi, 2016)**.

II.2 Proteins

II.2.1 Generalities about proteins

Proteins were first identified in 1838 by the Dutch chemist Gerhard Mulder (1802-1880). The term protein comes from the Greek prôtos which means first, essential. This probably refers to the fact that they are indispensable to life and often make up the majority of the dry weight of organisms (more than 60% of the dry weight of the cell). In biochemistry, a protein is simply defined as a complex molecule (macromolecule) composed of one or more chains of amino acids, themselves linked by peptide bonds. The peptide bond is made between the acid group (COOH) of one amino acid and the amino group (NH_2) of the other **(Petsko, Ringe, & Charmot, 2008)**. An amino acid is a functional compound comprising a carboxylic acid group - COOH an amine group $-NH_2$ carried by the same carbon atom Cα (it is a chiral carbon: it contains four different groups) which also carries a hydrogen atom and a radical -R.

Such protein compounds are called α-amino acids **(MERABET, 2014)**. Amino acids differ from each other by their radicals. Therefore, theoretically an infinite number of amino acids can be combined. However, it can be seen that in humans, as in many species, only twenty different amino acids make up proteins. The radical can contain an aliphatic group (-H, -CH_3...), an additional function (- OH: hydroxyl or -SH: sulfhydrile), a second acid function (-COOH) or a second amine function (-NH_2), it may also contain an aromatic ring (the case of phenylalanine and tyrosine) or a heterocycle (the case of tryptophan and histidine) **(Berthelot, 1860)**.

II.2.2 Functional properties of proteins

Functional properties are defined as all the physical and chemical properties affecting the behaviour of proteins in a formulation during the production, storage and consumption of food products **Kilara (1984)**. The knowledge of the functional properties of an ingredient makes it possible to improve food manufacturing processes because it gives access to its field of use and makes it possible to predict its main effects in a formulation; it also makes it possible to compare different competing ingredients. In general, the techno-functional properties of proteins are classified into three groups, according to the interactions involved (**Cheftel, Cuq, & Lorient, 1985**):

a) Hydration properties such as solubility or water retention depend on protein-water interactions;

b) Surface properties govern protein-fat and protein-air interactions, in particular foaming and emulsifying powers ;

c) Texturing properties such as gelation or coagulation, depend on protein-protein interactions.

These properties mainly involve the following chemical bonds:

- The hydrogen" bonds which establish interactions between an electronegative atom having an electron doublet and a hydrogen atom: this is for example the case between peptide chains and glutamine residues; hydrogen bonds also govern the interactions of proteins with water and influence their solubility.

- The electrostatic interactions that are established between anionic amino acids (glutamic acid and aspartic acid) and cationic amino acids (lysine, histidine, arginine and tyrosine): this type of binding is highly dependent on pH.

- ***The hydrophobic interactions*** that are established between branched chain amino acids (valine, leucine, isoleucine), aromatic acids (tryptophan and phenylalanine) of proteins and fats. They are responsible for emulsifying and foaming properties.

- ***The covalent bonds*** (disulphide bridges, g-glutamyl-e-lysine and lysinoalanine type isopeptides...) which ensure a certain rigidity to the protein matrix.

II.2.2.1 Functional properties related to hydration mechanisms

II.2.2.1.1 Protein-water interactions

The native structure of proteins is a consequence of the interactions of amino acids with water and some properties can be interpreted as the result of the interaction between proteins and water of thermodynamically favourable (wettability, swelling, water retention and solubility) or unfavourable (foaming, emulsification) interactions. Other properties reflecting the interactions of protein polymers with water are viscosity, gelation and coagulation **(Srinivasan Damodaran, 1997)**. The importance of water dynamics in relation to protein and adaptation to the protein environment was investigated by **Mattos (2002)**.

II.2.2.1.2 Water holding capacity

In food applications, water holding capacity is related to the ability to retain water against gravity, and includes bound water, hydrodynamic water, capillary water and physically trapped water. The amount of water associated with proteins is closely related to amino acid profile and amino acids and increases with the number of amino acid-laden residues **(Kuntz Jr & Kauzmann, 1974),** conformation, hydrophobicity, pH, temperature, ionic strength and protein concentration **(Srinivasan Damodaran, 1997)**.

II.2.2.1.3 Solubility

The hydrophilicity/hydrophobicity balance, which depends on the amino acid composition, especially at the surface of the protein, influences the solubility of the protein. Solubility is related to the presence of a low number of hydrophobic residues, high charge and electrostatic residues, with repulsion and ionic hydration occurring at pH above and below isoelectric pH. Denaturation affects the solubility of proteins by altering the hydrophobicity/hydrophilicity ratio of the surface. The effects of salting and release, related to the surface characteristics of the proteins, also affect the solubility of the proteins, which influences thickening, foaming, emulsification and gelation **(Srinivasan Damodaran, 1997).**

II.2.2.2 Functional properties related to protein structure and rheology

II.2.2.2.1 Viscosity and thickness

Several authors have shown that protein solubility, hydrodynamic properties, hydrophobicity and microstructure play an important role in the rheological properties of proteins **(Hall, 1996; Krause, Bagger, & Schwenke, 2001)**. **Añón, Sorgentini, and Wagner (2001)** suggest that the apparent viscosity of commercial and laboratory soybean isolates depends on the interaction between soluble and insoluble proteins and between hydrated particles. Viscosity increases exponentially with protein concentration probably due to increased interactions of hydrated proteins, water absorption and swelling **(J. Kinsella & Shetty, 1979). Añón, et al. (2001)** show that total water/impregnated water when the trend is 1, with several different protein concentrations, produces an increase in apparent viscosity. **Malhotra and Coupland (2004)** show the effect of surfactants and pH on the viscosity of soy protein isolates, an increase in the solubility of the protein isolate by the addition of a surfactant implies higher viscosity values. Swelling and water absorption capabilities have been suggested as a contributing factor to viscosity **(Sousa, Morgan, Mitchell, Harding, & Hill, 1996)**. Partial denaturation of proteins also increases the viscosity of proteins due to the hydrodynamic surface produced by protein unfolding **(SCHWENKE, et al., 1990).**

II.2.2.2.2 Gelation

A gel can be defined as an intermediate state between solid and liquid. In food systems, the liquid is water and the molecular network is formed by proteins, polysaccharides or a mixture of both. Proteins are more effective gelling agents than carbohydrates because large molecules are capable of forming three-dimensional cross-links **(Moure, Sineiro, Domínguez, & Parajó, 2006)**. Gelation is favoured by the size of the proteins, as large molecules form extended networks through three-dimensional networking, and by the flexibility and denaturation capacity of the proteins **(Srinivasan Damodaran, 2017).** In order to form gels, partial gelation occurs at the boundary between aggregation and solubility **(HEGG, 1982)**. In addition, denaturation is desirable, as the deployment of the structure results in long chains without covalent breaks. Other influencing factors are pH, ionic strength, reducing agents, urea, temperature, the presence of non-proteins and mechanical forces applied to the system **(Srinivasan Damodaran, 1997; Sathe, 2002)**. Heat-resistant proteins can be denatured by shear stress or by alcoholic solutions. The properties of the gels are a consequence of the interactions between the solvent and the molecular network. Proteins from non-polar residues

tend to form coagulating gels while those containing hydrophilic amino acids form transparent gels. **(Shimada & Matsushita, 1980).**

II.2.2.3 Functional properties related to the surface of proteins

Emulsions and foams are two-phase systems, with one phase dispersed in a continuous aqueous medium. Both are commonly found in food systems and their formation is significantly affected by protein surface activity. Emulsifying or foaming agents reduce interfacial tension and facilitate the formation of stable oil-water and air-water interfaces. Low molecular weight surfactants (phospholipids, mono and diglycerides or monoesters) are more effective than high molecular weight surfactants (proteins and gums) in reducing interfacial tension **(Srinivasan Damodaran, 1997)** because the conformational characteristics of proteins limit their adsorption, which usually occurs through the sequential attachment of polypeptides or non-polar residues to the surface. Despite the lower efficiency of proteins compared to low molecular weight surfactants in reducing surface tension, emulsions and foams formed with proteins are more stable. Proteins exhibit different surface activity, related to their conformation and ability to deploy at interfaces determined by internal factors (flexibility, conformational stability, distribution of hydrophilic and hydrophobic residues in the primary structure) and external factors (pH, ionic strength, temperature, possible competitive adsorption of other proteins or lipids in the interface) **(Damodaran, 1997)**

II.2.2.3.1 Foaming capacity

In food products, proteins are the main active surface-active agents needed to stabilize the dispersed gas phase. Foam formation requires a large interfacial surface area to facilitate the incorporation of air into the liquid phase and the formation of an interfacial film resistant to internal and external forces. The foaming capacity is therefore determined by the ability of the proteins to reduce surface tension, molecular flexibility and physico-chemical properties (hydrophobicity, net charge and charge distribution, hydrodynamic properties)**(Graham & Phillips, 1976**). Good foaming proteins should adsorb rapidly during whipping and boiling, have a rapid change in conformation, reorganize in the air/water interface with reduced surface tension and form a cohesive viscoelastic film via an intermolecular interaction system **(Hettiarachchy & Ziegler, 1994**). The stability of the foam indicates its ability to stabilize against gravity and mechanical stress **(Damodaran, 1997).**

II.2.2.3.2 Emulsifying activity

The presence of surfactants and their physico-chemical properties determine the formation of stable emulsions. Proteins are preferred to low molecular weight surfactants for emulsification in foods. The ability of proteins to act as emulsifiers varies according to the properties of the proteins, the main factors affecting the properties of emulsions are molecular weight, hydrophobicity, conformation, charge and physico-chemical factors such as pH, ionic strength and temperature **(J. E. Kinsella & Morr, 1984)**. Solubility plays an important role, highly insoluble proteins are not good emulsifiers and can generate coalescence. But also according to **Kato and Nakai (1980)**, the emulsifying properties correlate well with the presence of hydrophobic residues in surface proteins. The presence of salts and the pH value influence the stability of the emulsion **(Tsaliki, Pegiadou, & Doxastakis, 2004)**. Denaturation could also improve the emulsifying properties of proteins, thanks to an increased hydrophobic surface and flexibility **(Dickinson & Hong, 1994)**. Emulsion stability is influenced by several interdependent physical processes: cream formation, flocculation or aggregation and coalescence, that affect the separation phase **(Damodaran, 1997)**.

II.3 Vegetable beverages

II.3.1 Generalities on vegetable beverages

The food market increasingly reflects consumer demand for healthy food products. A clear example of this trend can be the so-called vegetable milks, which are mainly based on nuts and cereals and have a history in both Eastern and Western cultures. European sales of soymilk and other non-dairy products are increasing by more than 20% per year; Spain is the EU country where the market for vegetable drinks has grown the most **(Monitor, 2006)**. Similarly, total U.S. sales of soymilk, almond milk, rice and other crops reached $1.3 billion in 2011 **(Facts, 2012)**. The best known and most popular vegetable milk is derived from soya, but the demand for almond, rice, oat and coconut milk is increasing due to the awareness of allergy and intolerance issues and the lactose-free, cholesterol-free and low-calorie positioning of these products **(Stone, 2011)**. A wide range of vegetable milks based on nuts and cereals are currently available on the market in a wide range of formulations: flavoured, sweetened/unsweetened, low-fat and/or fortified. People with lactose intolerance and/or allergies to cow's milk are the primary consumers of these types of beverages, but they are also in high demand by people without health problems, such as vegans and vegetarians **(Bernat, Cháfer, Chiralt, & González-Martinez, 2014)**. Moreover, the development and

further increase in demand for these products would have an additional advantage, which could be of economic interest to many countries: the raw material (nuts and cereals) generally do not require particular soil and climatic conditions, they are able to adapt to different climates **(Osca, 2007).** This could contribute to rural development in developing countries and enable these crop products to achieve a high level of price competitiveness on the world market **(Bernat, et al., 2014).**

II.3.2 Main sources of vegetable beverages

All commercial vegetable milks share characteristics such as the absence of lactose, animal proteins and cholesterol. Taking into account the raw materials, their nutritional value and their health properties, plant milks can be classified into two main differentiated groups: nut milks and cereal milks **(Moure, et al., 2006).** Both types of products are state of the art thanks to new knowledge of their compounds impact on some chronic diseases, such as cardiovascular disease, type 2 diabetes mellitus, obesity and certain cancers. These metabolic diseases are linked to our daily lifestyle, including an unbalanced diet rich in energy, free of fiber and bioactive compounds, such as micronutrients and phytochemicals **(Fardet, 2010).** All of these limited nutrients are readily available in cereals and nuts. In addition to nuts and cereals, other raw materials have been used industrially, such as tubers and plants (hemp). However, these milk-based products are not well accepted in some countries.

II.3.3 Nut beverages

Because of their composition, nuts and nut products have recently attracted the attention of many food, nutrition and health specialists. Walnuts are rich in mono- and polyunsaturated fatty acids (PUFAs), plant proteins, dietary fibre, phytosterols, polyphenols, vitamins and minerals **(Phillips, Ruggio, & Ashraf-Khorassani, 2005**). Most of these compounds have antioxidant properties and are proven to have a beneficial effect on the plasma lipid profile and the inflammatory process **(Carlson, Eisenmann, Norman, Ortiz, & Young, 2011; Egert, Kratz, Kannenberg, Fobker, & Wahrburg, 2011);** Epidemiological studies have established a link between the frequent consumption of nuts and a risk of cardiovascular disease, diabetes or all causes of mortality **(Kelly & Sabaté, 2006**). In addition, **T. Y. Li, et al. (2009)** observed that increased nut consumption was significantly associated with a more favourable plasma lipid profile. In addition, walnuts have a high K/Na ratio, which contributes to maintaining well-balanced electrolytes in the human body. Carbohydrates in nuts are complex (low glycemic index), which also contributes to maintaining blood glucose levels at healthy levels. Thus, in addition to providing nutrients and bioactive antioxidants,

nut milks can be a useful dietary tool to reduce risk factors for several diseases, such as metabolic syndrome, diabetes or cardiovascular disease **(Moure, et al., 2006).**

II.3.4 Processes for the production of vegetable milks

The production process of vegetable milk is based on five main steps: grinding, water extraction, filtration, homogenization and treatment for the elimination of pathogens (**Figure 6**). The steps are for the most part the same. The packaging of nuts consists of washing and selection, plus a pre-treatment which is usually a bleaching to facilitate both the peeling of nuts and the initial reduction of the microbial load but also in some cases reduce the anti-nutritional factors that may be contained in these seeds. Despite the fact that almost 50% of the content of a nut consists of lipids, vegetable milks contain lower values of lipids which vary from 3 to 5% (w/v) in vegetable milk than in cow's milk. Moreover, since these milks must be used for the same purposes as dairy cows, the ratio (monounsaturated fatty acids + polyunsaturated fatty acids)/Saturated fatty acids) is much higher than animal milks and therefore they are healthier (**Bernat, et al., 2014**).

Almond milk stands out from other nut milks as a suitable alternative to cow's milk because, in addition to its lipid profile, it has a low Na/K ratio and a balanced ratio of Ca/P (**Luengo, 2009**). The main problem found during production or storage and milk derived from nuts or these cereals are related to the low stability of the liquid dispersion, generally with low viscosity, which promotes the separation of the phases of unstable fat globules caused by the phenomena of flocculation and coagulation in a short period of time. In addition, fibres and insoluble materials will also separate, either by sedimentation or flotation. The use of heat treatment and homogenization under pressure, the addition of amphiphilic compounds and hydrocolloids during milk processing could contribute to the development of an excellent product with desirable sensory attributes (**Bernat, et al., 2014**). The use of UHPH allows for a longer shelf life of the product, since the greatest physical stability is obtained mainly due to a reduction in the size of the fat globule which prevents coalescence. Sometimes, homogenization pressures are also able to reduce the microbial load of the product before heat treatment, if they are higher than 200 MPa (**Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007; Valencia-Flores, Hernández-Herrero, Guamis, & Ferragut, 2013**)

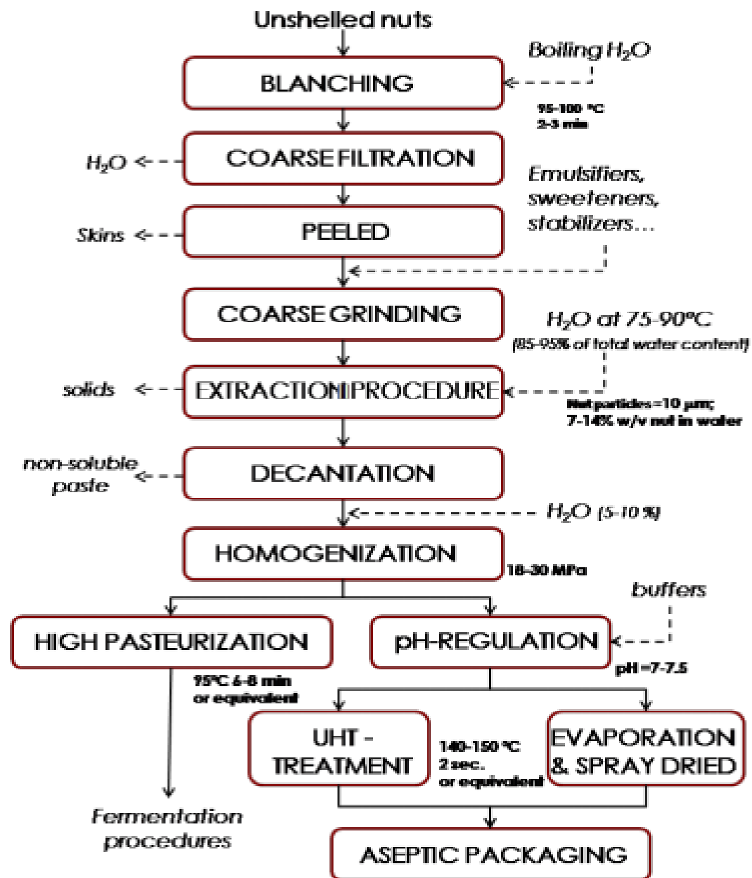

Figure 6: Standard manufacturing diagram of vegetable beverage **(Bernat, et al., 2014).**

Experimental part

Chapter II: *Effect of precipitation solvent on some biological activities of polysaccharides from Pinus halepensis Mill.*

Chapter II: Effect of precipitation solvent on some biological activities of polysaccharides from Pinus halepensis Mill.

Abstract

This study was designed to evaluate, for the first time, the effect of the precipitation solvent (Acetone, Ethanol, and Propanol) on the antioxidant, anti-inflammatory and anticoagulant activities of the polysaccharides extract from Aleppo pine seeds. The antioxidant activity was evaluated with different tests (ABTS, DPPH, metal chelation, ferric reducing power, antiperoxidation and ORAC tests), the anti-inflammatory activity was assessed with three tests (denaturation protein inhibition, antiprotéinase and anti-hemolytic tests). Finally, the anticoagulant activity was tested by endogenous and exogenous ways. The three extracts (AP: acetone polysaccharides extract, EP: ethanol polysaccharides extract and PP: propanol polysaccahrides extract) have exhibited a very interesting activities but with different degrees. The AP extract was most effective in almost all antioxidant activities (antiradical ABTS and DPPH, metal chelation, reducing power and ORAC), in two *in vitro* anti-inflammatory and the anticoagulant activities. However, for the lipid antiperoxidation activity, it was the PP extract that gave better activity. The best antiproteinase activity was expressed by the EP extract. These results indicate that polysaccharides of Aleppo pine seed may be considered as a source of bioactive polysaccharides and the precipitation solvent of the polysaccharides has a major effect on the intensity of the bioactivity of these polysaccharides.

Keywords : *Polysaccharides, Pinus halepensis Mill., antioxidants, anti-inflammatory, anticoagulant, solvent precipitation.*

Chapter II: Effect of precipitation solvent on some biological activities of polysaccharides from Pinus halepensis Mill

I. Introduction

A particular focus has been placed on the research of new sources of plant molecules, in order to develop new principles or to discover structural analogues of existing molecules **(Deters, Lengsfeld, & Hensel, 2005)**. During the last few years, they have attracted considerable attention and increasing studies have started to develop natural polysaccharides-based biomaterials for various applications. This is due to their biocompatibility, low toxicity, unique physical properties, and specific therapeutic properties **(Renaud, Belgacem, & Rinaudo, 2005).** This primary metabolite possessed several biological activities like as anticoagulant, antiviral, antioxidant, antitumor, anti-inflammatory, anticomplementary, antiseptic **(Kadri, et al., 2014; Yong-Guang, Ding-Long, Yu-min, & Min-xia, 2012). Villares, Mateo-Vivaracho, and Guillamón (2012)** have reported that more than 300 polysaccharides have been developed and are in clinical trials as antiviral, antitumor and antidiabetic agents. These macromolecules can therefore be of vegetable, animal, microbial or fungal origin. However, very little work is done on the use of the raw polysaccharide extract of the Pinaceae family, unlike other metabolites of the same family such as polyphenols and flavonoids.

The seed of *Pinus halepensis* Mill. is an oleaginous seed largely distributed in the Mediterranean basin. It was formerly very consumed especially in pastry making and it has a long tradition in folk medicine as an antidiabetic and to treat sexual problems. Most of the work carried out on this seed was focused on the phytochemical study and especially its lipid fraction **(Salma Cheikh-Rouhou, Besbes, Lognay, et al., 2008; S Cheikh-Rouhou, et al., 2006; Dhibi, et al., 2014; Kadri, et al., 2013; Wolff & Bayard, 1995)**. In our knowledge, there are no recorded studies on their polysaccharides.

The extraction of polysaccharides is based on the principle of precipitation, generally by solvents. The most widely used solvents in the literature are ethanol, acetone and propanol. But according to the literature, no study has proven the effect of these different solvents on the bioactivity of polysaccharide extracts. The principal purpose of this work is to identify new sources of polysaccharides (which are the Aleppo pine seeds) that may become drug substitutes synthetic and to find the best solvents allowing the precipitation of maximum polysaccharides with maximum purity and with the best bioactivity. For this purpose, the *in vitro* antioxidant, anti-inflammatory and anticoagulant activities were tested for the three

extracts (AP (acetone polysaccharides), EP (ethanol polysaccharides) and PP (propanol polysaccharides)) recovered by the three different solvents (acetone, ethanol and propanol).

II. Material and methods

II.1 Material

II.1.1 Plant material

The seeds of Aleppo pine (*Pinus halepensis* Mill.) were obtained from the Collo forest located in Skikda province of Algéria. They were cleaned; dried in an oven at 40 ° C for 2 days and then finely crushed using an electric grinder (KIKA Labortech- nik M20, Germany) to obtain a fine powder (250 µm) which was delipidated by the Soxhlet method with petroleum ether.

II.1.2 Red blood cell suspension

Blood was obtained by venipuncture from healthy volunteers collected in heparinized tubes and centrifuged at 3500 rpm for 15 min. Plasma and buffy coat were removed. Red blood cells (RBCs) were suspended in 10 volumes of 0.9% NaCl and centrifuged at 2500 rpm for 5 min. The RBCs were washed three times with the same solution. During the last washing, the packed cells were re-suspended in 10 volumes of phosphate buffered saline (PBS, pH 7.4) and utilized for the cytotoxicity and anti-hemolytic tests **(Oyedapo & Famurewa, 1995).**

II.2 Methods

II.2.1 Preparation of extracts

The protocol of **Chen, et al. (2012)** was used with some modifications; three different extractions were carried out. For each one, 25 g of powder was extracted with bi-distilled water (300 ml) for 1 hour at 60 °C. After centrifugation at 6000 rpm and 4 °C for 15 minutes, the supernatant was collected and filtered. The filtrate was concentrated to a third of the volume, and then three volumes of solvent (ethanol, acetone or propanol) have been added. The mixtures have been left to precipitate for 48 hours at 4 °C, and then centrifuged again. The pellets were recovered and suspended in bi-distilled water at a ratio of 1:20 (w/v) and deproteinized using $CaCl_2$ methods (Huang, 2008). After filtration, the obtained filtrate was subjected to a second precipitation with (ethanol, acetone or propanol) (1:3 v/v for 48H at 4 °C). After centrifugation (6000 rpm and 4 °C for 15 minutes), the pellet was lyophilized and stocked.

II.2.2 Chemical composition and yield

The extraction yields before and after deproteinization was expressed as residual solid extract obtained from fat-free powder. Ash, moisture and fat levels were estimated by **AOAC (1998b)** methods, the protein and sugar content were determined before and after deproteinization using **Bradford (1976)** and **Dubois, Gilles, Hamilton, Rebers, and Smith (1956) r**espectively. Concentrations of protein and sugar were deduced from a standard curve using BSA and glucose respectively.

II.2.3 FT-IR analysis of polysaccharide extracts

The FTIR spectra of the various samples were performed using a spectrometer SHIMADZU FTRI 8400, FT-IR (IRAffinity-1S Shimadzu, Japan). 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} **(Yuan, et al., 2015**).

II.2.4 Cyto-toxicity assay

To 1 mL of RBC suspension, a volume of 1 mL of extracts (AP, EP and PP) at increasing concentrations (1, 2, 3 and 4 mg/ml) was added. After 10 min of incubation at room temperature, the samples were centrifuged at 3000 g for 10 min and the resulting supernatant was removed and used to evaluate their hemolytic activity using a spectrophotometer at 540 nm. RBC lysis in the presence of distilled water was considered as 100 % hemolytic activity **(Pagano & Faggio, 2015**). Hemolysis in the presence of extracts was calculated relative to this control hemolysis

Hemolysis %= 100x (A_0-A_1/A_0).

Where A_o was the absorbance of control (distilled water without extract) and A_1 the absorbance in the presence of extract.

II.2.5 Antioxidant activities

II.2.5.1 Free radical scavenging activities

- ***DPPH free radical scavenger***

Briefly, 180 µL of the 0.1 mM DPPH solution in methanol is added to 20 µL of acetone polysaccharides (AP), ethanol polysaccharides (EP) and propanol polysaccharides (PP)

solution at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/mL). The mixtures have been vigorously shaken and incubated in darkness for 30 min. The absorbance was measured at 517 nm using micro-plate reader (BioTek, EIx800, USA) against a control containing 180 µL DPPH solution and 20 µL methanol **(Oktay, Gülçin, & Küfrevioğlu, 2003).**

- ***ABTS free radical scavenger***

Solutions of ABTS (7 mM) and potassium persulfate (2.45 mM) were mixed and incubated in the dark at room temperature for 12–16 h. The product was diluted in ethanol for optimal absorption ±0.7 at 734 nm. The reduction between $ABTS^{\cdot+}$ and test samples was monitored by a decrease in absorption at 734 nm during 30 min **(Re, et al., 1999)**.

Radical scavenging activity was calculated as follows:

% radical scavenging activity = 100 x (A_0– A_1/A_0)

Where A_0 is the absorbance of control solution and A_1 is the absorbance in the presence of plant extract. IC_{50} was determined from a graph in which scavenging activity was plotted against varying concentrations g/ml) of extract using a linear regression curve.

II.2.5.2 Metal chelating activity

The chelation of ferrous iron by extracts was evaluated by the ferrozine method, for this purpose, 250 µL of the different extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/mL) were put in competition with 50 µL of ferrozine to chelate the iron contained in 25 µL of $FeSO_4$ at 2 mM. After incubation for 5 min in the darkness, the absorbances were measured at 562 nm with a micro-plate reader **(Gulcin, Buyukokuroglu, & Kufrevioglu, 2003)**. The results have been expressed in terms of IC_{50} and the percentage of inhibition of ferrozine-Fe^{2+} complex formation was given by the following formula:

% inhibition= 100 x ((A_0-(A_1 - A_2)/ A_0).

Where A_0 was the absorbance of the control, A_1 was the absorbance without $FeCl_2$, A_2 was the absorbance with $FeCl_2$. The control contains $FeCl_2$ and ferrozine without extracts.

II.2.5.3 Lipid peroxidation

The TBARS protocol from **Ohkawa, Ohishi, and Yagi (1979) and Pandey, Chaurasia, Tiwari, and Tripathi (2007)** was used with minor modifications. 0.5 mL of egg yolk homogenate (10% in v/v distilled water) and 0.1 mL of different extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/mL) solution were mixed in a test tube and the volume was made up to 1 mL with distilled water. 0.05 mL of $FeSO_4$ (0.07 M) was added to the previous mixture and incubated for 30 min at 37 °C to induce lipid peroxidation. Then, 1.5 mL of acetic acid (20%, pH 3.5), 1.5 mL TBA (0.8% (w/v) Prepared in 1.1% sodium dodecyl sulfate) and 0.05 mL TCA (trichloroacetic acid 20%) were added, vortexed and heated at 100 °C in a water bath for 60 minutes. After cooling, 5 mL of 1-butanol were added then the mixture is centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant (organic phase) was measured at 532 nm. The results have been expressed in terms of IC_{50} and the percentage of inhibition of lipid peroxidation was calculated by the following formula:

% inhibition= 100 x $(A_0-(A_t-A_{t0})/ A_0)$

A_0 is absorbance of mixture without extract, A_t is the absorbance of test and A_{t0} is the absorbance of extract blank (prepared by replacing the TBA with sodium dodecyl sulphate).

II.2.5.4 Ferric reducing power

The reducing power of the polysaccharide extracts (AP, EP and PP) were tested by mixing them at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/mL) in 1 mL methanol with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL potassium ferricyanide at 1%. The mixtures were incubated for 20 min at 50 °C and then 2.5 m of 10% TCA was added to the mixtures. The all was centrifuged for 10 minutes at 3000 rpm. 2.5 mL of the supernatant was then mixed with 2.5 mL of methanol and 0.5 mL of $FeCl_3$ at 0.1%. Finally, the absorbance was measured at 700 nm **(Oyaizu, 1986)**. The results have been expressed in terms of EC_{50}.

II.2.5.5 Oxygen Radical Absorbance Capacity (ORAC)

The ability of extracts to inhibit the consumption of 2', 7' dichloroflurescein (DCF) was used to measure the ORAC value. for this purpose, a mixture of 50 µL of samples (extracts or trolox), 100 µL of DCS and 100 µL of AAPH was prepared in the wells of a microplate (the

extracts and all the products used were dissolved in salt phosphate buffer (10 mM, 150 mM NaCl at pH, 7.4). fluorescence spectrometer (Victor2 Wallac-Perkin-Elmer) was used to record the fluorescence every 1 min for 90 min at 485 nm-excitation and 535 nm emission.

ORAC values are expressed in terms of moles of Trolox equivalent (TE) per mole of antioxidant (pure) or per gram of polysaccharides extracts using the trolox calibration curve that determines the time required to achieve 50% fluorescence desintegration relative to trolox concentrations **(Ishimoto, et al., 2012)**.

II.2.6 Assessment of in vitro anti-inflammatory activity

II.2.6.1 Test of Inhibition of albumin denaturation

The inhibition of BSA denaturation test was used to study the possible anti-inflammatory capacity of polysaccharide extracts, for this purpose, 2 mL of 1% aqueous BSA solutions containing the extracts with different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/mL) was incubated at 37 ℃ for 20 min, then a heat treatment at 70 ℃ was applied for 20 min, turbidity was measured at 660 nm after cooling and to express the percentage of protein denaturation the following equation was applied **(Sakat, Juvekar, & Gambhire, 2010)**.

$$\% \text{ inhibition} = 100 \times (A_0 - A_1/A_0)$$

Where A_0 is the absorbance of control (heated BSA without extract) solution and A_1 is the absorbance in the presence of plant extract.

II.2.6.2 Test of antiproteinase action

The method of Oyedapo, et al. (1995) was used to study the antiproteinase activity which is also involved in the inflammatory process, one volume of polysaccharide extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/ml) was added to 2 volumes of Tris HCl (20 mM pH 7.4) containing 0.3 mg/mL of trypsine, the mixture was incubated for 20 minutes . Then the reaction was stopped by adding 2 volumes of 70% perchloric acid. The absorbance of the supernatant in the mixture was recorded at 210 nm after centrifugation. The buffer was used as white and the percentage of inhibition of the proteinase inhibitory activity was calculated as follows:

$$\% \text{ inhibition} = 100 \times (A_0 - A_1/A_0)$$

Where A_0 is the absorbance of control solution and A_1 is the absorbance in the presence of plant extract.

II.2.6.3 Membrane stabilization by hypotonicity induced hemolysis

Hemolytic activity was evaluated as described previously by **Oyedapo, et al. (1995)**. A volume of 5 mL of hypotonic PBS (10mM, 50mM NaCl, pH= 7.4) containing extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 µg/ml) was added to 0.5 mL of RBC suspension and then the samples were incubated for 10 min at 37 °C and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was then measured at 540 nm using a micro-plaque reader to determine the hemoglobin released from the lysed erythrocytes. The percentage of hemolysis inhibition was calculated for each sample according to the following law:

$$\% \text{ inhibition} = 100 \text{ x } (A_0 - A_1/A_0)$$

Where A_0 is the absorbance of positive control solution and A_1 is the absorbance in the presence of plant extract.

The negative control represents the blank where no hemolysis is induced; (0.5 mL of the RBC suspension with 5 mL of isotonic PBS) and the positive control where hemolysis is induced by a hypotonic phosphate buffer (10mM, 50mM NaCl, pH= 7.4) without extracts.

II.2.7 Anticoagulant activity

All tests were performed on plasma from citrated tubes recovered from healthy subjects (5 different donors). All tests were performed in triplicate.

II.2.7.1 Evaluation of Activated Partial Thromboplastin Time (APTT) (the endogenous way)

A volume of 50 µL of plasma was mixed with 10 µL of a solution of the different polysaccharide extracts at different concentrations (100, 50, 25 µg/ml) before adding 50 µL of APTT reagent. Then, the reaction mixture was incubated for 3 min at 37 °C. To trigger the coagulation cascade, 50 µL of $CaCl_2$ (0.025 M) were added. The coagulation time was recorded by a coagulometer and Heparin was used as the standard **(J. A. G. Rodrigues, et al., 2011)**.

II.2.7.2 Evaluation of Prothrombine Time (PT) (the exogenous way)

Briefly, 50 µL of plasma was added to 10 µL of a solution at different concentrations of different polysaccharide extracts and then incubated at 37 °C for 3 min. Then, 50 µL of 0.025 M $CaCl_2$ was added to the mixture to trigger the reaction of the coagulation cascade. The coagulation time was again recorded using a coagulometer (**J. A. G. Rodrigues, et al., 2011**).

II.3 Statistical analysis

All experimental results were expressed as mean ± SD. The differences between the groups were determined using the JMP software using analysis of variance (ANOVA) followed by the Tukey's test. The differences were considered significant at $p<0.05$.

III. Result and discussion

III.1 Chemical composition and yields

Polysaccharide extracts from Aleppo pine seeds was obtained by precipitation, using three different solvents (acetone, ethanol and propanol). According to the **Table 01**, the results revealed that extraction yields ranged from 2.204±0.04% (AP) to 2.04±0.05% (PP). The statistical analysis showed that both extracts (AP and EP) had significantly ($p<0.05$) a better yield than the PP extract. In fact, extraction yields depend on solvents **used (Azeredo & Oliveira, 1996)**. In this case, the extraction yield can be explained by the lower levels of sugars in Aleppo pine grains as reported by **Nabil Kadri, et al. (2015).** Although a comparable extraction yield was obtained with black cumin seeds (2%) (**Manjegowda, Rajagopal, & Dharmesh, 2017**). The effect of the three solvents used (acetone, ethanol, propanol) on total sugar and protein levels, was studied by measured them before and after deproteinization). The results indicate a high level of total sugar in AP extract before and after deproteinization with 70.543±0.48 and 65.43±0.38 % respectively compared to EP and PP extracts. Also, we can notice that AP, EP and PP extracts contained the same quantities of moisture and ash. Overall, the lipids were found in trace in all extracts. The protein content in AP extract was higher (13.636±0.20 %) than EP (12.333±0.08 %) and PP (12.31±0.09 %) extracts. However, deproteinization by calcium chloride eliminated the same amount of protein (about 88%) from all extracts, in close agreement with previous observations **(Huang, 2008).**

III.2 FT-IR analysis of polysaccharide extracts

The three infrared spectra (A, B and C) of **Figure 07** show almost identical gaits. The most marked peaks for the extract AP, EP and PP respectively are: peaks 3325, 3350 and 3316 cm^{-1} represent hydroxyl (OH) groups **(Branden & Tooze, 2012)**, peaks 2968, 2960 and 2956 cm^{-1} design C-H groups (**Branden, et al., 2012; J.-J. Shi, et al., 2016**), peaks 1647, 1643 and 1640 cm^{-1} express the presence of uronic acid by its COO or C=O groups **(Abdelmalek, et al., 2015; Yuan, et al., 2015)**, peaks 1542, 1544 and 1548 cm^{-1} are only impure (water, proteins or some polyphenols) **(J.-B. Lee, et al., 2015; J.-J. Shi, et al., 2016).** The presence of OCH_3 groups (pectin methyl ester) is demonstrated by peaks 1402, 1415 and 1404 cm^{-1} **(Abdelmalek, et al., 2015; Yuan, et al., 2015)**. Sulphate esters (SO) are also found (peaks 995, 988 and 995 cm^{-1}) **(Abdelmalek, et al., 2015)** and finally the presence of α-glycosidic bonds was demonstrated by peaks 850, 839 and 848 cm^{-1} **(Yuan, et al., 2015)**. The OH, CH, COO, COO, OCH_3 groups as well as sulphate groups, uronic acid and glycosidic bonds are characteristic groups of polysaccharides and are present in all three extracts, the difference may therefore lie in the quantity and location of these groups.

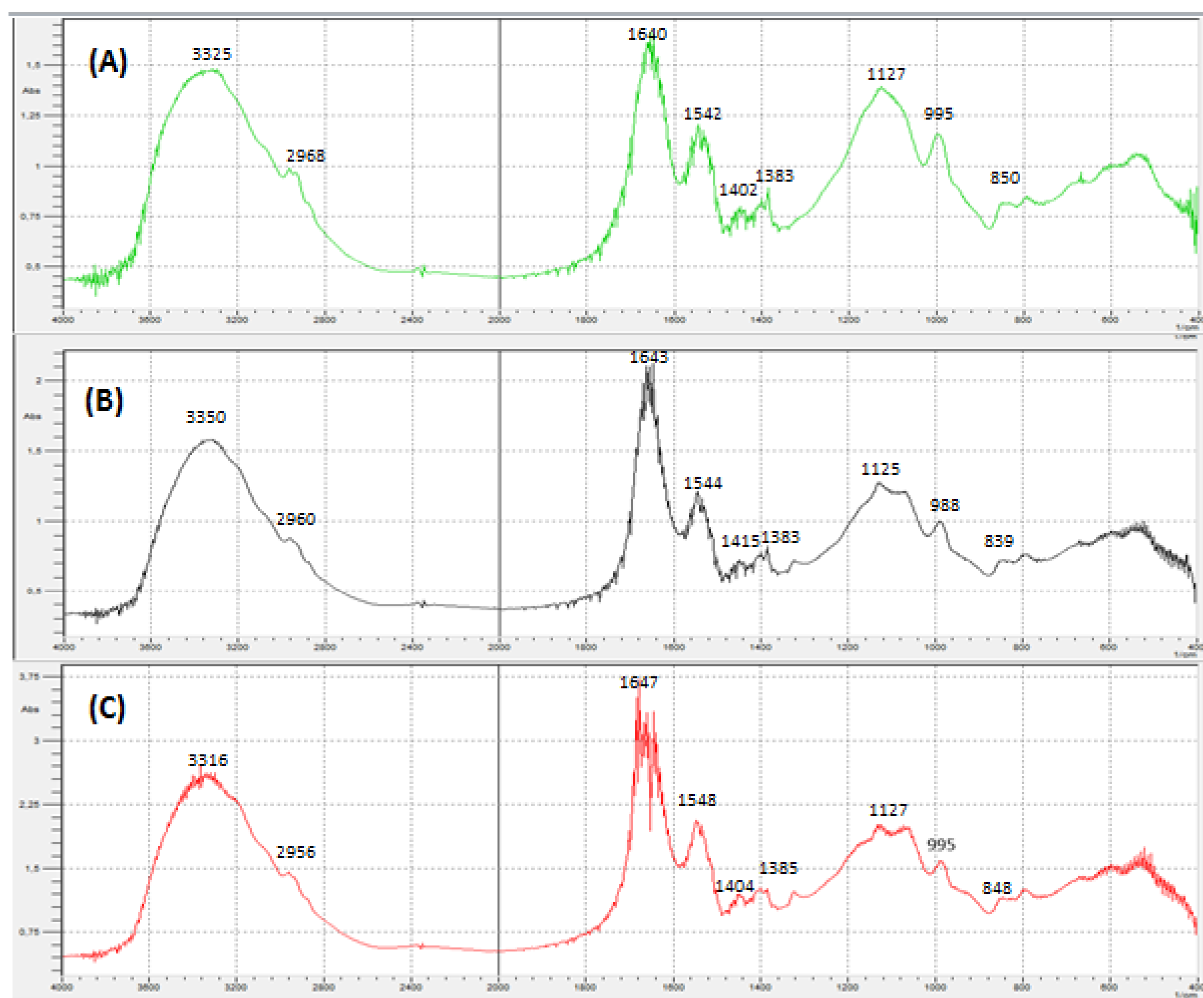

Figure 7: FT-IR spectrometry of polysaccharides extract (AP, EP, PP) from Pinus halepensis.Mill seeds. (A): Acetone polysaccharides extract, (B): Ethanol polysaccharides extract, (C): Propanol polysaccharides extract.

III.3. Cyto-toxicity essay

Red blood cells are one of the most widely used models for the study of *in vitro* toxicity **(Pagano, et al., 2015).** The results showed that no hemolysis was induced by any of the three polysaccharide extracts at all concentrations tested (no significant difference between the hemolysis percentages of the negative control and those obtained with the different concentrations of the different polysaccharide extracts). This implies that the polysaccharide extracts of Aleppo pine seeds do not present any toxicity even at high concentrations (4 mg/ml). The same results were obtained by **Ktari, et al. (2017)** for the polysaccharides of fenugreek seeds.

Table IV: Yields and composition of Acetone, ethanol and propanol crude polysaccharides of Pinus halepensis.Mill seeds.

	AP	EP	PP
Yields (%)	2.204±0.04[a]	2.123±0.07[a]	2.04±0.05[b]
Ash	1.33±0.32[a]	1.27±0.12[a]	1.39±0.21[a]
Moisture (%)	5.326±0.07[a]	5.396±0.02[a]	5.31±0.06[a]
Fat (%)	-	-	-
Carbohydrates before deproteinization (%)	70.543±0.48[a]	67.953±0.72[b]	65.71±0.38[c]
Carbohydrates after deproteinization (%)	65.43±0.38[a]	63.02±0.68[b]	60.89±0.39[c]
Loss of carbohydrates (%)	7.25±0.08[a]	7.276±0.03[a]	7.33±0.06[a]
Proteins before deproteinization (%)	13.636±0.20[a]	12.333±0.08[b]	12.31±0.09[b]
Proteins after deproteinisation (%)	1.54±0.15[a]	1.37±0.02[b]	1.413±0.10[b]
Loss of proteins (%)	88.323±0.80[a]	88.57±0.34[a]	88.486±0.88[a]

Values are means ± S.D. (n = 3). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract.

III.4 Antioxidant activities

To investigate the antioxidant activity of polysaccharide extracts of Aleppo pine seeds, six assays have been selected. The results of the total antioxidant activity estimated from the evaluation of DPPH and ABTS radical scavenging, metal chelation and lipid antiperoxidation activities, they were expressed as an IC_{50}. The reducing power was expressed as an EC_{50} (represents the absorbance of 0.5) and the oxygen radical absorbance capacity is expressed in ORAC value (equivalent in µM trolox equivalent).

III.4.1 Free radical scavenging activity (DPPH and ABTS)

The ABTS and DPPH tests were widely used to evaluate the ability of molecules to scavenge free radicals. As shown in **Table 02**, the highest DPPH scavenging activity was displayed by the AP extract with IC_{50}= 79.90±1.26 µg/ml, comparatively to other extracts.

Obtained results showed that *Pinus halepensis* Mill. seeds are a rich source of polysaccharides with antioxidant activity compared to other studies. Recently, **Ktari, et al. (2017)** reported an antioxidant activity of 73.0% at 10 mg/mL of fenugreek seeds. In addition, **Trigui, et al.**

(2018) obtained a DPPH inhibition percentage of 63.25% at concentration of 1 mg/mL polysaccharides from black cumin seeds Similary. **Sila, et al. (2014)** found an IC_{50}= 2.81 and 2.59 mg/mL for polysaccharides from almonds and pistachios respectively.

In the case of ABTS scavenging activity, the AP extract was the most effective compared to other extracts (IC_{50}= 57.29±0.46 µg/ml), which highlights the presence of potential antioxidants in this extract. These results revealed that the polysaccharides of AP extract contain many hydroxyl groups, with high hydrogen donating capacity. However, obtained results were more potent to those of other tested polysaccharides extracts such as *Plantago asiatica* seeds polysaccharides (IC_{50}=0,7 mg/ml) **(Ye, Hu, & Dai, 2011),** quinoa seeds polysaccharides (IC_{50} =1.108 mg/ml) **(Y. Hu, et al., 2017)** and of *sorghum bicolor* seeds polysaccharides (IC_{50} =20 mg/ml) **(Slima, et al., 2018)**. Many authors **(Floegel, Kim, Chung, Koo, and Chun (2011); Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Byrne (2006))** reported a good correlations between the results of the antioxidant activities using ABTS or DPPH radicals, which is in agreement with our studies. The difference revealed in the antioxidant activities of the three extracts may be due to the fact that ABTS test was more specific than the DPPH test, being exclusively an electron donor antioxidant. Whereas DPPH test combines the evaluation of both the hydrogen-donating capacity and reducing abilities. In addition, several studies reported that the antioxidant activity of polysaccharides can be influenced by several parameters (molecular weight, sulphate content, uronic acid content and the glycosidic bonds they contain) **(Zheng, Li, & Wang, 2014)** and the presence of carboxyl groups **(Luo, et al., 2016)**. Thus, it may be supposed that the three extracts may present different composition or different amount.

III.4.2 Metal chelating activity

Iron is another element which can induce oxidative damage to living tissues by the generation of OH•. Therefore, effective Fe^{2+} chelators afford protection against lipid peroxidation **(Ktari, et al., 2017).**

Different results were obtained regarding ferrous ion chelating activity, where PP was slightly less active than AP (IC_{50}=27.20±0.88 µg/ml) and PP samples (IC_{50}=28.10±0.41 µg/ml). **Sila, et al. (2014)** reported a metal chelating activity with an IC_{50} of 3.39 mg/Ml for pistachio polysaccharides and 0.22 mg/mL for almonds and pistachio polysaccharides respectively and **Trigui, et al. (2018)** showed that black cumin seeds exhibited an IC_{50} of 0.78 mg/ml. Indeed,

the metal chelating activity test showed less difference between extracts than what was observed in ABTS test. This may supposed the structure-antioxidant activity relationship, which is reported in several studies.

Also, according to the literature, compounds chelating metal ions generally have functional groups such as SH, COOH, OH, PO_3H_2, CO, NR_2, O and S **(Jiang, et al., 2014).** These groups do not act in the same way and not with the same efficacy **(Qi, et al., 2005).**

III.4.3 Lipid peroxidation

Unlike the ABTS and DPPH activities, AP and EP extracts demonstrated a weak lipid peroxidation inhibition activity. PP extract was effectively inhibited the lipid peroxidation initiated by iron-oxygen complexes with an IC_{50} of 4.88±6.04 µg/ml, EP extract showed moderate activity against inhibition of lipid peroxidation with an IC_{50} of 39.51±6.13 µg/ml. Although the lowest activity was obtained with AP extract (IC_{50}=142.3±5.52 µg/ml). The statistical analysis revealed a significant difference between the IC_{50}s of the three extracts. Similarly, based on the findings of **Sila, et al. (2014),** the polysaccharide of almonds and pistachio may prevent the lipid peroxidation performed by the beta carotene bleaching test with the IC_{50} of 4.46 mg/mL and 3.39 mg/mL respectively.

III.4.4 Ferric reducing power

The reducing capacity of natural compounds is a good indicator of antioxidant potential, resulting from their ability to hydrogen atoms and/or electron transfer (Ktari, et al., 2017). The ability of polysaccharide extracts to reduce the Fe^{3+}/ferricyanide complex was evaluated by following the formation of the Perl's index of the blue complex which absorbs at 700 nm **(Ferreira, Baptista, Vilas-Boas, & Barros, 2007).**

AP marked a significant reductive power with an EC_{50} of 46.40±5.21 µg/mL **(Table 02)**. On the other hand, a lower reducing activity of EP and PP was recorded (EC_{50} of 164.5±18.15 µg/mL and 173.7±0.47 µg/mL respectively). which are also very interesting compared to other results found by other authors. In fact, EC_{50} of 7.76 mg/mL was found for polysaccharides from *Plantago asiatica* seeds **(Ye, et al., 2011)** and 4-5 mg/mL for peony seed dreg polysaccharides **(J.-J. Shi, et al., 2016).**

The reducing properties are associated with the reductones possessed by the extract, which on the one hand carry out their activity by giving a hydrogen atom which breaks the chain of free

radicals **(Gordon, 1990).** On the other hand, they prevent the formation of peroxides by reacting with some peroxide precursors **(Qi, et al., 2005).**

III.4.5 ORAC value

The oxygen radical absorbance capacity is the only antioxidant activity that combines and evaluates both degree and time of inhibition in a single amount **(Yao, Shi, & Ren, 2014)** and **(Yao, et al., 2014)** and where the operating mechanism is H-atom transfer reactions from the phenols to AAPH-derived peroxyl radicals **(Dairi, et al., 2014)**. In fact, it allows detecting the trapping of physiological radicals (OH and ROO°) which are most involved in lipid oxidation **(Gamal-Eldeen, Amer, Helmy, Talaat, & Ragab, 2007)**.

ORAC of the three extracts (AP, EP and PP) were tested and a very good results have been shown with the AP extract having the best ORAC value (1.93±0.0 µM trolox equivalent) followed by the two extracts (EP and PP) which showed no significant difference with the ORAC values of 1.09±0.39 µM trolox equivalent and 1.19±0.31 µM trolox equivalent respectively. Knowing that for this activity, the concentration of 30 µg/mL has been tested, so this value is very important. **Lu, You, Lin, Zhao, and Cui (2013)** reported an ORAC value of 132.14 µM equivalent trolox for *Laminaria japonica* but which has been evaluated at 1g/ml.

All these observations confirmed that the chemical assays showed only the ability of polysaccharide extracts to neutralize free radicals by electron or hydrogen atom transfer, and lack a biological significance because the peroxidation occur in a complex system where different mechanism of antioxidant implication may be explored **(Dairi, et al., 2014)**. This could explain the difference observed sometimes between chemical assay results and lipid model oxidation results. The lipid systems used revealed different mechanism by which antioxidants may act against lipid peroxidation. Indeed, the result of this study confirmed the difference in kind and structure of polysaccharide present in the tested samples and a relation between the hydro-solubility of the precipitant agent used and the antioxidant activity observed.

Table V: Antioxidant activity of crude polysaccharide extracts of *Pinus halepensis* seeds

Antioxidant activity	AP	EP	PP
DPPH (IC_{50} µg/mL)	79.90±1.26^{b}	82.02±1.16^{b}	85.97±1.71^{a}
ABTS (IC_{50} µg/mL)	57.29±0.46^{c}	99.54±0.58^{b}	676±1.01^{a}

Metal chelation (IC_{50} µg/mL)	27.20±0.88[b]	28.10±0.41[a,b]	28.75±0.33[a]
Lipid peroxidation (IC_{50} µg/mL)	142.3±5.52[a]	39.51±6.13[b]	4.88±6.04[c]
Ferric reducing power (EC_{50} µg/mL)	46.40±5.21[b]	164.5±18.15[a]	173,7±0.47[a]
ORAC value (µM trolox equivalent) (30µg/ mL d'extrait)	1.93± 0.0[a]	1.09±0.39[b]	1.19±0.31[b]

Values are means ± S.D. (n = 3). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract.

III.5 Anti-inflammatory activities

III.5.1 Inhibition of BSA denaturation

Inflammatory and anti-inflammatory processes involve many molecules, most of which are proteins. The denaturation of these proteins causes them to lose their biological properties, which can trigger or accentuate inflammation **(Brown & Mackey, 1968)**. This denaturation often involves the alteration of the bonds they constitute (hydrogen, electrostatics, hydrophobic and disulfide) **(Grant, Alburn, & Kryzanauskas, 1970).**

The polysaccharide extracts had a good anti-denaturation activity of the BSA, in first position the AP extract with an IC_{50} of 153±0.2 µg/ml, then the EP extract with an IC_{50} of 335.2±2.14 µg/mL and finally the PP extract with an IC_{50} of 1159±0.06 µg/mL with significant differences.

The exact mechanism of protein denaturation inhibition is still not very well known **(Bailey-Shaw, Williams, Green, Rodney, & Smith). Chandra, Chatterjee, Dey, and Bhattacharya (2012)** reported that protein denaturation increases the viscosity of the medium, so the denaturation protective effect of BSA can be supported by the viscosity change. On the other hand, for the global biological pathway, the BSA NMR analysis performed by **Rösner, Williams, Jung, and Kraus (2001)**. **Williams, Vasquez, Milan, Zebitz, and Kraus (2002)** showed that the latter contained two active sites with the amino acids threonine, lysine and tyrosine to which the bioactive molecules could bind to activate and regulate signal transduction. **Duganath, Kumar, Kumanan, and Jayaveera (2010)** report that this effect could also be due to the binding of the bioactive molecules to plasma proteins and thus protect them from any aggression.

III.5.2 Anti-hemolytic activity

The erythrocyte hemolysis test has long been used for the strong analogy between the erythrocyte and lysosome membranes; both membranes have a lipid bilayer rich in protein (50%) and oligosaccharides. The stabilization or destabilization of one necessarily results in the stabilization or destabilization of the other **(Omale & Okafor, 2008).**

Table 03 shows that the three extracts (AP, EP and PP) present very high antihemolytic activity with IC_{50}s of 8.01±1.097, 31.25±2.42 and 103.4±2.82 µg/mL respectively. This mean that extracts are very effective with decreasing degrees (AP>EP >PP).

The exact mechanism of erythrocyte membrane stabilization is not yet well known. However, the literature reports several hypothetical mechanisms; for example, the extract could influence the surface volume ratio by increasing the erythrocyte membrane or reducing cell volume by interacting with membrane proteins. Therefore, this protective effect may be due to the ability of the extracts to modify the flow of calcium in the erythrocyte **(Shinde, et al., 1999)** or by binding to membrane components, in particular membrane proteins, thus contributing either to the regulation of the intracellular water volume by controlling the movement of sodium and potassium ions through protein channels **(Umapathy, et al., 2010)** or by inducing a subsequent modification of the charges on the membrane surface, which may prevent physical interaction with the aggregating agents or promote charge repulsion **(Oyedapo, et al., 1995).**

III.5.3 Antiproteinase activity

Leelaprakash and Dass (2011) reported that during the inflammatory reaction, leukocyte proteinases are very important in the development of tissue lesions and proteinase inhibitors would ensure a reduction of these lesions and would therefore be considered as an anti-inflammatory.

The EP extract exerted the best anti-proteinase activity with an IC_{50} of 24.19±3.17 µg/ml, then the PP extract with an IC_{50} of 348.2±3.42 µg/mL and finally in the last position the AP extract with an IC_{50} of 2032±1.78 µg/ml. This effect could be explained by the fact that polysaccharide extracts compete with casein by binding to the active sites of trypsin and the difference observed between the three extracts could be due to the difference in affinity of their compounds to the active sites.

Ibrahim, Mahmoud, and Asker (2014) also found good anti-inflammatory activity *in vitro* against COX-1 and COX-2 of polysaccharide from *Adansonia digitata.*

Table VI: In vitro anti-inflammatory activities of crude polysaccharide extract of Pinus halepensis seeds.

Anti-inflammatory activities	**AP**	**EP**	**PP**
Inhibition of BSA denaturation (IC_{50} µg/ml)	153±0.2[c]	335.2±2.14[b]	1159±0.06[a]
Anti-hemolytic activity (IC_{50} µg/ml)	8.01±1.097[c]	31.35±2.42[b]	103.4±2.82[a]
Antiproteinase activity (IC_{50} µg/ml)	2032±1.78[a]	24.19±3.17[c]	348.2±3.42[b]

Values are means ± S.D. (n = 3). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract.

III.6 Anticoagulant activities

The anticoagulant activity of polysaccharide extracts of Aleppo pine seeds was measured by two *in vitro* tests. One of which is the activated partial thromboplastin time that makes it possible to explore the activity of factors II, V, VII, IX, X, XI, XII of the endogenous pathway and the common coagulation pathway and the other which explores factors II, V, VII and X of the extrinsic pathway and the common coagulation pathway **(Batty & Smith, 2010).**

Table 04 shows that the three extracts exhibited high partial activated thromboplastin times compared to the negative control (41±2 s) with a remarkable dependency dose. At the three concentrations used, the difference is significant between the three extracts. In fact, the acetone extract has a higher activity than the ethanol extract and the latter is more active than the propanol extract. For PT activity, the extracts exhibited less activity but in the same order of effectiveness with the same dose of dependence. However, their activity was less than that of heparin, which is a reference molecule for both APTT and PT. These results are better than those found by **Arivuselvan, Radhiga, and Anantharaman (2011)** with the polysaccharides of brown algae (*Turbinaria ornata*) which showed 170 and 6 seconds for APTT and PT respectively at a concentration of 125 µg/ml.

several authors **(Nishino, Aizu, and Nagumo (1991); Pereira, Melo, and Mourão (2002); Zhang, et al. (2008))** reported that anticoagulant activity was highly dependent on sulphate content, the binding sites of some sugars and their molecular size.

In addition, **Fonseca, et al. (2008)** confirms that the change in sulphation proportions and positions in polysaccharide chains could be critical importance to coagulation system activators and inhibitors.

Table VII: anticoagulant activities (APTT and PT) of crude polysaccharide extracts (AP, EP and PP) of Pinus halepensis seeds.

	AP	**EP**	**PP**	**Positive control (heparin)**
	APTT (s)			
100 µg/ml	210±5^{b}	170±7^{c}	150±9^{d}	1700±12^{a}
50 µg/ml	156±4.5^{b}	110±2^{c}	90±8^{d}	854±9^{a}
25 µg/ml	98±8^{b}	66±5.5^{c}	57±3^{d}	509±6^{a}
	PT(s)			
100 µg/ml	90±6^{b}	82±5^{c}	67±2.5^{d}	1300±19^{a}
50 µg/ml	66±4^{b}	58±4^{c}	49±3^{d}	699±16^{a}
25 µg/ml	54±3.5^{b}	47±2^{c}	39±1.5^{d}	402±10^{a}

Values are means ± S.D. (n = 3). APTT (s) activated partial thromboplastin time (seconde) PT(s):Prothrombine Time (seconde). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract.

IV. Conclusion

This study was designed to evaluate, the effect of solvent precipitation (acetone, ethanol, propanol) of the polysaccharides extract from Aleppo pine seed on different biological activities (antioxidant, anti-inflammatory and anticoagulant activities)**.** The results recorded showed that Aleppo pine seed polysaccharides are very active without any toxicity. However, the polarity of precipitation solvents plays a crucial role in yields, purity levels as well as activities. The AP extract was most effective in almost activities. This supposes that acetone is the best precipitating agent giving the most active polysaccharides with perhaps more functional groups. The polysaccharides of Aleppo pine seeds can be a nutraceutical agent for the very interesting activities they have presented but the precipitation solvent of polysaccharides play a crucial role on their bioactivity.

Chapter III:

Optimizing functional properties and chemical composition of Pinus halepensis Mill. Seeds protein concentrates

Chapter III: Optimizing functional properties and chemical composition of *Pinus halepensis Mill.* Seeds protein concentrates

Abstract

Vegetable proteins are widely used in many food formulations due to their physico-chemical properties, low cost and availability. The main objective of this work is to study the chemical composition and properties of a protein concentrate of *Pinus halepensis*.Mill seeds (PHPC) and mainly to optimise the effect of pH, NaCl concentration and phosphate buffer (PB) molarity on functional properties (solubility, emulsifying activity index (EAI) and foaming capacity (FC)) of this concentrate by response surface methodology (RSM). The chemical composition was determined in terms of proteins, sugars, lipids, ash and moisture. The physico-chemical characteristics were studied by their water and oil holding capacity (OHC, WHC) and their surface hydrophobicity (SH). Finally, the functional properties of PHPC were studied in terms of solubility, EAI, FC, minimum gelling concentration (MGC) and finally heat coagulability (HC). A PHPC yield of 36.66±0.7% was obtained. The WHC and OHC was 3.89g water/g PHPC and 3.54g oil/g PHPC respectively and a SH of 87.09±0.78 was obtained. The optimization results showed that the optimal conditions for solubility, EAI and FC were: pH:10.88, NaCl:0 g/l, PB:0.078 M; pH:12, NaCl:0.55 g/l, PB:0.1M and pH:2, NaCl:0, PB:0 M respectively, having given a solubility of 87.13±0.14%, an EAI of 36.82±0.34 and a FC of 182.72. Then, the desirability of the three responses (solubility, EAI and FC) which was pH:12, NaCl: 0.55g/l and PB of 0.1M was used to assess the stability of EAI and FC, to determine the MGC and HC. This study shows that Aleppo pine seeds are a good source of functional proteins, potentially applicable in the food industry and that pH, NaCl concentration and PB molarity have a major influence on functional properties.

Chapter III: Optimizing functional properties and chemical composition of Pinus halepensis Mill. Seeds protein concentrates

I.Introduction

Vegetable proteins are a very good alternative to animal proteins whether for food or cosmetic application, because of their low cost, abundance and diversity of their sources (legumes, cereals and oilseeds), their adequate quality and nutritional value, their ease of digestion, their non-toxicity and finally for their functionality **(S. Damodaran, 2000; I. M. Rodrigues, Coelho, & Carvalho, 2012; Soria-Hernández, Serna-Saldívar, & Chuck-Hernández, 2015**).

In America, 60 % of the population relies heavily on the protein content of food when choosing their product, because among the three primary metabolites (carbohydrates, proteins and fats), proteins are the most beneficial for their health. Most adults perceive proteins as the most energy-efficient ingredient that is very healthy and improves muscle tone. They are macronutrients most considered in weight management diets **(Cheatham, 2014**).

In recent years, oilseed proteins have made a very significant contribution to protein intake in the diet. In 2004/2005, 380 million tonnes of oleaginous plants were produced and 207 million tonnes of protein meals were produced worldwide **(Ash, Dohlman, & Davis, 2006**).

The most commonly used proteins of oleaginous origin are that of soybean, peanut and rapeseed for their functionalities in food processing (additives and the protein film industry). With the awareness of their usefulness and therefore the increase in needs, new sources have been developed, such as cashew nut **(Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009**), milk weed **(Hojilla-Evangelista, Evangelista, & Victor Wu, 2009)** and almost all oilseeds. *Pinus halepensis* Mill seeds, come from a very abundant tree belonging to the Pinaceae family which can found on all the Mediterranean relief especially in Algeria and Tunisia **(Maestre, Cortina, Bautista, & Bellot, 2003**). The bible's manual of medicinal plants reports that they have been used extensively in pastry making, especially in Tunisia, and are also used to cure diabetes and sexual weakness in the eastern region of the Mediterranean **(Schiller, 2014**). **N. Kadri, et al. (2015)** studied the chemical composition of its seeds and found a protein percentage of 26.62 ± 0.129 which is a very high percentage especially compared to other species *Pinus pinea* L., *Pinus pinaster* and *Pinus canariensis*.

Functional proteins are those that when added to food, confer nutritional, sensory, physico-chemical and organoleptic properties (color, texture, flavor...). Functional properties could be classified according to their physico-chemical mechanisms as follows: hydration properties

(water/oil retention and solubility) rheological properties (viscosity, elasticity, aggregation and gelation), and protein surface properties (emulsifying and foaming activities, surface hydrophobicity and whipping) **(Moure, Sineiro, & Domínguez, 2001)** .

Food applications of proteins is limited by their low solubility **(Moure, et al., 2001)**, it is known that the pH, presence or absence of salts and its concentration and thus the ionic strength of the medium, as well as electrostatic repulsions influence the functional properties of proteins **(Soria-Hernández, et al., 2015)**. For this purpose and taking into account that according to the databases consulted, no studies were carried out on the functional properties of Aleppo pine seed proteins, the physico-chemical characteristics of *P. halepensis* Mill. seed concentrated proteins (PHPC) (approximate composition, water and oil holding capacity (WHC and OHC), surface hydrophobicity (SH)) were determined and the solubility, emulsifying activity index (EAI) and foaming capacity (FC) conditions were optimized using the Box Behnken Design (BBD) by the response surface methodology, to study the effect of the parameters considered (pH, NaCl concentration and phosphate buffer molarity) on each of the responses and the relationship between solubility and functional activities. Then the heat coagulability (HC) and the minimum gelling concentration (MGC) were determined under optimal conditions.

II. Material and methods

II.1 Plant material

The seeds of Aleppo pine (*Pinus halepensis* Mill.) were obtained from the Collo forest located in Skikda province of Algéria in May 2018. They were cleaned with bidistilled water; dried in an oven at 40 ° C for 2 days and then finely crushed using an electric grinder (KIKA Labortech- nik M20, Germany) until it became a fine powder (< 250 µm) which was delipidated by the Soxhlet method with petroleum ether.

II.2. Preparation of the protein concentrate

A mass of 10 g of delipidated powder was macerated under stirring for 20 min at room temperature. After filtration and centrifugation at 4 °C for 20 min at 6000 rpm, the supernatant was filtered again and its pH was adjusted to 6 (with 0.1 M HCl), $CaCl_2$ was added gradually until a concentration of 1 M, then centrifuged at 6000 rpm for 20 min. Finally, the recovered pellet was washed with distilled water and freeze-dried **(Rotimi E Aluko, McIntosh, & Katepa-Mupondwa, 2005).**

II.3. Chemical composition and yield

The extraction yield was expressed by the ratio of the amount of extract to the amount of defatted powder used. Ash, moisture and fat were determined according to **AOAC (1998a)** methods, the protein content was determined by the **Bradford (1976)** method and the carbohydrates content was carried out by the **Dubois, et al. (1956)** method using BSA and glucose for calibration curves respectively.

II.4. Water and oil holding capacity (WHC/OHC)

The**Tan, Ying-Yuan, and Gan (2014)** method was used to determine the capacity of the extract to retain water or oil. For this purpose, 100 mg of extract was suspended with the same amount of water or sunflower oil (1.5 ml), vortexed for 1 minute, and then centrifuged at 3000 rpm for 20 min. The water and oil retention capacity was expressed in gram of water or oil retained per gram of extract.

II.5. Surface hydrophobicity (SH)

The bromophenol blue (BPB) binding method was used to study the surface hydrophobicity of the protein concentrate. A volume of 1 ml of protein concentrate suspension (5 mg/ml in

20mM PB at pH 7) was added to 200 µl of BPB solution (1 mg/ml in distilled water). The mixture was vortexed for 10 min and directly centrifuged at 3000 rpm for 15 minutes. Finally, the supernatant absorbance was read at 595 nm. A control containing 1 ml of PB (20 mM, pH 7) and 200 µl of BPB solution was used **(Mune & Sogi, 2016)**. The surface hydrophobicity is according to the following formula:

SH (%) = BPB bound (%) = (Absorbance control – Absorbance sample)/Absorbance control X 100

II.6. Optimization of solubility conditions of PHPC and its functional properties

Before optimization of solubility conditions, three parameters (pH, NaCl concentration and PB molarity) were studied separately in the single-factor experiment, keeping the variables that were not studied constant each time in order to limit overall experimental work. After statistical analysis of the results of this preliminary study, three variables were selected as significant factors and three levels were used for each one. The study intervals were also determined for each parameter and then the response surface based on the Box Behnken Design was designed to obtain the conditions giving the best solubility, EAI and FC.

II.6.1. Protein solubility

A mass of 100 mg of PHPC was dispersed in 10 ml of different solutions prepared at the pH, NaCl concentration and molarity of PB determined according to the design of experiment. The dispersions were vortexed well for 15 minutes then centrifuged at 3000 g for 20 min. The protein content of the supernatant was determined by the Lowry method **(Peterson, 1977)** and the solubility was calculated as follows:

Solubility (%) = Protein content of supernatant x 100 /Total protein content.

Total protein content represents 100% solubility and is determined in 3% NaOH (Chao, Jung, & Aluko, 2018).

II.6.2. Emulsifying properties

The emulsifying properties of the PHPC were determined using the method reported by **Boye, et al. (2010).** A volume of 45 ml of protein solution (0.5% in different solution of pH, NaCl and PB) was added into 15 ml of sunflower oil. After homogenization of the emulsion with an ultra turrax (IKA T25, Staufen, Germany) for 1min at 20000 rpm, 50 µL of the prepared

solution were diluted in 5 ml of sodium dodecyl sulfate (SDS) at 0.1%. Finally, the absorbance was recorded at 500 nm. The EAI was calculated using following equation:

$$EAI\ (m^2/g) = 2 \times 2{,}303 \times A_0 \times DF / C \times \varphi \times 10000$$

Where A_0 is the absorbance of the emulsion after emulsification, DF is the dilution factor, C is the weight of the protein per volume (g/mL), φ is the volume fraction of the oil in the emulsion.

II.6.3. Foaming properties

Foaming capacity of PHPC was determined according to the method of **Shahidi, Han, and Synowiecki (1995).** A volume of 20 ml of protein concentrate solution at 0.1% (W/V) was homogenized using a Moulinex_R62 homogenizer to incorporate the air for 1 min at room temperature (25 ± 1°C). The FC was expressed as percentage of volume increase after homogenization, which was calculated according to the following equation:

FC (%) = ((volume after whipping−volume before whipping) / volume before whipping) ×100.

a. Experimental design

To optimize the factors affecting solubilization, foam capacity and emulsifying activity, the response surface methodology (RSM) with Box Behnken Design was studied using Minatab 17 (statistical analysis system Inc., SAS) software and the experimental values obtained for solubility, EAI and FC were compared to their values predicted based on the t-test ($p<0.05$) (Table 07). In this study, fifteen tests were performed with the different values of pH (2, 7, 12), NaCl concentration (0, 0.275, 0.55 g/ml), and PB concentration (0, 0.05, 0.1 M) as shown in **Table 01**. The values were coded as follows: (+1) maximum value, (0) central value and (-1) minimum value. The experimental data were adjusted to a second order polynomial model and expressed by following equation:

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

B_0 (constant coefficient); Bi, Bii, Bij (regression coefficients for intercepting, linear, quadratic and interaction terms, respectively); xi and xj (independent variables); k (number of optimized factors).

b. Validation of model

In order to draw conclusions from the validation of the model, the Minitab software provides the optimal conditions of the three factors (pH, NaCl, PB) from the three responses designed. The optimums responses obtained were used to test solubility, FC and EAI. Finally, the experimental optimums of each obtained response were verified by comparing them with the predicted values.

After optimization and validation of the experimental design, a compromise solution was obtained by using the desirability function. The desirability is an important function when multiple response optimizations were carried out because it's not possible to optimize each one in separate way. For that, the overall solution must be included in optimal region leading to a certain degree of compliance with the proposed criteria for each variable of the system; namely, a compromise solution must be found. Desirability (d) always takes values between 0 and 1, where D = 0 for an undesirable response, and d=1 represents a completely desirable value (Candioti, De Zan, Camara, & Goicoechea, 2014). The stability of the functional properties studied (FC and EAI) after 15, 30, 45 and 60 min and other functional properties (MGC and HC) were tested at the optimal conditions obtained by desirability.

II.7. Stabilization of foaming and emulsifying properties

To study the kinetic of the fo.aming and emulsifying activities depending on the time (15, 30, 45 and 60 min), the PHPC solution was prepared with the optimum of pH, NaCl and PB concentrations. The stability is expressed as a percentage of remaining of these two properties **(Boye, et al., 2010; Shahidi, et al., 1995).**

II.8. Minimum gelling concentration (MGC)

The method of **O'Kane, Vereijken, Gruppen, and Van Boekel (2005)** was used to determine the MGC with a slight modification. A volume of 5 ml of PHPC solution was prepared at the concentrations of 4-18% (w/v) and then heated in water bath at 95° C for 10 minutes (in sealed tubes to avoid evaporation). After cooling, the tubes were placed at 4 °C for 12 hours and then inverted. The MGC is the smallest concentration from which the contents of the inverted tube do not flow.

II.9. Heat Coagulability (HC)

For Heat Coagulability (HC), the solubility method described above was used, the suspension of PHPC under optimal conditions was vortexed and the proteins of the supernatant were

measured by the Lowry method. An aliquot of the supernatant was heated in a water bath at 100 °C for 30 min. After cooling and centrifugation at 3000 rpm for 15 min, a filtration was carried out on Whatman No. 2 filter paper, and concentration of proteins in the filtrate were again determined by the same method **(Voutsinas, Nakai, & Harwalkar, 1983).** The HC of the sample was calculated from the following equation:

$$\% \text{ Heat Coagulability} = Ps - Pf / Ps \times 100$$

Where:

Ps = % protein in supernatant

Pf = % protein in filtrate

III. Results and discussion

III.1 Proximate composition

As shown in **Table 02**, the extraction yield of PHPC was 36.66±0.7%, of which approximately 69.33±0.3% are proteins. Among the impurities, we found sugars representing 2±0.2%, which can be justified by the presence of glycoproteins also reported by **N. Kadri, et al. (2015)** and minerals (ash) found in the proportion of 4.9%. However, the lipids were found only in trace form, which confirms the good delipidation of the powder before extraction. The moisture test revealed a level of 2.4±0.2% which is comparable to lyophilized extract dried by other methods such as Bambara concentrate in which the moisture content is of the order of 4**% (Adeleke, Adiamo, & Fawale, 2018).**

Table VIII: Proximate chemical composition of PHPC.

	Rate (%)
Yield	36.66±0.7
Ash	4.9±0.3
moisture	2.4±0.2
proteins	69.33±0.3
carbohydrats	2±0.02
Fats	-

Data are the mean± SD of three analyses.

III.2 Water and oil holding capacities (WHC/ OHC) and surface hydrophobicity

III.2.1 Water and oil holding capacities (WHC/ OHC)

The Water and oil holding capacities of PHPC were evaluated and the results are represented in **Table 03**. The amount of water and oil that binds BPB depends on the polar and non-polar, ionized or deionized groups of proteins **(Ghribi, et al., 2015)** and these properties mean that these proteins can be used as an additive to improve food quality **(Tontul, Kasimoglu, Asik, Atbakan, & Topuz, 2018)**.

In our study, the WHC of PHPC was found at 3.89 g water/g PHPC **(Table 03)**. This is in

agreement with the WHC range of products with water retention capacity (1.49-4.71) **(Kaur & Singh, 2007).** This capacity can be explained by the large particle size of the extract as well as the capacity of the sugar and fibers found in the concentrate as impurities which are known for this capacity **(Zhao, et al., 2012)**. The capacity of this extract is greater than that of most protein extracts reported by the bibliography, for example, WHC chickpea protein concentrates were found at 3.65 for freeze-dried extract and in agreement with that found for rapeseed protein isolate (3.85 g water/ g extract) **(Yoshie-Stark, Wada, Schott, & Wäsche, 2006).** Therefore, this extract can be used as an additive for viscous foods **(Aletor, Oshodi, & Ipinmoroti, 2002)**.

The oil retention capacity was found in 3.54 g oil / g extract **(Table 03)**, This is in the range of literature values (1.1- 4.1) (Kaur, et al., 2007). This good capacity can be explained by the hydrophobic properties of PHPC and the non-polarity of the side chains of its amino acids. This extract can therefore be used as an additive to confer an organoleptic quality to a fatty food such as dairy products **(Ghribi, et al., 2015).**

III.2.2 Surface hydrophobicity (SH)

The SH informs us about the surface-active properties of the extract. The **table 03** shows that SH of PHPC studied by binding to the BPB has been found in order of 87.09±0.78 %. It is higher than that found by **Tontul, et al. (2018)** (60.98%), this means that our PHPC can have promising surface-active properties.

Table IX: Water and oil holding capacities (WHC/ OHC) and surface hydrophobicity of PHPC.

Parameters	
Water holding capacity (g of water/g of PHPC)	3.89±0.06
Oil holding capacity (g of oil/ g of PHPC)	3.54±0.02
Surface hydrophobicity (%)	87.09±0.78

Data are the mean± SD of three analyses.

Table X: solubility, emulsifying activity and foaming capacity of PHPC on distilled water

Functional properties	
Solubility	27.02± 0.52
EAI	20.89± 0.24
Foaming capacity	61.66± 0.66

Data are the mean± SD of three analyses.

III.3 Optimization by RSM

III.3.1 Model analysis

Combination of the three studied factors (pH, concentration of NaCl and PB molarity) and the value of the corresponding response obtained in different experiments were presented in **Table 01**. It indicated that solubility was ranged from 21.18 to 87.04, the EAI from 18.84 to 34.48, while FC varied from 16.66 to 123.33. The values of the experimental results are consistent with the predicted values for the three responses.

III.2.2 Analysis of response surface

RSM based on BBD was applied to disclose optimal levels for the studied parameters (pH, NaCl concentration and PB molarity). Surface response models were the best method which illustrates the effects of independent variables and their interactions on the solubility of PHPC, their emulsifying activity and their foaming capacity. Experimental data were fitted to second order polynomial model.

Table XI: Box–Behnken design matrix and experimental and predicted data.

Run	pattern	Variables			Solubility	Pred Formula solubility	EAI	Pred Formula EAI	FC	Pred Formula FC
		pH (X1)	C NaCl (X2)	PB M(X3)						
1	0−−	7	0	0	28	33.9310905	21.37	21.9767438	61.66	60.40375
2	−0−	2	0.275	0	44.11	49.4575682	24.177	24.515224	91.66	91.6725

3	+0−	12	0.275	0	26.14	24.5587616	18.731	19.3399277	50	62.485
4	0+−	7	0.55	0	21.4	13.3703086	23.84	22.1893426	16.66	2.08875
5	−−0	2	0	0.05	87.04	77.4362757	26.6	25.4061574	123.33	122.91375
6	+−0	12	0	0.05	81.6	77.4337243	27.71	26.7413426	106.66	102.03125
7	000	7	0.275	0.05	27.54	29.5491319	19.13	19.018366	41.66	41.66
8	000	7	0.275	0.05	29.12	29.5491319	18.84	19.018366	41.66	41.66
9	000	7	0.275	0.05	29.52	29.5491319	19.79	19.018366	41.66	41.66
10	−+0	2	0.55	0.05	43.08	46.7025309	34.48	35.4261389	58.33	64.56875
11	++0	12	0.55	0.05	57.4	67.5474691	24.14	25.3563611	93.33	93.74625
12	0−+	7	0	0.1	36.11	43.9489095	20.27	21.8257562	31.66	39.57125
13	−0+	2	0.275	0.1	21.18	24.406169	28.74	27.6611376	75	62.505
14	+0+	12	0.275	0.1	73.85	70.1473624	24.91	24.1018413	100	99.9875
15	0++	7	0.55	0.1	29.63	23.8896914	30.76	30.2481574	30	31.25625

C NaCl : concentration of NaCl ; PBM : Phosphate buffer molarity; EAI: emulsifying activity index; FC: foaming capacity. The codded values were (+): maximum value, (0): central value and (-): minimum value.

III.4 Solubility

III.4.1 Analyze of the model of solubility

In **table 04**, it has been shown that for solubility, all linear parameters have been significant; X1 and X3 ($p < 0.05$) and X2 ($p < 0.01$) therefore highly significant. Their quadratic parameters are also very highly significant ($p < 0.01$), as well as for the quadratic parameter X1, X3. However, all other parameters are not significant ($p > 0.05$) and the only significant interaction parameter is the X1X3 ($p < 0.01$). Taking into account only the significant parameters with $p < 0.05$, the predictive equation has been deduced.

$$\text{Solubility} = -0.18664 + 0.00909\ X1 - 0.01387\ X2 + 0.00844\ X3 + 0.03997\ X1X1 + 0.02111\ X2X2 - 0.02329\ X3X3 + 0.03649\ X1X3$$

Table 04 shows also, the variance analysis of the experimental results. The F value of the model was 27.04, this being said that the model is significant. The determination coefficient (R^2) was 0.9643 which means that only 3.57% of the variations could not be explained, and that 96.43% were attributed to the independent variables of solubility of PHPC.

However, the value of R^2 is not always synonymous with a good regression model, it must be comparable to the adjusted R^2, which is verified in our case as shown in **table 04** (adjusted R^2 = 0.9287). In addition, the value of lack of fit was 0.087 (whose value must be insignificant ($p>0.05$) compared to the pure error). Finally, the low value of S which is the standard variation error (0.009) implies that the values obtained are close to the adjusted line. All these values and significance indicate that this model is well and truly validated and that it could work for the prediction of the solubility of PHPC.

Table XII: Analyze of variance (ANOVA) for the experimental results obtained by solubilty.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	7	0,018057	0,002580	27,04	0,0002
Linear	3	0,002770	0,000923	9,68	0,0075
X1	1	0,000662	0,000662	6,93	0,0344
X2	1	0,001539	0,001539	16,13	0,0053
X3	1	0,000570	0,000570	5,98	0,0440
Square	3	0,009962	0,003321	34,81	0,0001
X1X1	1	0,005900	0,005900	61,84	0,0006
X2X2	1	0,001645	0,001645	17,24	0,0044
X3X3	1	0,002002	0,002002	20,99	0,0032
Interaction	1	0,005325	0,005325	55,82	0,0003
X1X3	1	0,000668	0,005325	55,82	0,0006
Error	7	0,000644	0,000095		

Lack of fit	5	0,000024	0,000129	10,83	0,0873
Pure error	2	0,018725	0,000012		
Total	14				
S	**0.0097674**				
R-sq	**0.9643**				
R-sq (adj)	**0.9287**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

III.4.2 Response surface of solubility

Figure 8 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of PHPC solubility.

The solubility depends mainly on pH because the linear and quadratic effect are significant and highly significant with ($p < 0.001$ and $p < 0.05$) respectively. As mentioned above, the three parameters studied (pH, NaCl and PB) are factors that significantly influence solubility and the interaction effect between pH and PB is also significant.This is perfectly in agreement with the results of the preliminary study. The solubility of PHPC can be improved by varying the pH values; it increases as pH approaches the extreme pH values of 2 and 12. Our results are similar to those found for the protein extracts from other plant matrices, as demonstrated by **H. Hu, et al. (2017)** for walnut protein concentrate, **(Tontul, et al., 2018)** for chickpea protein isolate and **Chao, et al. (2018)** for pea isolate.

Solubility also depends strongly on NaCl concentration but with a negative effect and very significant linear and quadratic effects ($p<0.01$). The preliminary study showed that the salt concentration increases the solubility of the protein extract to the concentration of about 0.280 g/l and then decreases it to the minimum at 0.55 g/l, this result is very logical and can be explained by the phenomenon of salting in and salting out found by several authors such as **Deng, et al. (2011)** for the protein isolate of *Ginkgo biloba* seeds. The optimum occurred without NaCl can be explained by the fact that at this pH and this molarity in phosphate buffer, the solubility reaches its maximum and the addition of NaCl does not affect it and this is confirmed by the very high significance of these two parameters interaction. These

phenomena depend also on the conformational differences characteristic of proteins **(H. Hu, et al., 2017)** and effect of NaCl on the ionic strength of the medium **(Inyang & Iduh, 1996).**

It was found that the molarity in PB has also a positive influence on solubility because its linear effect is significant ($p<0.05$) and its quadratic effect is highly significant. This confirms the results of the preliminary study where we found that more the molarity of the phosphate buffer increases, more soluble the PHPC are at lower pH in the range studied. This may be due to the fact that the phosphate buffer on the stability of the protein **(Pikal-Cleland, Rodríguez-Hornedo, Amidon, & Carpenter, 2000).** Therefore the higher the molarity the more stable the protein is and therefore more soluble.

For this model, the optimal solubility conditions are: pH: 10.88; NaCl: 0; PB: 0.078. The protein extract was solubilized under these conditions and the actual solubility obtained was 87.13%± 0.14 against a predicted value of 87.113% whose difference is not significant ($p<0.05$).

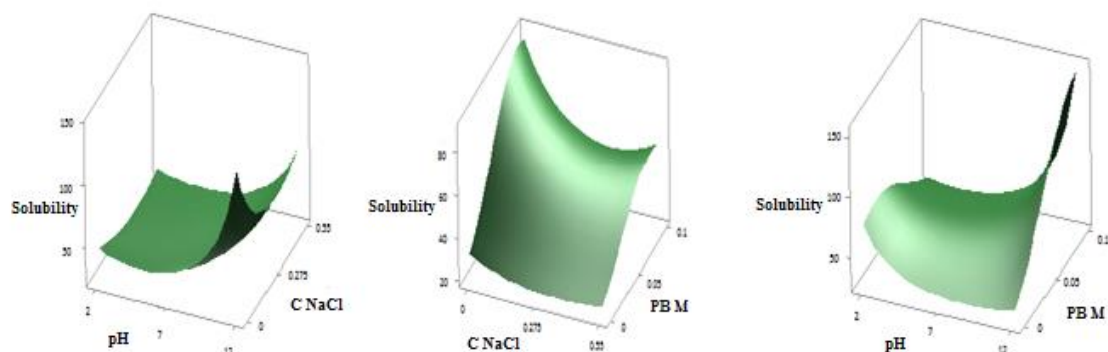

Figure 8: Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on solubility of PHPC.

III.5 Emulsifying activity index

III.5.1. Analyze of the model of emulsifying activity index

For the emulsifying activity index, it has been shown that all linear parameters, their quadratic and their interactions were very highly significant ($p<0.001$). The final predictive equation was obtained as follows:

$(EAI^\lambda-1)/(\lambda\times g^{(\lambda-1)}) = 4.058 - 2.961\ X1 + 2.903\ X2 + 2.395\ X3 + 4.845\ X1X1$

+4.969 X2X2 – 4.316 X1X2 + 2.495 X2X3

(λ = 3; g = 23,8189 is the geometric mean of EAI)

Table 05 shows the variance analysis of the experimental results of EAI. The F value of the model was 53.18, this being said that the model is significant. The determination coefficient (R^2) was 0.9815 which means that only 1.85% of the variations could not be explained, and that 98.15% were attributed to the independent variables of emulsifying activity index of PHPC and the value of adjusted R^2 (0.9631) is quite close to R^2.

In addition, the value of lack of fit was 0.06 which is not significant. Finally, the standard variation error was 1,09919. All these values and significance indicate that this model is well and truly validated and that it could work for the prediction of the emulsifying activity of PHPC.

Table XIII : Analyze of variance (ANOVA) for the experimental results obtained by emulsifying activity.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	7	449.795	64.2564	53.18	0.0001
Linear	3	183.440	61.1468	50.61	0.0004
X1	1	70.119	70.1186	58.03	0.0001
X2	1	67.419	67.4188	55.80	0.0003
X3	1	45.903	45.9031	37.99	0.0005
Square	2	166.959	83.4796	69.09	0.0001
X1X1	1	87.187	87.1870	72.16	0.0002
X2X2	1	91.694	91.6936	75.89	0.0005
Interaction	2	99.395	49.6976	41.13	0.0001
X1X2	1	74.496	74.4961	61.66	0.0002
X2X3	1	24.899	24.8990	20.61	0.0033
Error	7	8.457	1.2082		
Lack of fit	5	8.251	1.2082	16.01	0.0600

Pure error	2	0.206	0.1031
Total	14	458.252	
S	**1.09919**		
R-sq	**0.9815**		
R-sq (adj)	**0.9631**		

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

III.5.2. Response surface of emulsifying activity index

Figure 9 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of emulsifying activity index of PHPC. The parameters pH, NaCl and PB have significant effects ($p<0.05$) on emulsifying activity, and the interaction effects between pH and PB, pH and NaCl as well as PB and NaCl are also significant ($p<0.05$). Such as solubility, the emulsifying activity is also pH dependent because the linear and quadratic effects are very highly significant ($p< 0.001$) and it affects it in the same way as solubility (high activities at extreme pH and lower activities at neutral pH levels), as demonstrated by several authors such as **H. Hu, et al. (2017), Inyang, et al. (1996)** and **Tontul, et al. (2018).**

Although, most authors reported a more pronounced effect at basic pHs than at Acid pHs, contrary to the results obtained, whose optimum has been found at acid pH which can be explained by the interaction effect between pH and NaCl which is very highly significant ($p<0.05$).

The same observation for the salt concentration, which also affects emulsifying activity with a very high significance ($p<0.001$) and with a positive effect, the higher the salt concentration the higher the emulsifying activity increases. Our results are in agreement with those reported by other authors such as **Deng, et al. (2011), H. Hu, et al. (2017) and (Inyang, et al., 1996).** The phosphate buffer affects positively the emulsifying activity with a very high significance.

The optimal emulsifying activity conditions for this model are: pH: 2; C NaCl: 0.55 g/l; PB: 0.1 M. the EAI of the protein extract has been investigated under these conditions and the actual EAI obtained was 36.82±0.34 against a predicted value of 36.65 whose difference is not significant.

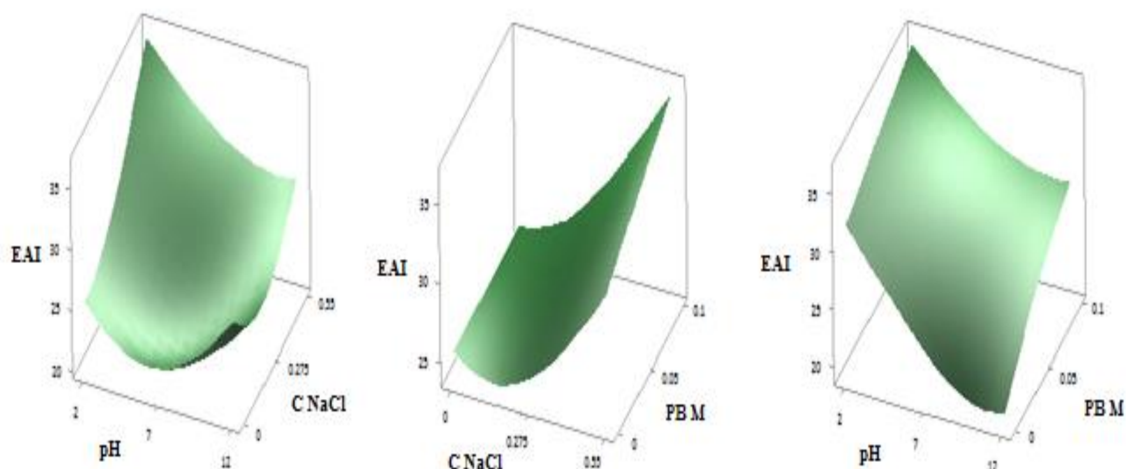

Figure 9: Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on emulsifying activity (EAI) of PHPC.

III.6 Foaming capacity

III.6.1 Analyze of the model of foaming capacity

The results of the analysis of variance of the pH effect, NaCl and PB on foaming capacity are represented in **table 06**.

The results show that for linear parameters, only X2 is significant ($p<0.01$). For quadratic parameters, X1X1 is very highly significant and X3X3 is significant, and finally the significant interaction parameters are X1X3 and X2X3. The equation of prediction was as follows:

$$FC = 3.7295 + 0.0008\ X1 – 0.2806\ X2 + 0.0518\ X3 + 0.8413\ X1X1 + 0.0492\ X2X2 - 0.2327\ X3X3 + 0.1538\ X1X2 + 0.2234\ X1X3 + 0.3137\ X2X3$$

The model of FC as shown in **table 06** is significant at F value of 19.763. The R-sq is at 0.9726 which means that only 2.74 % of the variation could not be explained by the model and then 97.26% were attributed to the independent variables used. This value of R-sq is very comparable to the value of R-sq adjusted (0.9234). Finally, the value of S is also very low (0.1555). However, for this response (FC) the value of lack of fit was significant ($0.012<0.05$) but this does not prevent the validation of the design given the validity of the other R^2 and adjusted R^2 parameters as well as S. Moreover, several authors have demonstrated that the significance of the value of lack of fit does not necessarily invalidate the design because it can

be due to the value of the pure error which can be very small or zero due to the accuracy of the repeat measurements (**Ahmad, Yusup, Bokhari, & Kamil, 2014; Bashir, Aziz, Yusoff, & Adlan, 2010; Marković, et al., 2018).** According to the obtained results, we can say that this model can be used for prediction of the effect of pH, NaCl and PB molarity on FC.

Table XIV: Analyze of variance (ANOVA) for the experimental results obtained by foaming capacity of PHPC.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	9	4.31315	047924	19.76	0.0023
Linear	3	0.65130	0.21710	8.95	0.0192
X1	1	0.00001	0.00001	0.00	0.9895
X2	1	0.62985	0.62985	25.97	0.0048
X3	1	0.02144	0.02144	0.88	0.3904
Square	3	2.97391	0.99130	40.87	0.0015
X1X1	1	2.97391	2.61342	107.74	0.0004
X2X2	1	0.00892	0.00892	0.37	0.5715
X3X3	1	0.19986	0.19986	8.24	0.0358
Interaction	3	0.68794	0.22931	9.45	0.0177
X1X2	1	0.09463	0.09463	3.90	0.1055
X1X3	1	0.19970	0.19970	8.23	0.0353
X2X3	1	0.39361	0.39361	16.23	0.0105
Error	5	0.12129	0.02426		
Lack of fit	3	0.00000	0.04043		0.0120
Pure error	2	4.43444	0.00000		
Total	14				
S	**0.1555747**				
R-sq	**0.9726**				
R-sq (adj)	**0.9234**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

III.6.2 Response surface of foaming capacity

Figure 10 (A, B, C) shows the three-dimensional response surface profiles of multiple non-linear regressions of Foaming capacity of PHPC. The NaCl parameters have a significant effect on foaming activity, and the interaction effects between pH and PB and PB and NaCl are also significant. The foaming capacity depends also on the pH with a very highly significant quadratic effect. On the concentration of NaCl with a very highly significant linear effect, and on the phosphate buffer with a significant quadratic effect, the interaction effects are significant for the pH-PBM and NaCl-PBM parameters. The effect of pH and NaCl on FC has also been reported by several authors **(H. Hu, et al., 2017; Inyang, et al., 1996)**

The optimal FC conditions for this model are: pH: 2; NaCl: 0 g/l; PBM: 0 M. the FC of the protein extract has been investigated under these conditions and the actual FC obtained was 183.55±2.03 against a predicted value of 182.72 with no significant differences ($p<0.05$).

The optimal conditions were determined by maximizing desirability using the Minitab prediction profiler. In order to verify the predictive capacity of the model, the results of the maximized conditions were used for a solubility test of the PHPC, their EAI and their FC. The optimal conditions obtained were: pH 12, NaCl concentration 0.55 g/l. molarity in PB 0.1 M. The experimental values for solubility, EAI and FC were 78.07±0.98, 30±0.52, 110±2 respectively with composite desirability value of 0.77, intermediate values of desiravility between (0-1) indicate more or less desirable response (Candioti, et al., 2014). These experimental results were in agreement with the predicted values corresponding to 77.4, 30.05 and 111 respectively (no significant difference).

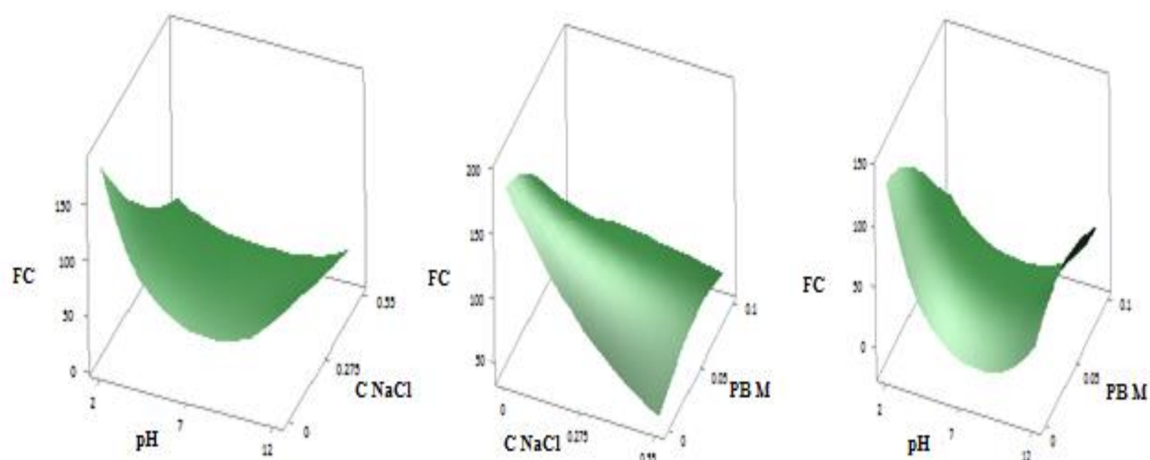

Figure 10: Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on foaming capacity (FC) of PHPC.

Table XV: Regression coefficient, standard error, and t test results of response surface for solubility, EAI and FC.

	Regression coeffients	Standard error	t- value	P- value
Solubility				
Constant	-0,18664	0,00564	-33,10	0,0002
X1	0,00909	0,00345	2,63	0,0344
X2	-0,01387	0,00345	-4,02	0,0053
X3	0,00844	0,00345	2,44	0,0440
X1X1	0,03997	0,00508	7,86	0,0001
X2X2	0,02111	0,00508	4,15	0,0044
X3X3	-0,02329	0,00508	-4,58	0,0032
X1X3	0,03649	0,00488	7,47	0,0003
EAI				
Constant	4,058	0,528	7,68	0,0001
X1	-2,961	0,389	-7,62	0,0001

X2	2,903	0,389	7,47	0,0003
X3	2,395	0,389	6,16	0,0005
X1X1	4,845	0,570	8,49	0,0002
X2X2	4,969	0,570	8,71	0,0005
X1X2	-4,316	0,550	-7,85	0,0002
X2X3	2,495	0,550	4,54	0,0033
FC				
constant	3,7295	0,0899	41,48	0,0023
X1	0,0008	0,0551	0,01	0,9895
X2	-0,2806	0,0551	-5,10	0,0048
X3	0,0518	0,0551	0,94	0,3904
X1X1	0,8413	0,0811	10,38	0,0004
X2X2	-0,0492	0,0811	0,61	0,5715
X3X3	-0,2327	0,0811	-2,87	0,0358
X1X2	0,1538	0,0779	1,98	0,1055
X1X3	0,2234	0,0779	2,87	0,0353
X2X3	0,3137	0,0779	4,03	0,0105

EAI: emulsifying activity index; FC: foaming capacity

III.7 Stabilization of foaming and emulsifying properties

The optimal desirability conditions of the three responses (solubility, EAI and FC) were used to study the stability of the emulsion and that of the foam formed. The results of stability percentages are represented in **table 08.**

A very good stability was exhibited for both the emulsion and the foam compared to that of sesame **(Inyang, et al., 1996)**. The stability of these two activities depends primarily on the solubility of the extract but also on the ionic strength of the medium.

Table XVI: Percentages of foam and emulsion stability of PHPC.

	15 min	30 min	45 min	60min
Emulsion Stability index (%)	75±2.3	68.5±1.5	53±2	51±0.8
Foam stability (%)	90±4.6	87±2.2	83±1.9	73±4.3

Data are the mean± SD of three analyses.

III.8 Minimum gelling concentration (MGC)

The MGC of PHPC under optimal conditions of functional properties was around 6% of PHPC, it's showing the very good gelling capacity of these proteins under these conditions. The parameters that can improve gelling are the increase in time, temperature, pH and protein concentration **(Coffmann & Garciaj, 1977). Sun and Arntfield (2010)** reported also that the salt concentration improves significantly the gelling properties of proteins. In our study, the high pH and high salt concentration may have influenced this capacity. In addition to the solubility of its proteins which is 77.39%, the minimum concentration obtained is more interesting than that reported by most other protein extracts. **O'Kane, et al. (2005), Coffmann and Garciaj (1977) and (Altschul, 1958)** obtained MGC of 16% for pea protein, 10% for mung bean protein and 8% for soy protein respectively.

III.9 Heat Coagulability (HC)

A relatively high HC was demonstrated by PHPC which was 24% compared to concentrate of *Brassica juncea* mustard seeds and *Sinapis alba* **(Rotimi E. Aluko, McIntosh, & Katepa-Mupondwa, 2005)** and canola isolate seeds **(Rotimi E Aluko & McIntosh, 2001)** as well as soybean isolate and pea isolate **(Voutsinas, et al., 1983)** which have not shown any coabulability to heat. Our result is comparable to the HC of sunflower isolate (22.5%). **Voutsinas, et al. (1983); Rotimi E Aluko, et al. (2001)** and **Rotimi E. Aluko, et al. (2005)** explain that HC depends mainly on the solubility as well as the surface hydrophobicity of the protein extract, which justifies the heat coagulation of our PHPC under the conditions used which solubilize them at 77.39%.

IV.Conclusion

In conclusion, this study confirmed that the functional properties are strongly related to the pH of the medium, its NaCl concentration and its PB molarity. In addition, the results show that Aleppo pine seeds are a good source of protein whose functional activities have been improved by the three parameters influencing them (pH, NaCl concentration and PB molarity). These proteins can therefore be used as food ingredients and the variation in pH, NaCl concentration and PB molarity can be employed as an effective processing method to improve the use of proteins as functional ingredients in food product formulations

Chapter IV:

Setting up a diagram process for the elaboration of a new plant-based beverage from Pinus halepensis seeds: selection of unit operations and their conditions

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Abstract

Plant-based beverages are an interesting alternative to animal milk. Aleppo pine seeds are a potential candidate for non-dairy milk production because of their high content in proteins and lipids as well as their availability. This study presents the setting up of a fabrication diagram from the seeds to the final product, including pre-extraction (seed conditioning), extraction and post extraction (physical and microbiological stabilization), thanks to a fractional factorial $2^{(7-4)}$ screening design. The 7 factors were soaking time of the seeds, grinding time, temperature and seed/water ratio during extraction, xanthan gum concentration, homogenization pressure and pasteurization temperature. The quality analysis of beverage consisted of chemical composition, color, stability and rheological behavior measurements. The results showed that the seed/water ratio and grinding time significantly increased the dry matter content including lipids and proteins while the extraction temperature increased the extraction of lipids but not proteins. Xanthan gum showed a stabilizing effect but only at low concentrations, and finally the homogenization pressure and the pasteurization temperature showed their effectiveness by decreasing particle size and in turn increasing the physical stability of the product.

Keywords:

Non-dairy milk, Mediterranean product, Formulation, Ultra-High-Pressure Homogenization, Pasteurization.

I.Introduction

Animal milk has always been described as an essential and mandatory food for its high protein, fat and mineral content **(Mesfine, Feyera, & Mohammed, 2015).** However, plant-based beverages commonly known as non-dairy milk or plant milk are increasingly finding their way onto the market. Sales of these products have grown by approximately 61% since

2012 (**Lopes, Duarte, Nunes, Raymundo, & Sousa, 2019).** This growth is due to the various nutritional benefits these products can offer. Indeed, aside their diverse tastes, plant-based beverages can have interesting contents in carbohydrates and proteins associated to essential fatty acids and can contribute to diabetes control, cholesterol reduction and cardiovascular disease prevention. That is why they are more and more consumed as an alternative to animal milk by people with lactose intolerance, vegetarians and vegans and also, by health and environmentally conscious consumers **(Kurajdova & Táborecka-Petrovicova, 2015; Lopes, Duarte, Nunes, Raymundo, & Sousa, 2019).**

The main sources of these beverages are almond, soy and coconut with market shares of 64%, 13% and 12% respectively. But given the enthusiasm of consumers to change and diversify beverage consumption, new types of vegetable drinks have appeared and have experienced a rapid growth in popularity, for example, pecan, quinoa, hazelnut and flax based beverages **(Kurajdova, et al., 2015).**

For this reason, researchers and industries are constantly looking for new plant-based sources that could be suitable for plant milk production **(Kurajdova, et al., 2015)**. Aleppo pine (*Pinus halepenis* Mill.) seeds could be a new potentiel source of plant material for beverage production. Indeed, seeds are highly nutritious and are a good source of sugars (5.6%), proteins (26.6%) and lipids (36.7%) with essential fatty acids, mainly unsaturated, with linoleic acid and oleic acid being the major ones **(Nabil Kadri, et al., 2015)**. Moreover, Aleppo pine is largely widespread throughout the Mediterranean basin found in both natural and urban environments **(Dhibi, Mechri, et al., 2012)**. In total, the forests of Aleppo pine occupy more than 3.5 million hectares **(Barbéro, Loisel, Quézel, Richardson, & Romane, 1998)**. Given the nutritional quality of the seeds and the availability of the trees, their valorization in the food industry is interesting from a nutritional and economic point of view. The study of the conception of a plant-based beverage using these seeds is therefore relevant. The process of plant milk production consists in extracting the nutritive coumpounds of the seed in water. However, the design of this process is a real challenge, since seeds and plant milk are complex in their structure. Seeds have many coats that may impair extraction efficiency and the resulting plant-based beverage is a complex colloidal system formed essentially by an oil-in-water emulsion and dispersed particles that is unstable**.** The most

common challenge about setting up such production is to maximize the yield of extraction for the economic viability while ensuring the beverage quality, i.e., physical and microbiological stability. Physical instability of theses beverages is characterized by creaming and sedimentation phenomena that is not acceptable to consumers **(Briviba, Gräf, Walz, Guamis, & Butz, 2016)**. To tackle this problem, several processes have been studied and adapted to the different plant-based sources. Various pre-extraction steps were conducted to prepare the seed for extraction, namely soaking, roasting and cooking **(Ahmadian-Kouchaksaraei, Varidi, Varidi, & Pourazarang, 2014; Ukwuru & Ogbodo, 2011).** For the extraction steps, different parameters were tested such as the ratio of seeds to water, the time **(Kizzie-Hayford, Jaros, Schneider, & Rohm, 2015**) and temperature of extraction **(Wilkens, Mattick, & Hand, 1967)**. Different strategies were adopted for enhancing physical stability after extraction by firstly choosing an apropriate separation step, either filtration or centrifugation. This step was followed by the formulation step, that consists of the addition of stabilizing food additives, among others **(Maghsoudlou, Alami, Mashkour, & Shahraki, 2016; Taherian, Fustier, & Ramaswamy, 2007)** and/or more importantly, by ultra-high pressure homogenization **(Bernat, Chafer, Rodríguez-García, Chiralt, & González-Martínez, 2015).** It is very important to carry out this stabilization step before the final microbiological stabilization by thermal treatment **(Bernat, et al., 2015; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012)**.

The main objective of this study is first to ascertain the potential of Aleppo pine seeds for the production of a plant-based milk alternative and to present a structured approach to creating a process diagram for this new product. In addition, this study aims to better understand the effect of each parameter (alone or coupled) of this complex process from extraction (pre-treatment, ratio, grinding time and temperature) to stabilization (formulation, homogenization and pasteurization) through a multi-criteria assessment of the quality of the beverage by means of a screening design testing 7 different parameters.

Chapter IV:Setting up a diagram process for the elaboration of a new plant-based beverage from Pinus halepensis seeds: selection of unit operations and their conditions

II. Material and methods

II.1. Raw material

Aleppo pine cones, harvested from the Collo forest in Skikda, Algeria, were heat treated at 180 °C to melt the resin that seals the cone scales and cause them to open and release the seeds trapped inside. These seeds were recovered, washed with tap water and then dried at 37 °C for 24 hours.

II.2. Chemical reagent

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, USA): Chloroform, methanol, soybean lecithin, guar gum, xanthan gum, starch, amylopectin and Bradford reagent.

II.3. Preparation and processing of *Pinus halepensis* seed beverages

To set up the general process of *Pinus halepensis* seed beverage presented in **Figure 11**, several pre-tests were conducted to identify the appropriate unit operations and the right range of parameter variation.

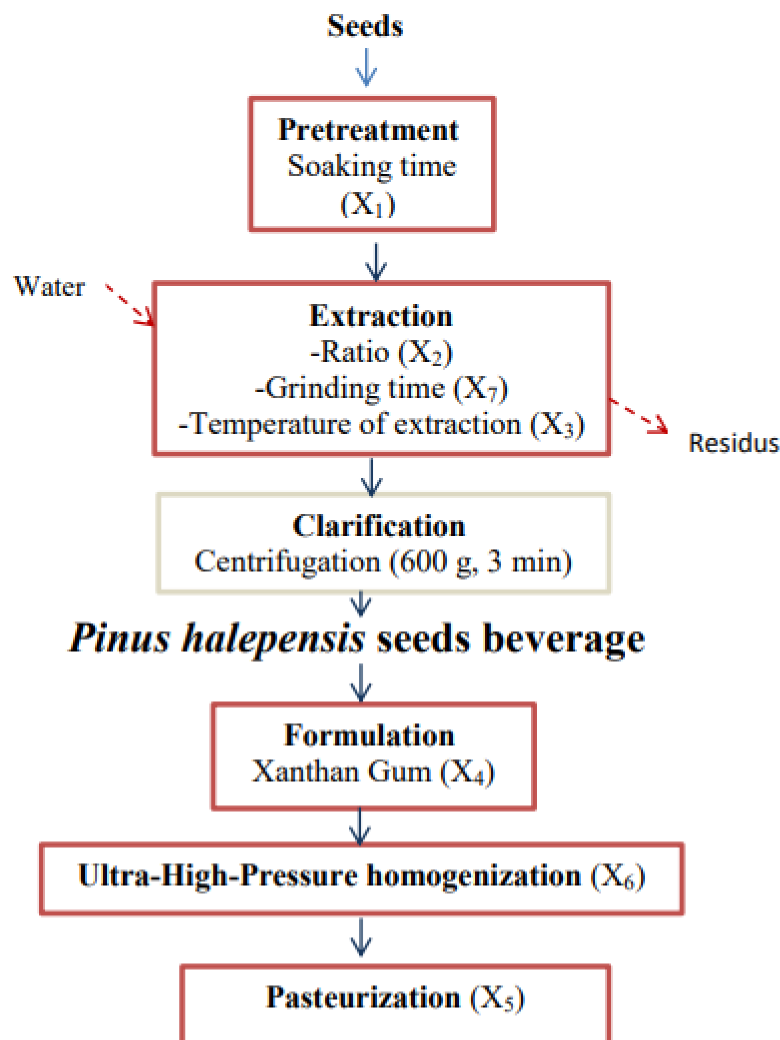

Figure 11: Manufacturing process of *Pinus halepensis* beverage.

II.3.1. Selection of unit operation and material

Before extraction, a pre-treatment by soaking was tested on the raw seeds at ambient temperature in beakers at a seed/water ratio of 1/10. After draining, the seeds were incorporated in water for extraction. Extraction was done in a Thermomix TM6 Vorwerk (Wuppertal, Germany) at the highest speed on the whole seed without dehulling. To start the extraction at the set temperature, the Thermomix was first pre-heated with the appropriate volume of water before the seeds were incorporated and ground. After extraction, different clarification options were tested. Filtration was firstly used with filters of different pore

diameters, but this operation failed in achieving good yield and purity (remaining black particles, results not shown). Therefore, centrifugation was tested for different combinations (acceleration: time) and was finally carried out at 600 g for 3 min using a Aventi JE centrifuge (Beckman Coulter, California, USA). After extraction, in order to stabilize the beverage by formulation, several food additives, soybean lecithin, guar gum, xanthan gum, starch and amylopectin, were tested at different concentrations. Xanthan gum was selected visually because it presented the best solubility and stabilizing property. Finally, physical and microbiological stabilization were also considered by applying ultra-high pressure homogenization with a homogenizer from A/S APV 1000/2000 (SPX FLOW Technology, Denmark) followed by pasteurization in a water bath using 25 mL Pyrex tubes.

II.3.2. Range of processing conditions for each step

Based on the operations selected and given the large number of potential factors affecting the beverage quality, we opted for the fractional factorial $2^{(7-4)}$ screening design to optimize production. Three central points were added to evaluate the repeatability of experiments. The range of processing conditions are presented in **Table XVII** and were used to define the factor $X_{1\rightarrow 7}$ variation of the screening design of experiments presented in **Table XVIII**. Soaking was done with a duration range of 0 to 4 h (X_1). Extraction was done from 20 to 100°C (X_3) at different seed/water ratios from 1/10 to 3/10 (w/w) (X_2) and ground from 20 to 120 s (X_7). Then, xanthan gum was added at room temperature from 0 to 0.4% under stirring (X_4). Finally, homogenization was done from 0 to 700 bars (X_6) and pasteurization in a water bath at 65 to 85°C (X_5). Duration of pasteurization treatment at 65 and 85°C was set to achieve a pasteurization value (P) of 100 min at T_{ref}=70°C. (P) was calculated from the time-temperature curve of the beverage in the tubes according to **Eq (1):**

$$P = \int_{t_0}^{t_{end}} 10^{\left(\frac{T(t)-T_{ref}}{z}\right)} \cdot dt \qquad \textbf{(Eq 1)}$$

Table XVII: Independent variables of milk fabrication integrated process and their levels in the screening design.

Independent variables	symbols	Level	
		-1	+1
Soaking time (h)	X_1	0	4
Ratio of extraction (/10)	X_2	1	3
Temperature of extraction (°C)	X_3	20	100
Grinding time (s)	X_7	20	120
Xanthan Gum (%)	X_4	0	0.4
Pressure of homogenization (Bar)	X_6	0	700
Temperature of pasteurization (°C)	X_5	65	85

Table XVIII: A fractional factorial 2 (7-4) screening design matrix with coded value.

	Soaking time (h) X_1	Ratio X_2	Temperature of extraction (°C) X_3	Xanthan gum (%) X_4	Temperature of pasteurization (°C) X_5	pressure of homogenization (Bar) X_6	Grinding time X_7
1	-1	-1	-1	1	1	1	-1
2	-1	-1	1	1	-1	-1	1
3	1	1	-1	1	-1	-1	-1
4	1	1	1	1	1	1	1
5	1	-1	1	-1	1	-1	-
6	1	-1	-1	-1	-1	1	1
7	-1	1	-1	-1	1	-1	1
8	-1	1	1	-1	-1	1	-1
9	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0

II.4. Analysis of Aleppo pine beverage

II.4.1. Chemical properties

Dry matter (DM) was determined by the AOAC method **(AOAC, 2005)**. The extraction yields were calculated as the ratio of the weight of DM of the final beverage over the initial DM of the seeds used. The pH was measured with a pH meter (Titroline 96; Schott-Geräte GmbH, Mainz, Germany). The lipids were quantified with the Folch method using chloroform/methanol (3:1) for extraction **(Corrales Hernández, et al., 2017).** Proteins were measured with the Bradford method **(Kruger, 2009).** Finally, aroma compounds were extracted with SPME (Solid Phase Micro-Extraction) and analyzed with GC-MS Agilent 6890/5973 series (Agilent Technologies, PaloAlto, CA, USA) equipped with a DB-WAX polar column (60 m, 0.25 mm, 0.25 μm phase film thickness, Agilent J&W GC column) using butanol as an internal standard.

II.4.2. Physical properties

II.4.2.1. Rheological behavior

Rheological measurements of *Pinus halepensis* seed beverage were performed using a Physica MCR301 rheometer (Anton Paar GmbH, Austria) equipped with a Couette flow measuring cell (Ref. DG27/T2000/SS), according to the procedure of **Dahdouh, et al. (2015)**. All measurements were conducted at 25°C. Consistency index, flow index and viscosity at a shear rate of 100 s^{-1} were deduced from the following formula (**Eq 2**):

$$\eta = K\gamma^{n-1} \qquad \textbf{(Eq2)}$$

where:

η is the apparent dynamic viscosity, K is the consistency index, γ is the shear rate and *n* is the flow index.

II.4.2.2. Particle size distribution

Particle size distribution was determined by laser light scattering using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK) and log-spaced array detector in a range between 0.01 μm and 3500 μm. This method involved loading the sample into a dispersant

unit HydroMV filled with water as suspension medium set for obscuration at 20 % and stirring of 1500 rpm **(Corredig, Kerr, & Wicker, 2001)**. Particle size distribution was expressed as $D_{(4:3)}$ (particle diameter means), DV_{90} (particle diameter of 90% of particles) and span (particle dispersion) indices.

II.4.2.3. Colloidal stability

The stability of the suspension was evaluated after storage of the sample for 48 hours at 4°C. A volume of 10 mL of *Pinus halepenis* beverage was placed in a 15 mL graduated tube and three phenomena were observed and measured for stability: creaming, dephasing and sedimentation. The ratio of the volume of creaming (c), phase shift (p) or sediment (s) to the total liquid volume was calculated and we decided to express it as indices of visual stability using the following equation **(Eq3) :**

$$\boldsymbol{VS = 100 - \sqrt{a^2 + p^2 + c^2}} \qquad \textbf{(Eq 3)}$$

II.4.2.4. Optical properties

The color of the beverage was expressed as a whiteness index (WI) by measuring it with the L*a*b* system using a CR400 colorimeter (Konica Minolta, Tokyo, Japan) and using following equation (Eq 4) **(Bernat, et al., 2015)**

$$\boldsymbol{WI = 100 - \sqrt{(100 - L*)^2 + a*^2 + b*^2}} \qquad \textbf{(Eq 4)}$$

II.5. Statistical analysis

The statistical analysis of the experimental design was carried out using the Statistica 7.1 software and the factors were considered significant at $p<0.1$. The hypothesis of linearity underlined by the experimental design chosen was validated by the analysis of the significance of curvature. The linearity is validated if the curvature, which is the difference between the theoretical mean proposed by the model and the experimental mean, does not exceed 5%.

III. Results and discussion

III.1. Composition of *Pinus halepensis* seeds

Aleppo pine seed composition was experimentally measured and contained 27.48 ± 0.17% proteins, 31.55 ± 0.11% lipids and 4.97 ± 0.17% carbohydrates. These values were close to results presented by **Nabil Kadri, et al. (2015)**. The dry matter (DM) of the raw seeds was 91.97%. DM of the seed soaked for 2 hours was 82.01% and 77.96% for the seeds soaked for 4 hours. The decrease of DM is due to the hydration kinetic. Hydration reached 9.93 % in 2 h and 14.01 % in 2 h which are significant, similar to almond **(Khazaei, 2008),** but quite low in comparison with rich starchy seeds like soybean **(de Lima, Kurozawa, & Ida, 2014)**

III.2. Results of the experimental design: effect of processing factors on the multi-criteria quality of the Aleppo pine seed beverage

The seeds were used to produce a plant-based beverage according to the chart presented in **Figure 11** and conditions of the experimental design presented in **Tables XVII and XVIII.** The quality analyses, for which the chemical and physical characteristics were measured on the 9 different beverages obtained, are given in **Table XIX.**

III.2.1. Chemical characteristics

III.2.1.1. Dry matter, fats and proteins

The statistical study showed that the ratio (X_2), grinding time (X_7) and temperature of extraction (X_3) were the only significant factors affecting dry matter, protein and fat contents. The ratio and temperature of extraction had a positive effect on all three responses. The temperature of extraction had a positive effect on lipids but a negative effect on DM and proteins. Based on the assumptions of this experimental design, no interaction effect was identified for these three responses. Since the curvature, which is the difference between the theoretical mean proposed by the model and the experimental mean, was insignificant, the DM, lipid and protein models can be assumed to have a linear response. The DM of the final beverage varied significantly between 2.2 and 9.4 % with a mean of 5.8 %. These values were close to those found in almond beverages (6.2%) **(Maria & Victoria, 2018)**, in melon seed beverages (9.9%) **(Akubor & Ogbadu, 2003),** and in almond and hazelnut beverages (6.6

and 5.9 % respectively) **(Bernat, et al., 2014)**. Lipid results varied between 1.2 and 7.2% with an average of 4.0%, which is comparable to the levels found in soy milk (3.2%), almond milk (3.4%), sesame milk (about 7%) and peanut milk (about 4%) **(C. Lee & Beuchat, 1992).** The level of proteins found in the beverage was between 0.5% and 2.3% with an average of 1.3%. These results are close to those found in melon seed milk which is around 2% and in soy bean and almond milks 1.2 et 0.7 % respectively **(Akubor, et al., 2003; Sharma, Kumari, Wongputtisin, Nout, & Sarkar, 2015).**

III.2.1.2. Yields

The yield depended only on the ratio X_2 and there were no interaction effects with other factors. In this case, the curvature was significant. Therefore, the yield was assumed a non-linear response of the ratio. The yield varied between 21.3% and 32.8 from ratio 1/10 to 3/10 with an average of 24.9%. These values were higher than the yields obtained for tiger nut beverage, which were between 11 and 19% (depending on seed size, grinding time and soaking temperature) but lower than that of 56% obtained during almond beverage production **(Maria, et al., 2018).**

III.2.1.3. pH

The results showed that the pH was influenced negatively by the ratio (X_2) and positively by the temperature of extraction. The interaction effect and the curvature were insignificant for this response. For all products, the pH was still quite neutral. The pH values obtained (with an average of 6.8) were comparable to those of cowpea milk (**Nnam, 2003**), melon seed milk (Akubor, et al., 2003), soymilk and almond milk **(Kundu, Dhankhar, & Sharma, 2018).**

III.2.1.4. Aromatic compounds

The aroma compounds most present in the beverage of Aleppo pine were α-pinene, β-myrcene and D-limonene. Significant factors with a positive effect were the soaking time (X_1) for α-pinene, the ratio (X_2) for α-pinene, β-myrcene and D-limonene and with a negative effect of the temperature of extraction (X_3) for α-pinene and β-myrcene, and of xanthan gum (X_4) for α-pinene, β-myrcene and D-limonene. Furthermore, since factor X_6 was significant

for the β-myrcene response, and factors X_2 and X_3 were also significant, there could be an interaction effect between these two factors: ratio and temperature of extraction (X_2X_3). Finally, curvature was significant for α-pinene and β-myrcene, which means that only the D-limonene model could be linear. Aroma compound values ranged from 0.04 to 1.21% for α-pinene, 0.01 to 0.46 % for β-myrcene and 0.02 to 0.06 % for D-limonene.

III.2.3. Physical characteristics

III.2.3.1. Stability

The factors influencing stability were (in descending order): the ratio (factor X_2) with a negative effect, pasteurization temperature (factor X_5) and grinding time (factor X_3) (the same positive effect) and then, the homogenization pressure (factor X_6) which also had a positive effect, temperature of extraction (Factor X_3) and finally, the soaking time (X_1) and xanthan gum (X_4) with the same negative effect. It makes sense that many factors affect stability. This is the most delicate point and the manufacturing challenge. Both the formulation factors (ratio and additive that should not be increased too much) and process temperature and homogenization are important.

All factors were significant, therefore there may be interaction effects between soaking time and ratio (X_1X_2) because X_5 (pasteurization temperature) was significant, between X_2X_3 and X_1X_2, ratio and temperature of extraction (X_2X_3) because X_6 (homogenization pressure) was significant and finally, between soaking time and temperature of extraction (X_1X_3) because X_7 (grinding time) was significant. However, the curvature was also significant which may be due to the non-linearity of this model. The following indicators can explain further the stability observations.

III.2.3.2. Granulometric distribution

Figure 12 illustrates the difference in the particle size distribution of the eleven assays. Most samples had about the same maximum diameter volume distribution (around 50 µm) except four samples, of which two samples (assay 3 and 2) had most of their maximum particle volume density around 500 µm. This result was influenced by the presence of xanthan gum at the highest concentration (0.4%), the minimum pasteurization temperature (65°C) and the minimum homogenization pressure (without homogenization). Moreover, two samples

presented the maximum of the particle volume density of the lowest size, around 10 µm. These samples were influenced mainly by the conditions of maximum pasteurization and homogenization.

Particle size $D_{(4:3)}$: Pasteurization temperature (X_5), xanthan gum (X_4) and homogenization pressure (X_6) were the factors that influenced the mean particle diameter in this order, X_5 and X_6 with a negative effect, and X_4 with a positive effect, but no significant interaction effect was observed. However, the curvature was significant, so the model can be non-linear. The diameter of the particles varied between 11.6 and 216.4 µm, the maximum diameter obtained was higher than the $D_{(4:3)}$ obtained by most vegetable beverages even without any treatment, for example hazelnut milk had a $D_{(4:3)}$ of 172.1. This difference was mainly influenced by the presence of xanthan gum **(Gul, Saricaoglu, Mortas, Atalar, & Yazici, 2017)**

Particle size $DV_{(90)}$: The 90% particle size was influenced the most negatively by pasteurization temperature, then, positively by xanthan gum, and then, negatively by homogenization pressure. No interaction effect has been revealed. However, the model could be non-linear because the curvature was significant.

Span: The particle dispersion (span) was influenced by ratio with a positive effect and by the homogenization pressure with a positive effect. The model seemed to be non-linear (significant curvature). The span values varied between 1.9 and 2.9. A study of hazelnut milk **(Gul, et al., 2017)** showed that these values varied between 1.9 and 3.2 depending on the concentration and pressure of homogenization.

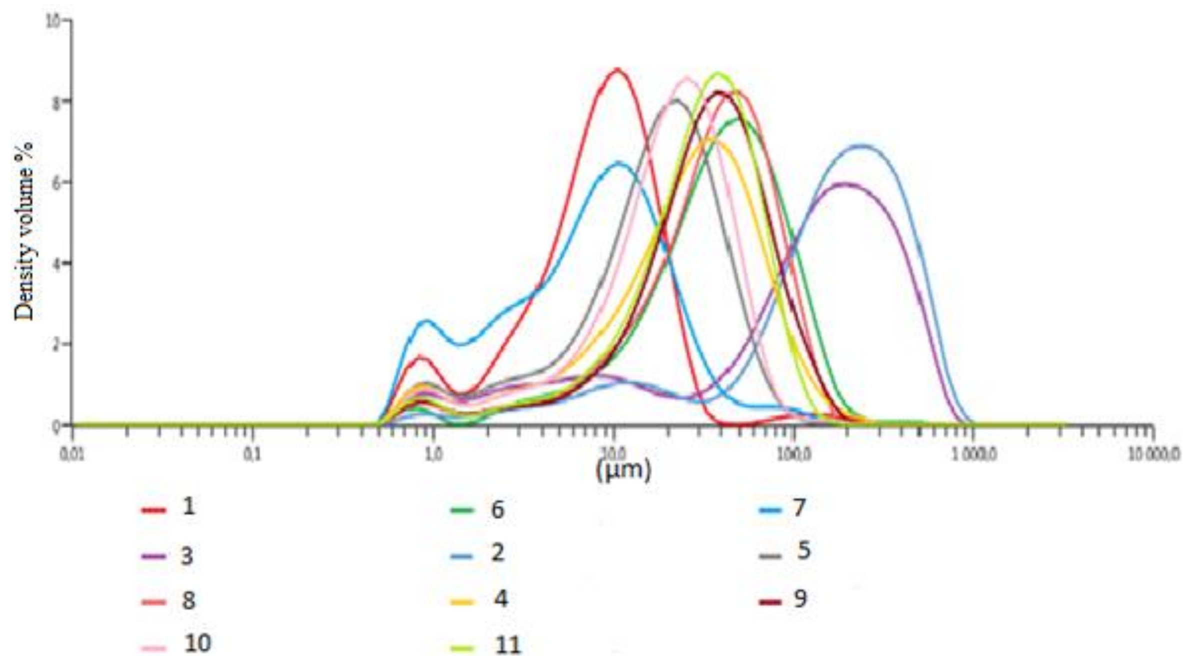

Figure 12: Particle size distribution curves of different Aleppo pine beverage samples.

III.2.3.4. Rheological behavior

Figure 13 describes the difference in rheological behavior of the eleven samples as a function of shear rates. Assays were grouped into two large groups: one group containing four samples (Assay 2, 4, 5, 6) with a viscosity close to 1 mp/s with Newtonian behavior (which are samples that do not contain xanthan gum); and another group with a higher viscosity (presence of xanthan gum) which decreased with the increase of the shear rate.

Viscosity: The viscosity was influenced firstly by xanthan gum (X_4), then in the following order by grinding time (X_7), temperature of extraction (X_3), pasteurization temperature (X_5) and finally, homogenization pressure (X_6). All these factors affected the viscosity with a positive effect. No interaction effects were observed. Indeed, the model could be assumed as being linear because the curvature was non-significant.

Consistency index: All factors were significant with the same degree: soaking time, ratio, temperature of extraction and grinding time had a negative effect and the rest of the factors had a positive effect. The model could be not linear because curvature was significant.

Flow index: The flow index was influenced by xanthan gum (X_4) (negative effect), then pasteurization temperature (X_5) (positive effect) and then homogenization pressure, (positive effect), no interaction effect has been revealed, and the model could be assumed as being linear since curvature was non-significant.

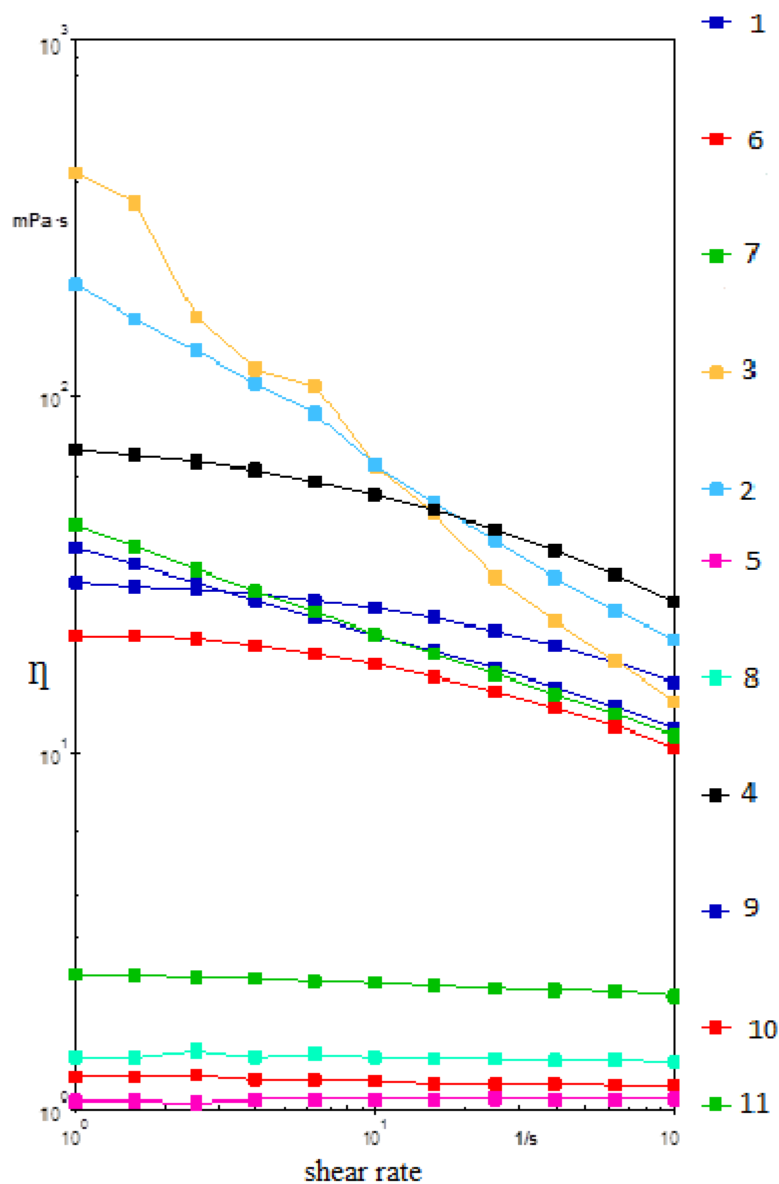

Figure 13: Rheological behavior of different Aleppo pine beverage samples.

III.2.3.5. Whiteness index

For the whiteness index (WI), the factors that were found to be significant are (in decreasing order of effect), temperature of extraction (negative effect), xanthan gum (%) (negative effect), homogenization pressure (negative effect), ratio (positive effect), and finally pasteurization temperatures (positive effect). The factor X_6 (homogenization pressure) was significant and the factors X_2 (ratio) and X_3 (temperature of extraction) were also significant. In this case, the result may reveal a possible interaction between the ratio (X_2) and the temperature of extraction (X_3), which must be confirmed by further experiments. However, the curvature was not significant, which means that the model could be linear. The WI of the beverage varied between 74.6 and 94.0 % with an average of 81.8 % which is close to the values of WI obtained on almond and hazelnut milks in the study of **Bernat, et al. (2015).**

Table XIX: Results of experimental design.

	Chemical properties								Physical properties							
	Nutritional quality					**Aromatic compounds**			**Stability**	**Grulometrical behavior**			**Rheological behvior**			**color**
	Dry matter (%)	Yields (%)	pH	Fats (%)	proteins (%)	alpha-pinene (%)	beta-myrcene (%)	D-limonene (%)	Stability index (%)	$D_{(4:3)}$	DV_{90}	Span	viscosity (Pa/s) at a shear rate of 100^{-1}	Consis-tency index (K)	Flow index (n)	Whiteness index (%)
1	2,46	21,5	6,73	1,2	0,91	0,531	0,057	0,019	100	11,99	19,032	1,95	16,566	0.033	0.862	78,6
2	3,02	21,33	7,24	2,49	0,54	0,041	0,003	0,021	94,29	216,42	461,721	2,46	19,533	0,213	0.477	74,65
3	8,96	28,07	6,27	5,5	2,03	1,004	0,189	0,047	33,4	172,85	402,738	2,94	13,633	0,398	0.241	84,99
4	9,03	28,58	6,45	6,92	1,86	0,6	0,153	0,052	100	35,45	74,329	2,54	26,4	0,079	0.788	79,23
5	2,18	21,5	7,21	1,63	0,53	0,507	0,164	0,032	94,29	21,45	43,260	2,21	1,07	0,001	1.004	83,03
6	3,92	21,63	6,83	2,03	1,32	0,832	0,117	0,04	100	50,82	102,484	2,27	1,156	0,001	1.014	82,96
7	9,42	28,35	6,5	5,6	2,34	1,215	0,466	0,065	91,6	11,57	23,428	2,86	2,043	0,002	0.969	94,03
8	7,57	28,3	7,09	6,46	1,33	0,823	0,215	0,062	81,22	43,58	86,405	2,06	1,416	0,001	0.921	77,38
9	6,18	32,8	6,54	3,51	1,23	0,489	0,057	0,024	97,5	41,05	82,144	2,14	11,8	0,038	0.749	80,42
10	6,04	32,73	6,73	3,83	1,31	0,475	0,081	0,024	98,25	24,66	47,662	2,02	10,55	0,023	0.847	78,58
11	6,49	32,86	6,53	3,63	1,19	0,457	0,102	0,032	81.8	36,01	69,611	1,95	11,3	0,046	0.693	80,89

III.3. Discussion about the main effects of each processing factor on the quality of Aleppo pine seed beverage

The effect of key factors in the Aleppo pine seed plant-based beverage manufacturing process and their analysis of variance on the chemical and physical quality are presented in **Table XX** and the Pareto charts of the most important responses (dry matter, stability and whiteness index) are shown in **Figure 14**.

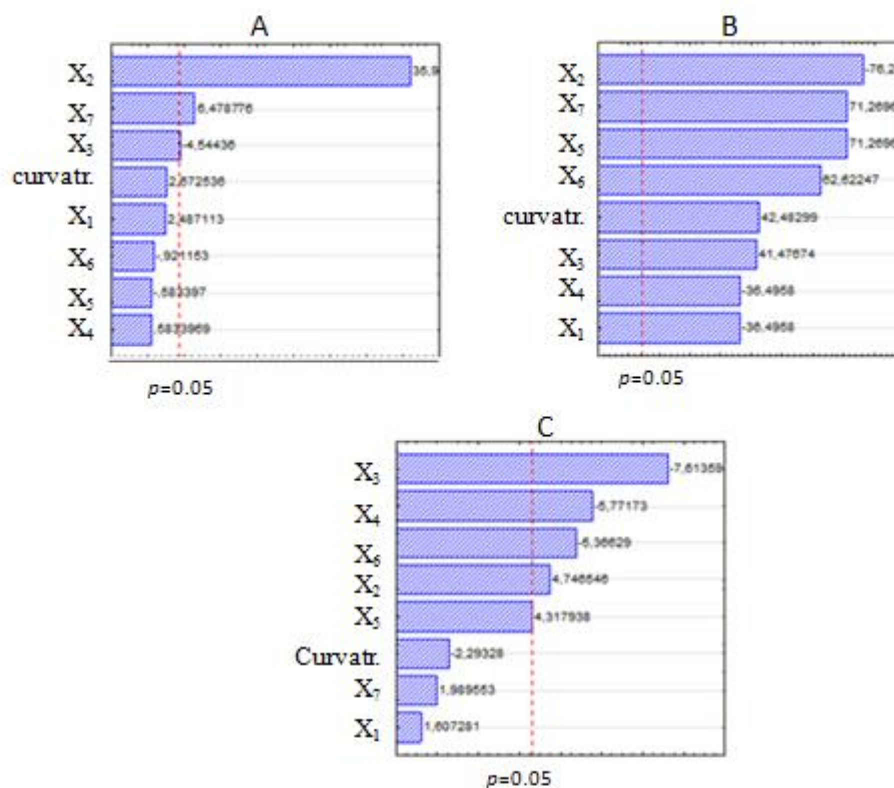

Figure 14: Pareto charts representing the effect of all investigated factors on dry matter (A), stability (B) and whiteness index (C).

III.3.1. Soaking time X_1

The soaking time did not exhibit any effect on the composition quality of the beverage (dry matter, yield, pH, fats and proteins) but it allowed more aromatic compounds (α-pine and D limonene) to be extracted. This may be due to the fact that soaking markedly hydrated the seeds which softened the plant matrix and enables a better release of aroma compounds in the

beverage during grinding. However, the longer the soaking time, the less the milk was stable and consistent. The soaking time negatively affected the stability and consistency

III.3.2. Ratio X_2

The ratio was positively proportional to the yield and the final DM as well as the protein, fat levels and aroma compounds (α-pinene, β-myrcene and D-limonene). As explained above, the DM was essentially influenced by the ratio. An increase in the ratio lowered the pH. This small but significant decrease may be due to the release of organic acids, a phenomenon which is not favorable for stability. The negative effect of ratio on the consistency index and the positive effect on the span can be explained by the fact that the more material there is, the more unstable the beverage. However, the ratio had no significant effect on particle size, viscosity and flow index. On the other hand, the higher the ratio, the higher the whiteness index, which leads to the conclusion that the whiteness index was also proportional to the amount of DM in the beverage.

III.3.3. Temperature of extraction X_3

The temperature of extraction had a positive effect on lipid extraction, but a negative effect on dry matter and this is explained by the fact that this parameter also has a negative effect on protein content. The study, carried out by **Ejoh, Djomdi, and Ndjouenkeu (2006)**, revealed that when the temperature was raised, the cell walls swelled and ruptured thus increasing the level of solids in the water. This observation is consistent with the positive effect of temperature extraction on all nutrients except on the proteins. Indeed, when beverages are heated to temperatures above 65°C, the deployed proteins expose their hydrophobic groups to the outside and aggregate together to form very high molecular weight molecules **(Donato, Guyomarc'h, Amiot, & Dalgleish, 2007)**. This phenomenon can explain a reduced concentration of protein in the milk because of a decrease in solubility. In addition, this change may have an impact on their emulsifying properties **(Claeys, et al., 2013).** The aggregation of proteins and the reduction of their solubility mean that when the mixture is centrifuged, the proteins settle and are eliminated with the solid residue, which may be the reason why grinding at high temperatures reduces the level of proteins in the beverage. The temperature of extraction was also inversely proportional to the concentration of aroma

compounds (α-pinene and β-myrcene). This may be due to the action of temperature on the destruction of aroma compounds. Also, the positive effect of the temperature of extraction on pH may be due to the destruction of acids. The temperature of extraction causes the whiteness to decrease, which may be due to the higher extraction of some particles from the seed coat.

The higher the temperature of extraction, the more stable the beverage regarding its positive effect on viscosity and negative effect on consistency.

III.3.4. Xanthan gum X_4

The amount of xanthan gum added had no effect on the chemical properties in general (dry matter, yield, lipids, proteins, pH) except that it lowered the level of aroma compounds (α-pinene, β-myrcene, D-limonene). It is known that the use of polysaccharides as thickeners often leads to the retention of aroma compounds and decreases their liberation in the headspace, which results from two main possible mechanisms: the slowing down of their diffusion caused by the increase of the viscosity of the solution and the inter and intramolecular interactions, absorption and encapsulation **(Secouard, Malhiac, Grisel, & Decroix, 2003).** The study by **Secouard, Malhiac, and Grisel (2003)** highlighted the same effect of xanthan gum on limonene. Contrary to most studies that prove that xanthan gum is an excellent stabilizer (**Taherian, et al., 2007**), our study showed that stability was inversely proportional to the concentration of xanthan gum. According to **Taherian, et al. (2007)** xanthan gum exerts its best stabilizing effect at low concentrations. In our results, the non-linearity of the stability toward xanthan gum may reveal that the beverage containing low concentration is much more stable than the beverage containing no xanthan gum or at high concentrations. Moreover, we also noticed in the preliminary study that the stability in the presence of xanthan gum also depended on the ratio (seeds/water). On the other hand, the more concentrated xanthan gum was, the more the particle size increased ($D_{(4:3)}$ and $DV_{(90)}$). The same particle magnifying effect was exhibited in the study of **(Mirhosseini, Tan, Hamid, Yusof, & Chern, 2009).** This may be due to the aggregating effect that can occur between the xanthan gum and other compounds of the beverage **(Taherian, et al., 2007)**, that can cause the observed increase of the viscosity and consistency. Xanthan gum has the ability to form a network by entering lipidic droplets with a film, which somehow immobilizes the

particles of the hydrocolloid system, so that the higher the concentration of xanthan gum, the more viscous and consistent the beverage was **(Taherian, et al., 2007)**. Finally, xanthan decreased the whiteness index and this is explained by the yellowish color of the xanthan gum that changed the color of the beverage at high concentrations **(Taherian, et al., 2007)**

III.3.5. Pasteurization temperature X_5

The pasteurization temperature had almost no effect on the chemical properties of the beverage (dry matter, yield, proteins, lipids, pH) but decreased the particle size ($D_{(4:3)}$ and DV_{90}). This is the opposite found in most studies where pasteurization increased particle size, caused by the aggregation of particles, especially proteins **(Bernat, et al., 2015).** This inverse result can be explained by the fact that there were many more lipids than proteins in the samples and that the temperature can cause the lipid droplets to break and therefore decrease in size. **(Cavazos-Garduño, et al., 2016).** Pasteurization temperature also increased the parameters of rheological behavior (viscosity, consistency index and flow index). This can be explained by the fact that the high temperatures induce a partial denaturation of the proteins and their aggregation. This denaturation increases the hydrodynamic volume resulting in a slight gelation. On the other hand, the rheological behavior can be the result of the hydration of the biopolymer chains of the soluble fibers **(Bernat, et al., 2015).** These phenomena may explain its positive effect on stability. It is the same for the whiteness index which increases with the temperature of pasteurization; this can be explained by the possible decrease in particle size. **(Bernat, et al., 2015).**

III.3.6. Homogenization pressure X_6

The homogenization pressure had no effect on the chemical properties of the beverage. However, it has a very significant positive effect on the stability and this may be due to its negative effect on the particle size behavior ($D_{(4:3)}$, DV_{90} and span) and its positive effect on the rheological behavior (viscosity, consistency index and flow index). Regarding color, the higher the homogenizing pressure, the lower the whiteness index. The homogenizing pressure also reduced whiteness because it decreases particle size by the reflection of light **(Bernat, et al., 2015)**. In addition, the homogenization pressure changes the conformation of the proteins, causing them to unfold, and cut the lipidic droplets of the beverage, which reduces the size of

these particles and at the same time increases the viscosity, and like the pasteurization temperature, makes the product more stable.

The homogenization pressure and the pasteurization temperature are the main factors improving the stability by their reducing effect of particle size which favors their dispersion in the aqueous phase while increasing the viscosity which prevents flocculation and coalescence.

III.3.7. Grinding time

The grinding time has shown a positive effect on the dry matter as well as on proteins and lipids. The more the mixture was crushed, the more compounds were extracted. It also had a positive effect on stability and viscosity and a negative effect on the consistency index. Similarly, a positive effect of grinding time on the dry matter of the tiger nut beverage was found in the study of **Ellis, Oduro, Saalia, and Asante (2014).** As in many studies (**Kundu, et al., 2018**), when the grinding time increases, the diffusion of the particles consisting essentially of lipids and fibers also increases by destroying more cell walls and releasing their contents.

Tableau XX: Analysis of variance for the experimental results obtained by different responses (regression coefficient and significativity)

	Chemical properties								Physical properties							
	Nutritional quality					Aromatic compounds			stability	Granulometric behavior			Rheological behavior			color
	DM	Yields	pH	Fats	Proteins	Alpha-pinene	Beta-myrcene	D-limonene	Stability index	Particle size $D_{(4:3)}$	Particle size DV_{90}	span	viscosity at a shear rate of 100^{-1}	Consistency index (k)	Flow index (n)	WI
Mean/Inter	**5.82*****	**24,90*****	**6.79*****	**3.97*****	**1.35*****	**0,69*****	0,17	0,04	**86.85*****	**70.52*****	**151,67*****	2,41	**10,227*****	**12.837*****	**0.77*****	**81.85*****
R^2	0.99	0.99	0.97^{-}	0.99	0.99	0.99	0.99	0.98	0.99	0.99	0.98	0.98	0.99	1	0.97	0.98
Curvatr.	0.41^{-}	**7,88*****	6.79^{-}	-0.32^{-}	-0.11^{-}	**-0,22*****	**-0,09***	-0,01	**10.98*****	**-36.61****	**-85,20****	**-0,37*****	0,98^{-}	**-12.80*****	-0.02^{-}	-1.89^{-}
Soaking time (X_1)	0.20^{-}	0.03^{-}	-0.10^{-}	0.04^{-}	0.07	**0,04***	-0,01^{-}	5x10^{-4-}	**-4.92*****	-0.37^{-}	4,02^{-}	0,07^{-}	0,33^{-}	**-12.71*****	-0.02^{-}	0.69^{-}
Ratio (X_2)	**2.92*****	**3,417*****	**-0.21****	**2.14*****	**0.53*****	**0,21*****	**0,08****	**0,01***	**-10.29*****	-4.65^{-}	-4,95^{-}	**0,18****	0,64^{-}	**-12.71*****	-0.05^{-}	**2.04***
Temperature of extraction (X_3)	**-0.37****	0,02^{-}	**0.20****	**0.39****	**-0.29*****	**-0,20*****	**-0,03***	-5x10^{-4-}	**5.60*****	8.70^{-}	14,75^{-}	-0,09	**1,87****	**-12.76*****	0.01^{-}	**-3.28***
Xanthan gum (X_4)	0.04^{-}	-0,03^{-}	-0.11^{-}	0.04^{-}	-0.02^{-}	**-0,15*****	**-0,07***	**-7x10^{-3*}**	**-4.92*****	**38.66*****	**87,78*****	0,06*	**8,80*****	**12.83*****	**-0.19*****	**-2.49***
Temperature of pasteurization (X_5)	-0.04^{-}	0,07^{-}	-0.06^{-}	-0.14^{-}	0.05^{-}	0,01^{-}	**0,03***	-2x10^{-4-}	**9.62*****	**-50.40*****	**-111,66*****	-0,02^{-}	**1,29****	**12.68*****	**0.12****	**1.86***
Pressure of homogenization (X_6)	-0.07^{-}	0,09^{-}	-0.01^{-}	0.17^{-}	-2.10^{-3-}	-2.10^{-3-}	**-0,03***	1x10^{-3-}	**8.45*****	**-35.05*****	**-81,11*****	**-0,21****	**1,15****	**12.68*****	**0.11****	**-2.31***
Milling time (X_7)	**0.52****	0,06^{-}	-0.03^{-}	**0.28****	**0.15****	-0,02^{-}	0,01^{-}	2x10^{-4-}	**9.62*****	8.05^{-}	13,81^{-}	0,12^{-}	**2,05****	**-12.76*****	-0,08^{-}	0.85^{-}

(p>0.1 ($^{-}$), p<0.1 (*), p<0.05(**), p<0.01(***))

IV. Conclusion

The design of the experiments allowed the impact of various key operations and factors of the production of Aleppo pine seed beverage on its quality to be studied. It was shown that all the factors had a significant impact on the quality parameters, chemical and/or physical. The soaking time had a negative effect on the stability and did not present any significant advantage regarding yield or beverage quality. During extraction, the ratio was a very important factor for the nutritional quality. However, the higher the ratio, the more unstable the product was. The grinding time and temperature of extraction were positive factors for nutritional quality and stability apart from the fact that the temperature of extraction decreased the protein extraction rate. Regarding the formulation step, the xanthan gum that was added to thicken and stabilize the beverage had a positive effect on viscosity but increased the particle size and showed its effectiveness only at low concentrations. Finally, ultra-high homogenization pressure and pasteurization temperature had almost the same positive effect on stability and this was due to their reducing effect on particle size and increasing effect on viscosity. Aleppo pine seeds are indeed an interesting source for the design of a plant-based beverage, because we could still have a beverage comparable to other commercialized plant-based beverages in terms of nutritional and organoleptic quality. To have an acceptable product based on these seeds, soaking is not really necessary. For the extraction, the ratio must be average so as to have a maximum of nutrients in a stable beverage. The grinding time must be high for extracting the maximum amount of nutrients. The temperature must be set at the average temperature of 60°C to extract the maximum of both lipids and proteins. For the formulation step, addition of xanthan gum is not necessary but it can increase viscosity and stability. Physical stabilization by homogenization pressure is a good alternative to stabilize this product and the result is not impaired when followed by a pasteurization process. In order to determine the optimal conditions for the design of the beverage, a complete experimental design must be applied to confirm this effect and better study the interaction effects of the most influential factors on its nutritional quality, its stability and its color and coupled to this, sensory analysis and a study of acceptability of this new product.

General discussion and conclusion

Aleppo pine seeds are very interesting seeds, very little or almost not used in traditional medicine as before, nor in industry and yet it is a very abundant matrix, which has a very good taste. Previous studies have focused on the characterization of the seed (lipid, protein and carbohydrate profile as well as its polyphenols and volatile compounds) it has been shown that they have a very interesting profile and could be a functional food. This is what sparked our curiosity to explore other avenues that could enhance the value of these seeds in the pharmaceutical or food industry.

In this work, we first worked and tried to valorize its polysaccharide fraction since it has never been studied before and because these molecules interest more and more researchers for their high activity and non-toxicity. To test some biological activities, the antioxidant activity was evaluated with different tests (ABTS, DPPH, metal chelation, ferric reducing power, antiperoxidation and ORAC tests), the anti-inflammatory activity was assessed with three tests (denaturation protein inhibition, antiprotéinase and anti-hemolytic tests) and finally, the anticoagulant activity was tested by endogenous and exogenous ways. On the other hand, the study was pushed further to see and compare which solvent (between ethanol, acetone and propanol) allowed us to have a better yield and better activities. It turned out that the polysaccharide extracts, whether extracted by ethanol, acetone or propanol, showed very good antioxidant, anti-inflammatory *in vitro* and anticoagulant activities at the microgram level, but for most of the activities the acetone extract always presented the best efficiency. This said, acetone is the best solvent to use for the precipitation of polysaccharides from Aleppo pine seeds and that the polysaccharides of these seeds can be very well used in pharmaceutical industry as a natural active ingredient, very effective at low concentration, without any toxicity, whose matrix is very available.

In a second time, as these seeds are rich in proteins, and as industries are constantly looking for new natural molecules to replace chemical food additives, in recent years researchers and industrialists focus a lot on vegetable proteins, especially oil seeds (which are rich in proteins) with very interesting functional properties, but on the other hand the problem often encountered is that the proteins are very poorly soluble, so we have tried to optimize its functional properties according to pH, NaCl and phosphate buffer to find the best conditions for Aleppo pine proteins to perform the best functional activities, and we have indeed greatly

improved its solubility by applying the optimal conditions. this said that Aleppo pine proteins have good functional properties at basic pH, without NaCl and in phosphate buffer.

In the third part, we wanted to design a food product based on its seeds since they had all the potential of other oilseeds from which vegetable milk is made (lipids and proteins), so we studied the effect of soaking, ratio, grinding time and temperature, of percentage in xanthan gum, the homogenization pressure and the pasteurization temperature on the chemical and physical quality of this beverage, to be able to identify the conditions that allow to give a better milk (nutritive, stable, white) and to understand a little bit the phenomena that explain these properties. In fact, it has been shown that soaking had almost no considerable effect on the quality of the milk, that the ratio was proportional to the nutritive value but a ratio of 3/10 made the milk unstable, the grinding time allows the extraction of a maximum of material, xanthan gum was added as a thickener and stabilizer, but was more effective at low concentrations, while the homogenization pressure and pasteurization greatly improved the stability of the milk. At the end, we were still able to have a white, stable and very nutritious milk of the same quality as the other vegetable milks, but the Aleppo pine seeds have a high potential. We were still able to have a white, stable and very nutritious milk of the same quality as the other vegetable milks. However, Aleppo pine seeds have a great potential for this application.

The results of his three studies show that these seeds are very exploitable and can be used in the pharmaceutical industry (for its antioxidant, anti-inflammatory and anticoagulant activities) and in the agro-food industry, whether it is the use of their protein fraction as a food additive due to their functional properties or the use of complete seeds in the manufacture of vegetable milk.

However, these are not complete studies on which we can make a statement, for which some perspectives have been put forward,

- Purify and characterize its polysaccharide extracts and experiment on antioxidant, anti-inflammatory and anticoagulant activities *in vitro*.
- Purify and characterize the protein extract and make an application as a food additive and see their effect in a food matrix.

- Optimize the factors most influencing the quality of milk to define an optimum and make a sensory analysis and acceptability of Aleppo pine seed milk to consider marketing.

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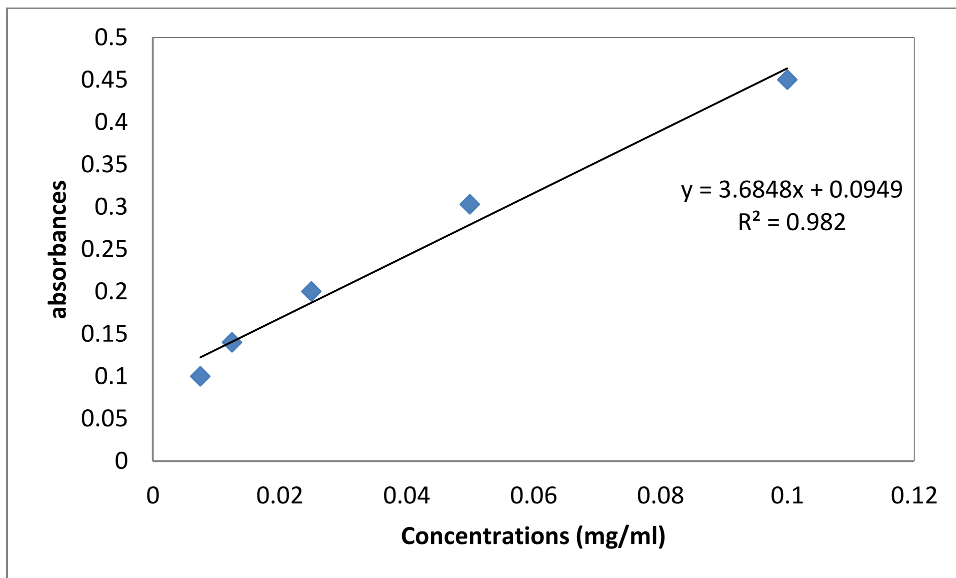
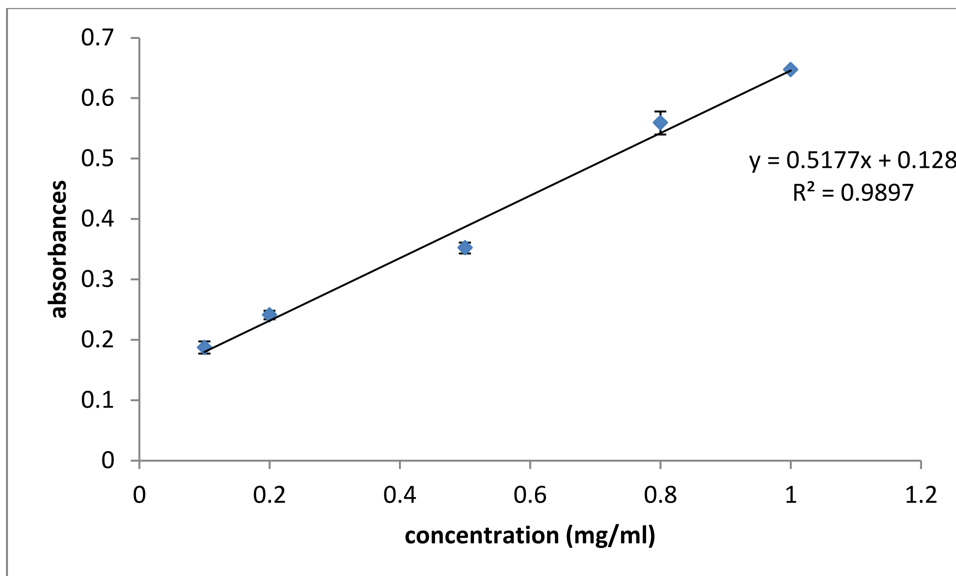
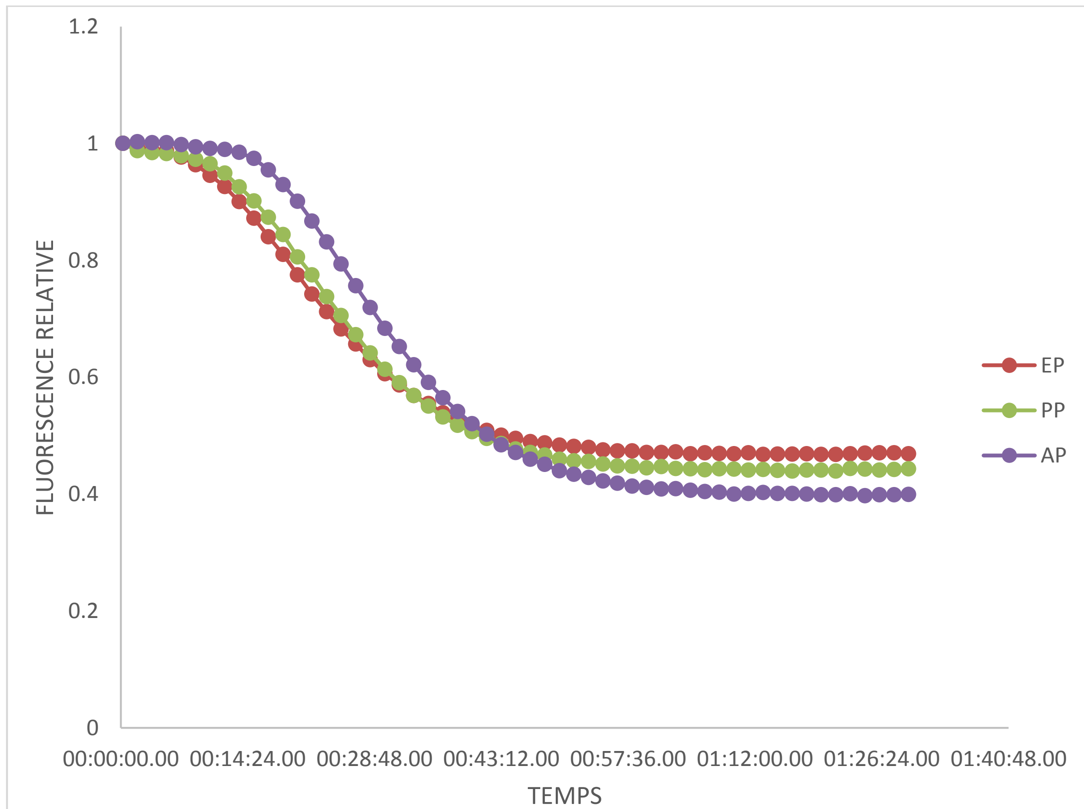


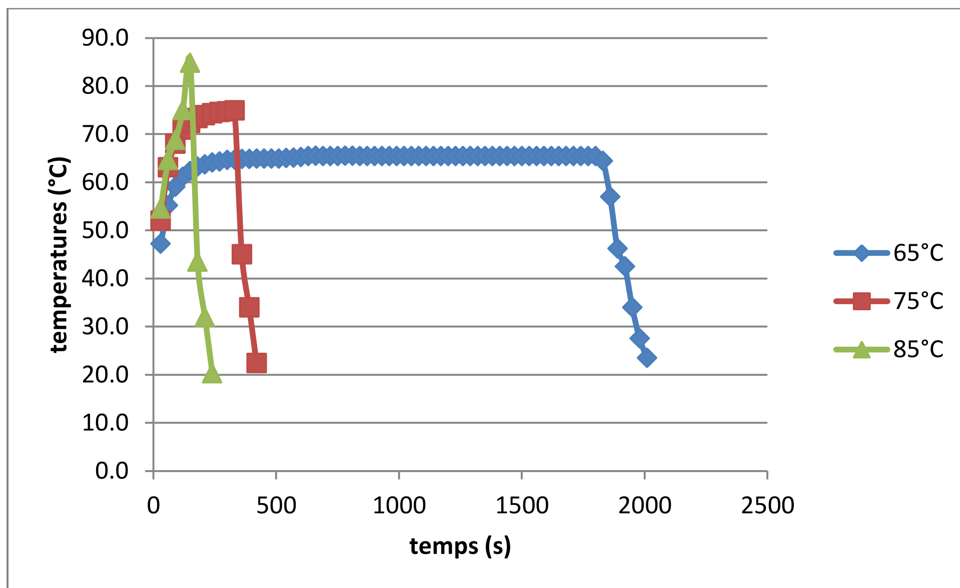
Appendix 1 : Carbohydrates calibration curve (using glucose as standard)



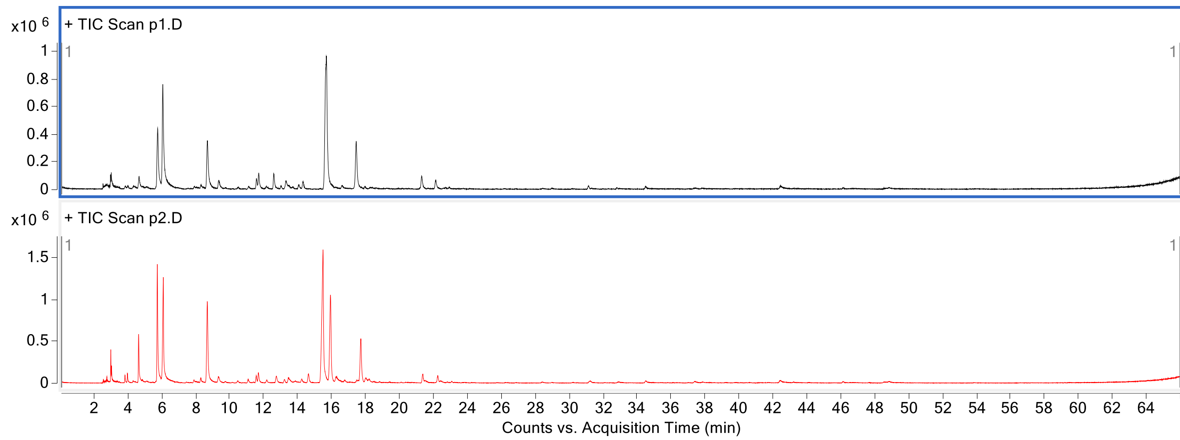
Appendix 2 : Proteins calibration curve (using BSA as standard)



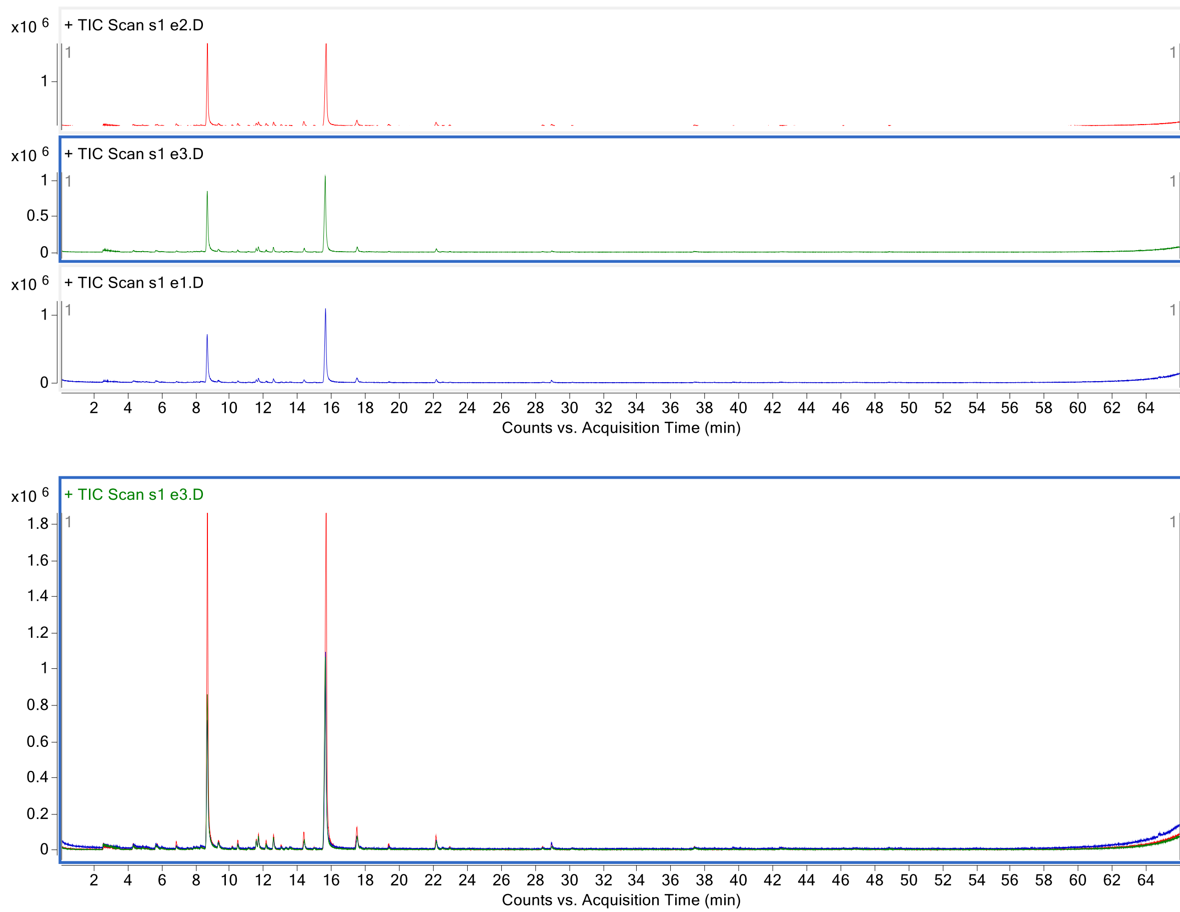
Appendix 3: ORAC assay measures antioxidant capacity



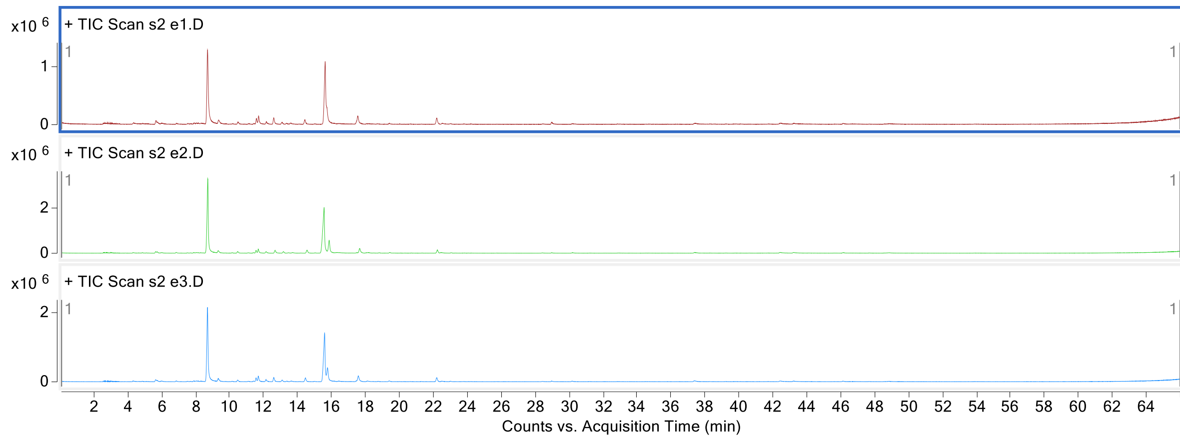
Appendixe 4: Pasteurization bar for *Pinus helepensis*.Mill seed beverage

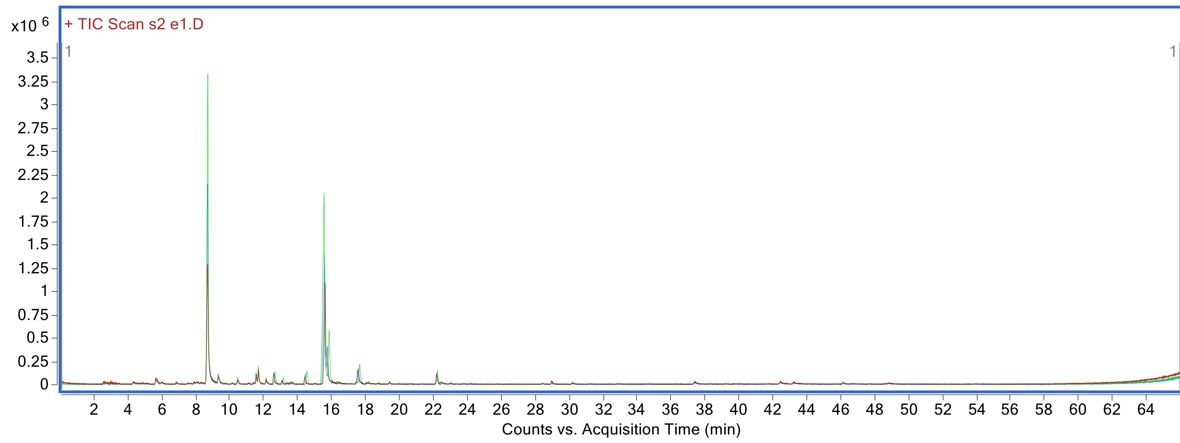


Appendix 5: chromatogram of aroma compounds of Aleppo pine seed powder

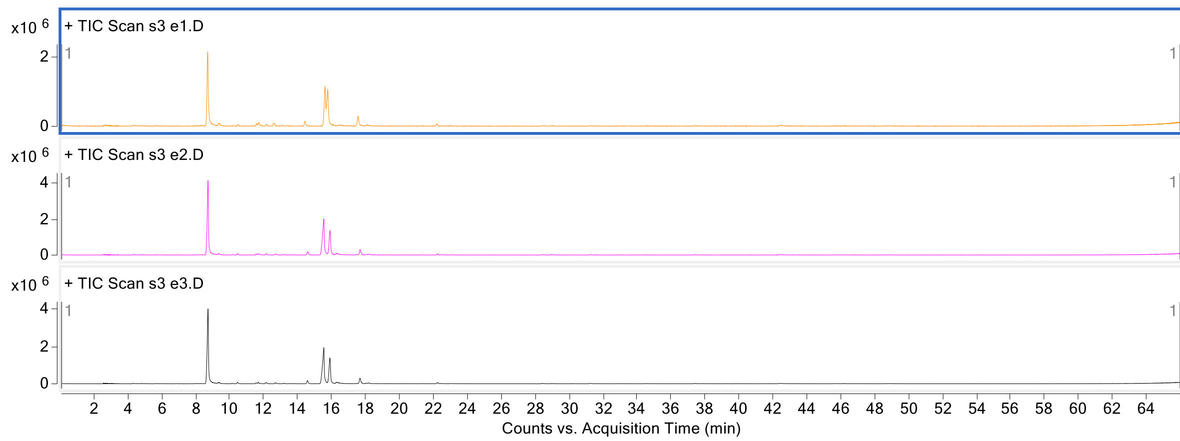


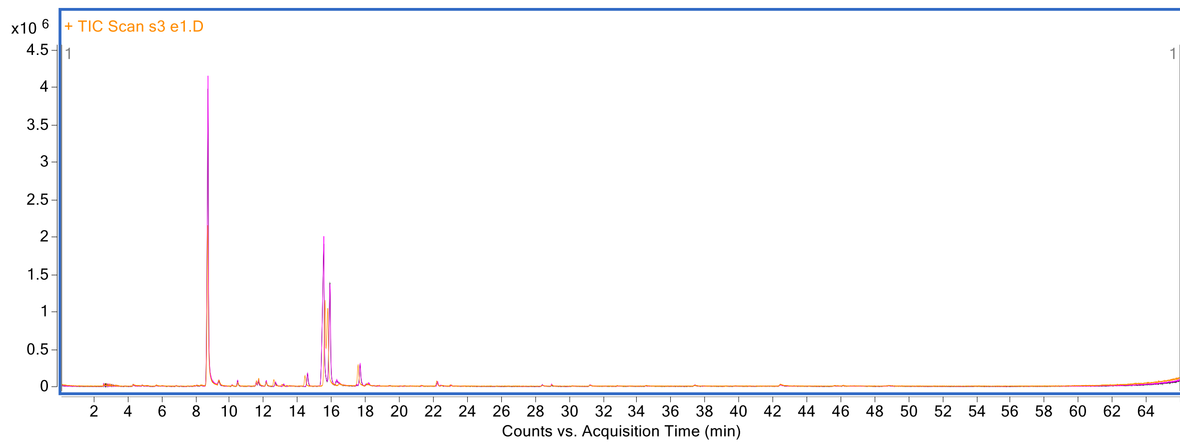
Appendix 6: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 1)



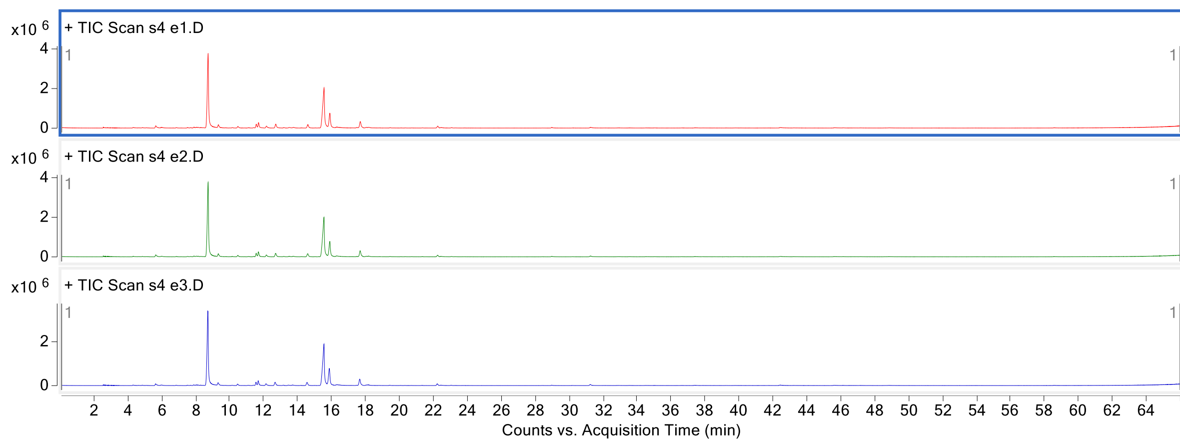


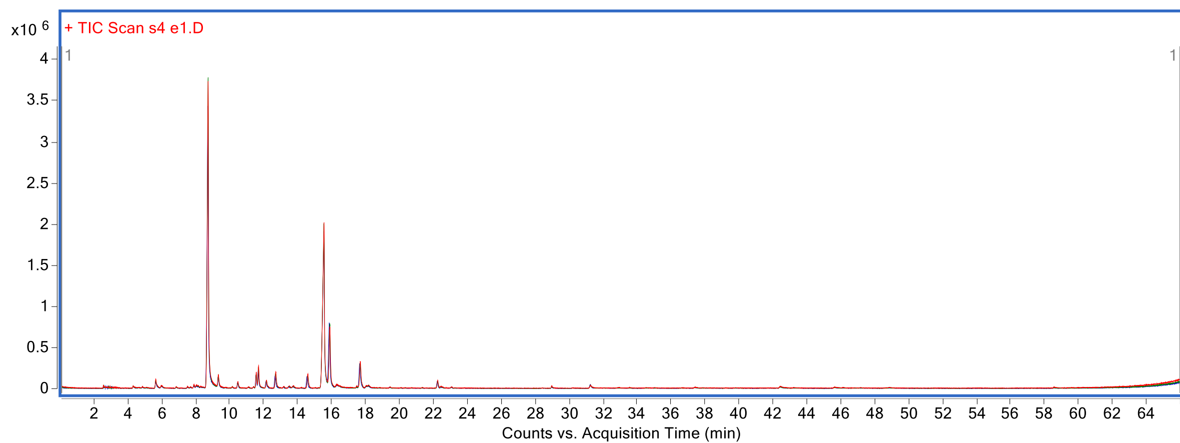
Appendix 7: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 2)



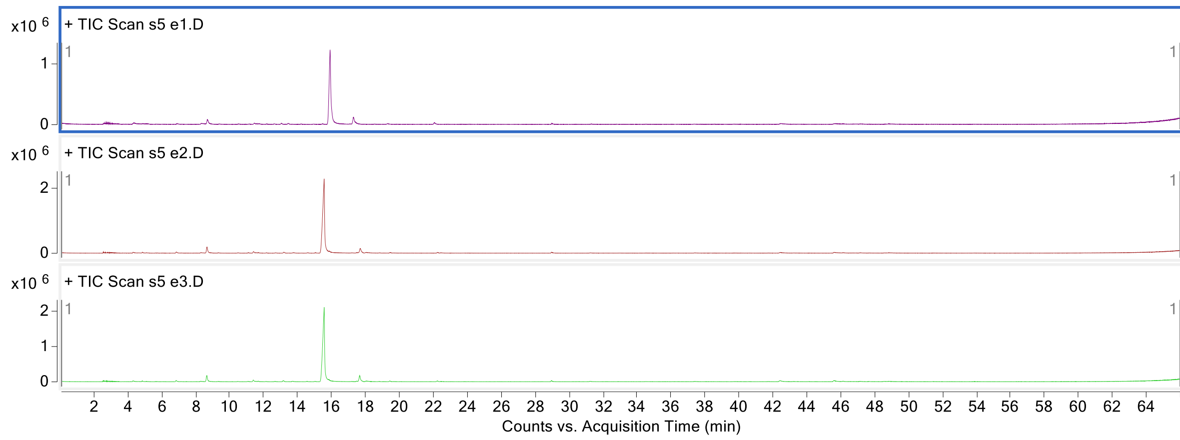


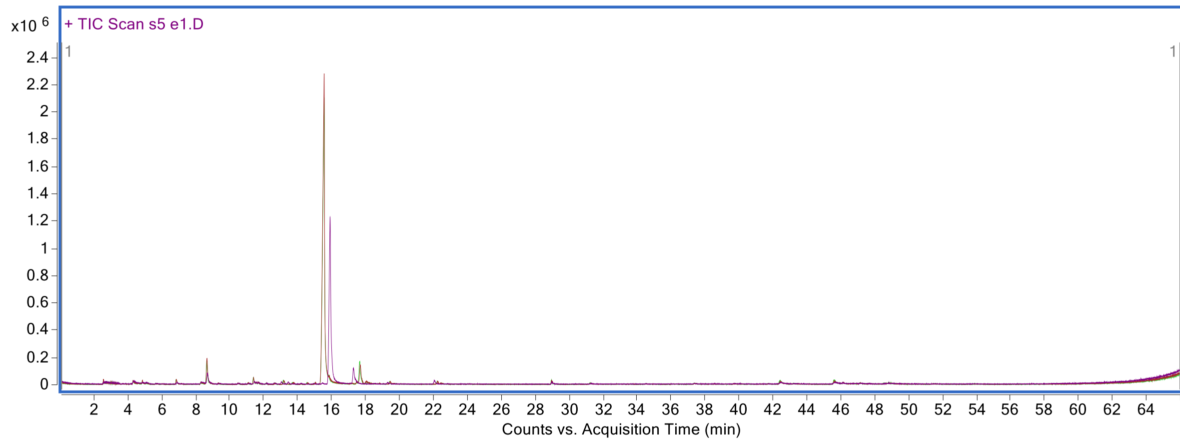
Appendix 8: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 3)



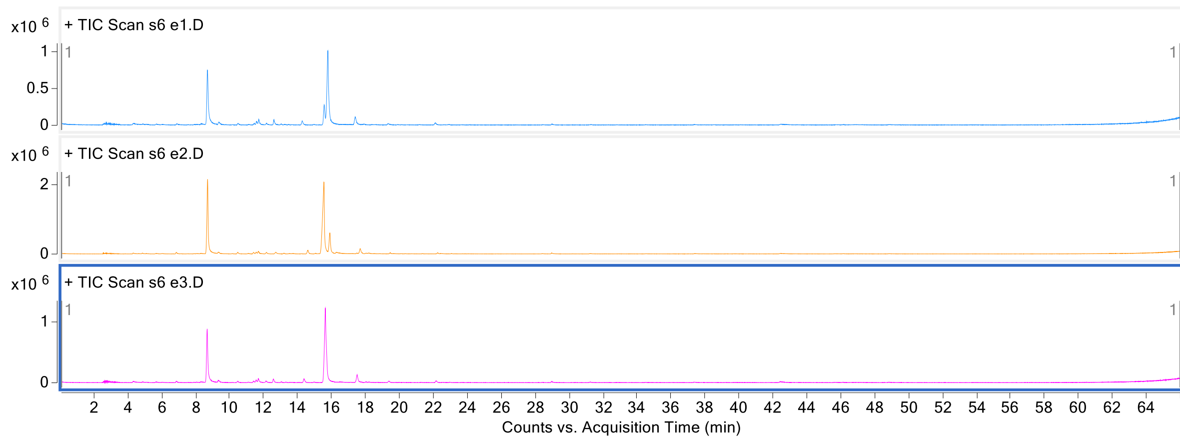


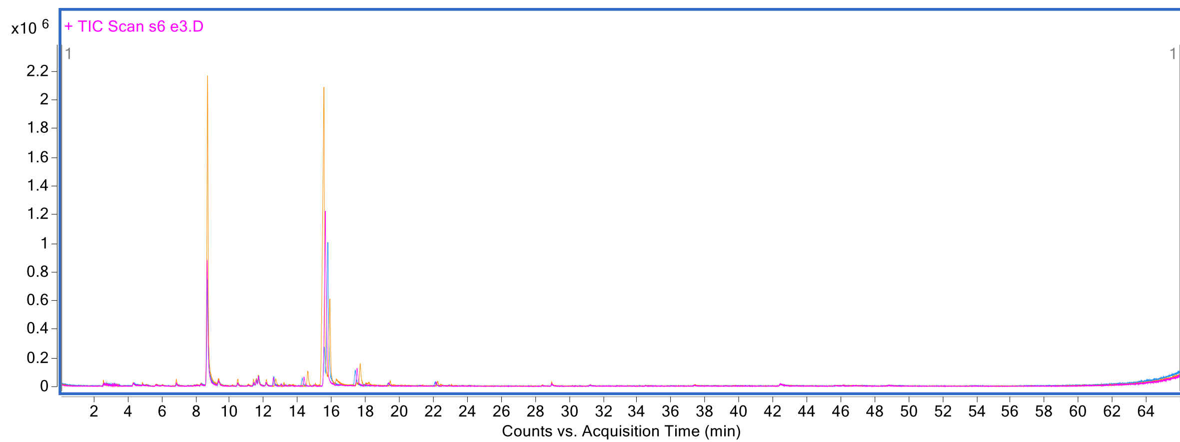
Appendix 9: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 4)



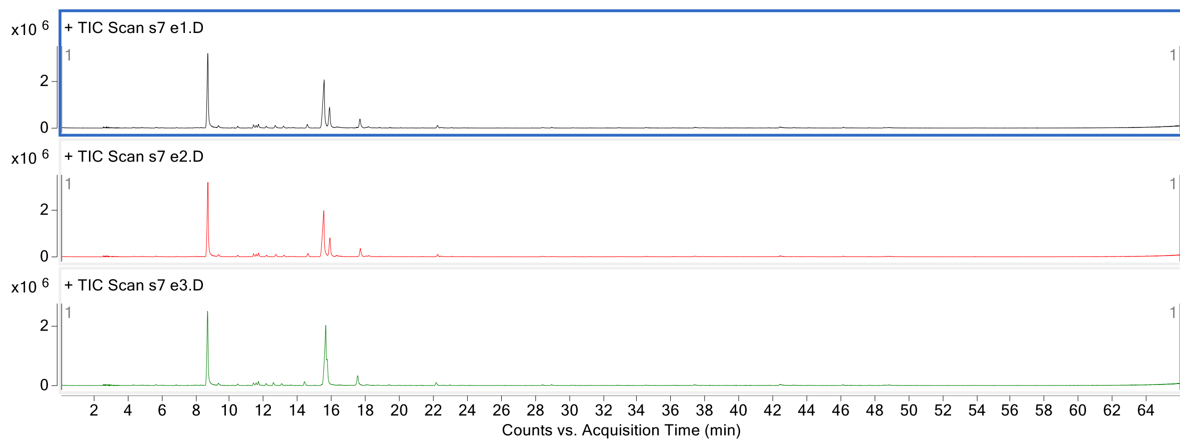


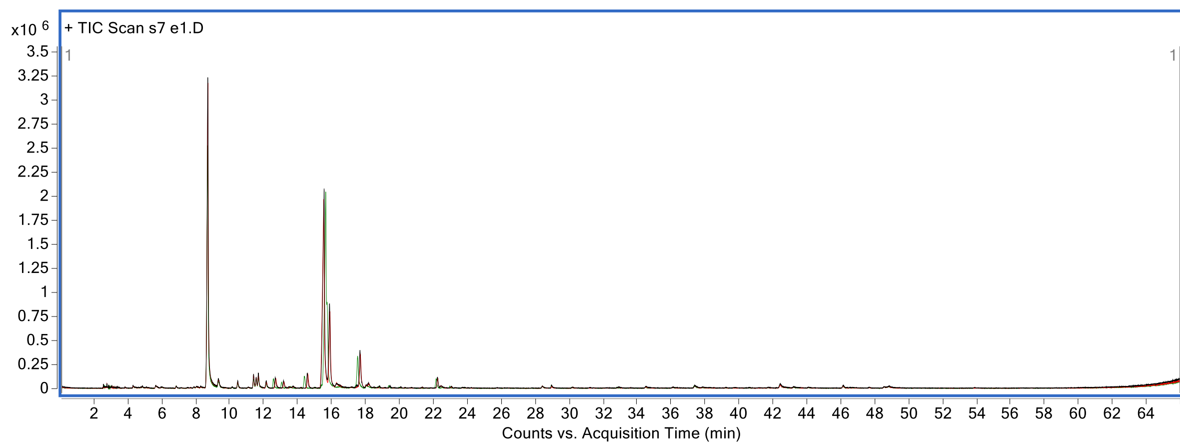
Appendix 10 : chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 5)



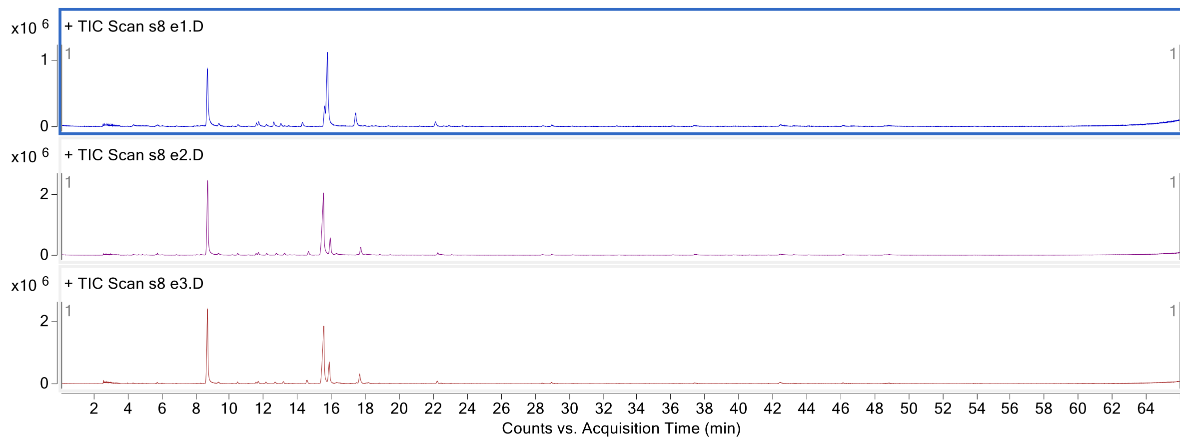


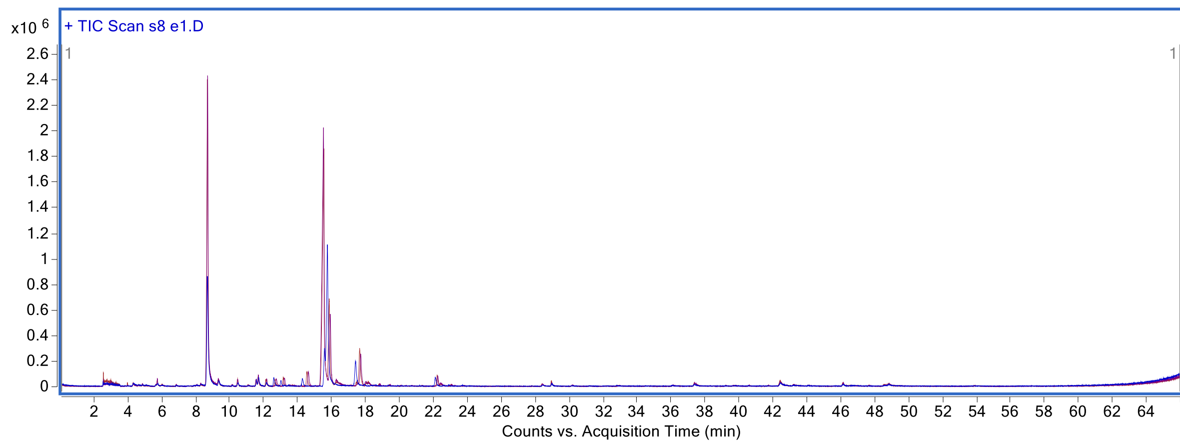
Appendix 11: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 6)



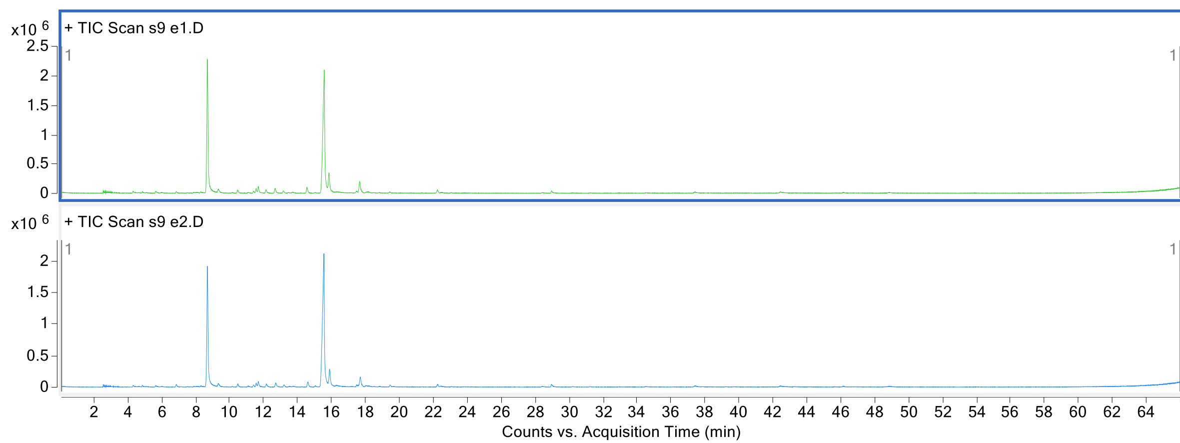


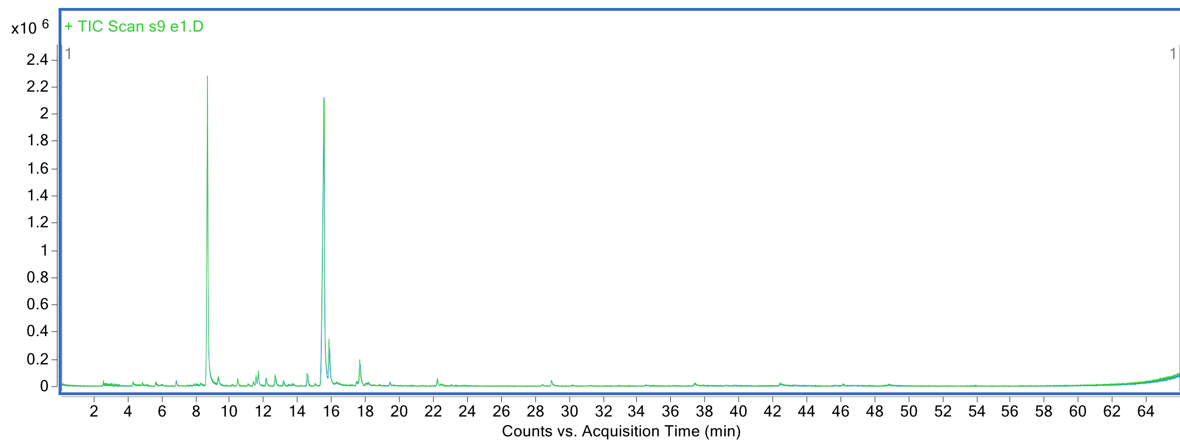
Appendix 12: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 7)



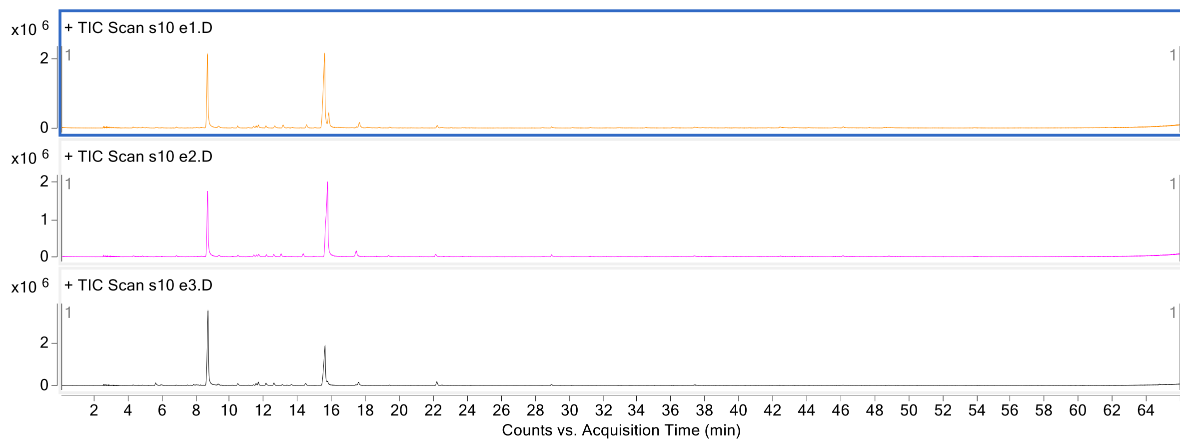


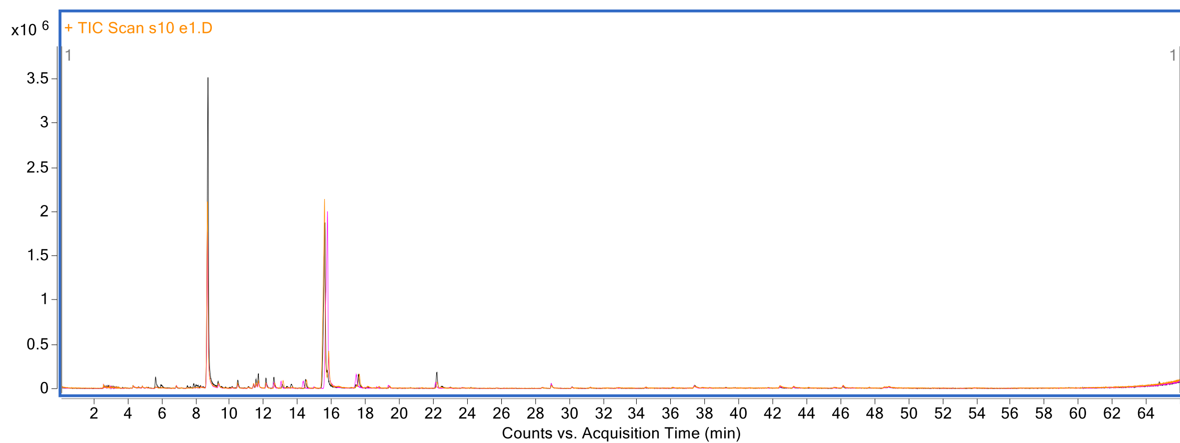
Appendix 13: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 8)



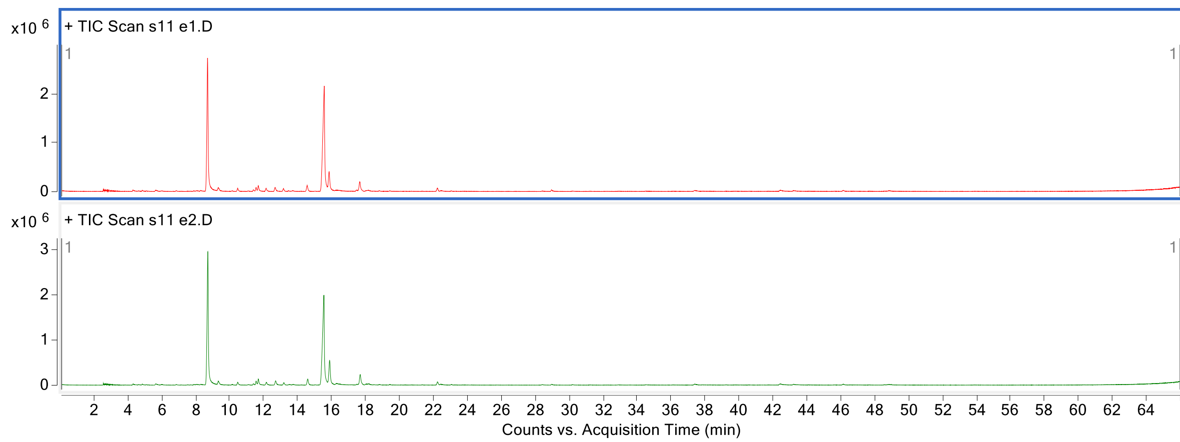


Appendix 14: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 9)





Appendix 15: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 10)



Appendix 16: chromatogram of aroma compounds of vegetable beverage of Aleppo pine seed (sample 11)

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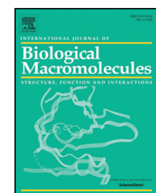
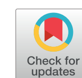


Effect of precipitation solvent on some biological activities of polysaccharides from *Pinus halepensis* Mill. seeds



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ABSTRACT

This study was designed to evaluate, for the first time, the effect of the precipitation solvent (Acetone, Ethanol, and Propanol) on the antioxidant, anti-inflammatory and anticoagulant activities of the polysaccharides extract from Aleppo pine seeds. The antioxidant activity was evaluated with different tests (ABTS, DPPH, metal chelation, ferric reducing power, antiperoxidation and ORAC tests), the anti-inflammatory activity was assessed with three tests (denaturation protein inhibition, antiproteinase and anti-hemolytic tests). Finally, the anticoagulant activity was tested by endogenous and exogenous ways. The three extracts (AP: acetone polysaccharides extract, EP: ethanol polysaccharides extract and PP: propanol polysaccharides extract) have exhibited a very interesting activities but with different degrees. The AP extract was most effective in almost all antioxidant activities (antiradical ABTS and DPPH, metal chelation, reducing power and ORAC), in two *in vitro* anti-inflammatory and the anticoagulant activities. However, for the lipid antiperoxidation activity, it was the PP extract that gave better activity. The best antiproteinase activity was expressed by the EP extract. These results indicate that polysaccharides of Aleppo pine seed may be considered as a source of bioactive polysaccharides and the precipitation solvent of the polysaccharides has a major effect on the intensity of the bioactivity of these polysaccharides.
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1. Introduction

A particular focus has been placed on the research of new sources of plant molecules, in order to develop new principles or to discover structural analogues of existing molecules [1]. During the last few years, they have attracted considerable attention and increasing studies have started to develop natural polysaccharides-based biomaterials for various applications. This is due to their biocompatibility, low toxicity, unique physical properties, and specific therapeutic properties [2]. This primary metabolite possessed several biological activities like as anticoagulant, antiviral, antioxidant, antitumor, anti-inflammatory, anticomplementary, antiseptic [3,4]. Villares et al. [5] have reported that >300 polysaccharides have been developed and are in clinical trials as antiviral, antitumor and antidiabetic agents. These macromolecules can therefore be of vegetable, animal, microbial or fungal origin. However, very little work is done on the use of the raw polysaccharide extract of the Pinaceae family, unlike other metabolites of the same family such as polyphenols and flavonoids.

The seed of *Pinus halepensis* Mill. is an oleaginous seed largely distributed in the Mediterranean basin. It was formerly very consumed especially in pastry making and it has a long tradition in folk medicine as an antidiabetic and to treat sexual problems. Most of the work carried out on this seed was focused on the phytochemical study and especially its lipid fraction [6–10]. In our knowledge, there are no recorded studies on their polysaccharides.

The extraction of polysaccharides is based on the principle of precipitation, generally by solvents. The most widely used solvents in the literature are ethanol, acetone and propanol. But according to the literature, no study has proven the effect of these different solvents on the bioactivity of polysaccharide extracts. The principal purpose of this work is

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to identify new sources of polysaccharides (which are the Aleppo pine seeds) that may become drug substitutes synthetic and to find the best solvents allowing the precipitation of maximum polysaccharides with maximum purity and with the best bioactivity. For this purpose, the *in vitro* antioxidant, anti-inflammatory and anticoagulant activities were tested for the three extracts (AP (acetone polysaccharides), EP (ethanol polysaccharides) and PP (propanol polysaccharides)) recovered by the three different solvents (acetone, ethanol and propanol).

2. Material and methods

2.1. Material

2.1.1. Plant material

The seeds of Aleppo pine (*Pinus halepensis* Mill.) were obtained from the Collo forest located in Skikda province of Algéria. They were cleaned; dried in an oven at 40 °C for 2 days and then finely crushed using an electric grinder (KIKA Labortech-nik M20, Germany) to obtain a fine powder (250 μm) which was delipidated by the Soxhlet method with petroleum ether.

2.1.2. Red blood cell suspension

Blood was obtained by venipuncture from healthy volunteers collected in heparinized tubes and centrifuged at 3500 rpm for 15 min. Plasma and buffy coat were removed. Red blood cells (RBCs) were suspended in 10 volumes of 0.9% NaCl and centrifuged at 2500 rpm for 5 min. The RBCs were washed three times with the same solution. During the last washing, the packed cells were re-suspended in 10 volumes of phosphate buffered saline (PBS, pH 7.4) and utilized for the cytotoxicity and anti-hemolytic tests [11].

2.2. Methods

2.2.1. Preparation of extracts

The protocol of Chen et al. [12] was used with some modifications; three different extractions were carried out. For each one, 25 g of powder was extracted with bi-distilled water (300 mL) for 1 h at 60 °C. After centrifugation at 6000 rpm and 4 °C for 15 min, the supernatant was collected and filtered. The filtrate was concentrated to a third of the volume, then three volumes of solvent (ethanol, acetone or propanol) have been added. The mixtures have been left to precipitate for 48 h at 4 °C, and then centrifuged again. The pellets were recovered and suspended in bi-distilled water at a ratio of 1:20 (w/v) and deproteinized using $CaCl_2$ methods [13]. After filtration, the obtained filtrate was subjected to a second precipitation with (ethanol, acetone or propanol) (1:3 v/v for 48H at 4 °C). After centrifugation (6000 rpm and 4 °C for 15 min), the pellet was lyophilized and stocked.

2.2.2. Chemical composition and yield

The extraction yields before and after deproteinization was expressed as residual solid extract obtained from fat-free powder. Ash, moisture and fat levels were estimated by AOAC [14] methods, the protein and sugar content were determined before and after deproteinization using Bradford [15] and Dubois et al. [16] respectively. Concentrations of protein and sugar were deduced from a standard curve using BSA and glucose respectively.

2.2.3. FT-IR analysis of polysaccharide extracts

The FTIR spectra of the various samples were performed using a spectrometer SHIMADZU FTRI 8400, FT-IR (IRAffinity-1S Shimadzu, Japan). 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} [17].

2.2.4. Cyto-toxicity assay

To 1 mL of RBC suspension, a volume of 1 mL of extracts (AP, EP and PP) at increasing concentrations (1, 2, 3 and 4 mg/mL) was added. After 10 min of incubation at room temperature, the samples were centrifuged at 3000*g* for 10 min and the resulting supernatant was removed and used to evaluate their hemolytic activity using a spectrophotometer at 540 nm. RBC lysis in the presence of distilled water was considered as 100% hemolytic activity [18]. Hemolysis in the presence of extracts was calculated relative to this control hemolysis

$$\text{Hemolysis}\% = 100 \times (A_0 – A_1 / A_0).$$

where A_o was the absorbance of control (distilled water without extract) and A_1 the absorbance in the presence of extract.

2.2.5. Antioxidant activities

2.2.5.1. Free radical scavenging activities

2.2.5.1.1. DPPH free radical scavenger. Briefly, 180 μL of the 0.1 mM DPPH solution in methanol is added to 20 μL of acetone polysaccharides (AP), ethanol polysaccharides (EP) and propanol polysaccharides (PP) solution at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL). The mixtures have been vigorously shaken and incubated in darkness for 30 min. The absorbance was measured at 517 nm using micro-plate reader (*BioTek*, *EIx800*, *USA*) against a control containing 180 μL DPPH solution and 20 μL methanol [19].

2.2.5.1.2. ABTS free radical scavenger. Solutions of ABTS (7 mM) and potassium persulphate (2.45 mM) were mixed and incubated in the dark at room temperature for 12–16 h. The product was diluted in ethanol for optimal absorption ± 0.7 at 734 nm. The reduction between $ABTS^+$ and test samples was monitored by a decrease in absorption at 734 nm during 30 min [20].

Radical scavenging activity was calculated as follows:

$$\%\text{radical scavenging activity} = 100 \times (A_0 – A_1 / A_0)$$

where A_0 is the absorbance of control solution and A_1 is the absorbance in the presence of plant extract. IC_{50} was determined from a graph in which scavenging activity was plotted against varying concentrations g/mL of extract using a linear regression curve.

2.2.5.2. Metal chelating activity. The chelation of ferrous iron by extracts was evaluated by the ferrozine method, for this purpose, 250 μL of the different extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) were put in competition with 50 μL of ferrozine to chelate the iron contained in 25 μL of $FeSO_4$ at 2 mM. After incubation for 5 min in the darkness, the absorbances were measured at 562 nm with a micro-plate reader [21]. The results have been expressed in terms of IC_{50} and the percentage of inhibition of ferrozine-Fe^{2+} complex formation was given by the following formula:

$$\%\text{inhibition} = 100 \times ((A_0 – (A_1 – A_2) / A_0).$$

where A_0 was the absorbance of the control, A_1 was the absorbance without $FeCl_2$, A_2 was the absorbance with $FeCl_2$. The control contains $FeCl_2$ and ferrozine without extracts.

2.2.5.3. Lipid peroxidation. The TBARS protocol from Ohkawa et al. [22] and Pandey et al. [23] was used with minor modifications. 0.5 mL of egg yolk homogenate (10% in v/v distilled water) and 0.1 mL of different extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) solution were mixed in a test tube and the volume was made up to 1 mL with distilled water. 0.05 mL of $FeSO_4$ (0.07 M) was added to the previous mixture and incubated for 30 min at 37 ° C to induce lipid peroxidation. Then, 1.5 mL of acetic acid (20%, pH 3.5), 1.5 mL TBA (0.8% (w/v) Prepared in 1.1% sodium dodecyl sulphate) and 0.05 mL TCA (trichloroacetic acid) (20%) were added, vortexed and heated at 100 ° C in a water bath for 60 min. After cooling, 5 mL of 1-butanol were added then the mixture is centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant (organic phase) was measured at 532 nm. The results have been expressed in terms of IC_{50} and

the percentage of inhibition of lipid peroxidation was calculated by the following formula:

$$\%\text{inhibition} = 100 \times (A_0 - (A_t - A_{t0})/A_0)$$

A_0 is absorbance of mixture without extract, A_t is the absorbance of test and A_{t0} is the absorbance of extract blank (prepared by replacing the TBA with sodium dodecyl sulphate).

2.2.5.4. Ferric reducing power. The reducing power of the polysaccharide extracts (AP, EP and PP) were tested by mixing them at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) in 1 mL methanol with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL potassium ferricyanide at 1%. The mixtures were incubated for 20 min at 50 °C and then 2.5 m of 10% TCA was added to the mixtures. The all was centrifuged for 10 min at 3000 rpm. 2.5 mL of the supernatant was then mixed with 2.5 mL of methanol and 0.5 mL of $FeCl_3$ at 0.1%. Finally, the absorbance was measured at 700 nm [24]. The results have been expressed in terms of EC_{50}.

2.2.5.5. Oxygen radical absorbance capacity (ORAC). The ability of extracts to inhibit the consumption of 2′,7′dichloroflurescein (DCF) was used to measure the ORAC value. For this purpose, a mixture of 50 μL of samples (extracts or trolox), 100 μL of DCS and 100 μL of AAPH was prepared in the wells of a microplate (the extracts and all the products used were dissolved in salt phosphate buffer (10 mM, 150 mM NaCl at pH, 7.4). fluorescence spectrometer (Victor2 Wallac-Perkin-Elmer) was used to record the fluorescence every 1 min for 90 min at 485 nm-excitation and 535 nm emission.

ORAC values are expressed in terms of moles of Trolox equivalent (TE) per mole of antioxidant (pure) or per gram of polysaccharides extracts using the trolox calibration curve that determines the time required to achieve 50% fluorescence desintegration relative to trolox concentrations [25].

2.2.6. Assessment of in vitro anti-inflammatory activity

2.2.6.1. Test of Inhibition of albumin denaturation. The inhibition of BSA denaturation test was used to study the possible anti-inflammatory capacity of polysaccharide extracts, for this purpose, 2 mL of 1% aqueous BSA solutions containing the extracts with different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) was incubated at 37 °C for 20 min, then a heat treatment at 70 °C was applied for 20 min, turbidity was measured at 660 nm after cooling and to express the percentage of protein denaturation the following equation was applied [26].

$$\%\text{inhibition} = 100 \times (A_0 - A_1/A_0)$$

where A_0 is the absorbance of control (heated BSA without extract) solution and A_1 is the absorbance in the presence of plant extract.

2.2.6.2. Test of antiproteinase action. The method of Oyedapo and Famurewa [11] was used to study the antiproteinase activity which is also involved in the inflammatory process, one volume of polysaccharide extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) was added to 2 volumes of Tris HCl (20 mM pH 7.4) containing 0.3 mg/mL of trypsin, the mixture was incubated for 20 min. Then the reaction was stopped by adding 2 volumes of 70% perchloric acid. The absorbance of the supernatant in the mixture was recorded at 210 nm after centrifugation. The buffer was used as white and the percentage of inhibition of the proteinase inhibitory activity was calculated as follows:

$$\%\text{inhibition} = 100 \times (A_0 - A_1/A_0)$$

where A_0 is the absorbance of control solution and A_1 is the absorbance in the presence of plant extract.

2.2.6.3. Membrane stabilization by hypotonicity induced hemolysis. Hemolytic activity was evaluated as described previously by Oyedapo and Famurewa [11]. A volume of 5 mL of hypotonic PBS (10 mM, 50 mM NaCl, pH = 7.4) containing extracts at different concentrations (3.125, 6.25, 12.5, 25, 50, 100 μg/mL) was added to 0.5 mL of RBC suspension and then the samples were incubated for 10 min at 37 ° C and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was then measured at 540 nm using a micro-plaque reader to determine the hemoglobin released from the lysed erythrocytes. The percentage of hemolysis inhibition was calculated for each sample according to the following law:

$$\%\text{inhibition} = 100 \times (A_0 - A_1/A_0)$$

where A_0 is the absorbance of positive control solution and A_1 is the absorbance in the presence of plant extract.

The negative control represents the blank where no hemolysis is induced; (0.5 mL of the RBC suspension with 5 mL of isotonic PBS) and the positive control where hemolysis is induced by a hypotonic phosphate buffer (10 mM, 50 mM NaCl, pH = 7.4) without extracts.

2.2.7. Anticoagulant activity

All tests were performed on plasma from citrated tubes recovered from healthy subjects (5 different donors). All tests were performed in triplicate.

2.2.7.1. Evaluation of activated partial thromboplastin time (APTT) (the endogenous way). A volume of 50 μL of plasma was mixed with 10 μL of a solution of the different polysaccharide extracts at different concentrations (100, 50, 25 μg/mL) before adding 50 μL of APTT reagent. Then, the reaction mixture was incubated for 3 min at 37 ° C. To trigger the coagulation cascade, 50 μL of $CaCl_2$ (0.025 M) were added. The coagulation time was recorded by a coagulometer and Heparin was used as the standard [27].

2.2.7.2. Evaluation of prothrombin time (PT) (the exogenous way). Briefly, 50 μL of plasma was added to 10 μL of a solution at different concentrations of different polysaccharide extracts and then incubated at 37 ° C for 3 min. Then, 50 μL of 0.025 M $CaCl_2$ was added to the mixture to trigger the reaction of the coagulation cascade. The coagulation time was again recorded using a coagulometer [27].

2.3. Statistical analysis

All experimental results were expressed as mean ± SD. The differences between the groups were determined using the JMP software using analysis of variance (ANOVA) followed by the Tukey's test. The differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Chemical composition and yields

Polysaccharide extracts from Aleppo pine seeds was obtained by precipitation, using three different solvents (acetone, ethanol and propanol). According to the Table 1, the results revealed that extraction yields ranged from 2.204 ± 0.04% (AP) to 2.04 ± 0.05 % (PP). The statistical analysis showed that both extracts (AP and EP) had significantly ($p < 0.05$) a better yield than the PP extract. In fact, extraction yields depend on solvents used [28]. In this case, the extraction yield can be explained by the lower levels of sugars in Aleppo pine grains as reported by Kadri et al. [29]. Although a comparable extraction yield was obtained with black cumin seeds (2%) [30]. The effect of the three solvents used (acetone, ethanol, propanol) on total sugar and protein levels, was studied by measured them before and after deproteinization. The results indicate a high level of total sugar in AP extract before and after

Table 1
Yields and composition of acetone, ethanol and propanol crude polysaccharides of *Pinus halepensis* Mill. seeds.

	AP	EP	PP
Yields (%)	2.204 ± 0.04[a]	2.123 ± 0.07[a]	2.04 ± 0.05[b]
Ash	1.33 ± 0.32[a]	1.27 ± 0.12[a]	1.39 ± 0.21[a]
Moisture (%)	5.326 ± 0.07[a]	5.396 ± 0.02[a]	5.31 ± 0.06[a]
Fat (%)	–	–	–
Carbohydrates before deproteinization (%)	70.543 ± 0.48[a]	67.953 ± 0.72[b]	65.71 ± 0.38[c]
Carbohydrates after deproteinization (%)	65.43 ± 0.38[a]	63.02 ± 0.68[b]	60.89 ± 0.39[c]
Loss of carbohydrates (%)	7.25 ± 0.08[a]	7.276 ± 0.03[a]	7.33 ± 0.06[a]
Proteins before deproteinization (%)	13.636 ± 0.20[a]	12.333 ± 0.08[b]	12.31 ± 0.09[b]
Proteins after deproteinization (%)	1.54 ± 0.15[a]	1.37 ± 0.02[b]	1.413 ± 0.10[b]
Loss of proteins (%)	88.323 ± 0.80[a]	88.57 ± 0.34[a]	88.486 ± 0.88[a]

Values are means ± S.D. ($n = 3$). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract. The yields with the same letter are not significantly diffrent (P = 0.05) according to the Tukey's test.

deproteinization with 70.543 ± 0.48 and 65.43 ± 0.38% respectively compared to EP and PP extracts. Also, we can notice that AP, EP and PP extracts contained the same quantities of moisture and ash. Overall, the lipids were found in trace in all extracts. The protein content in AP extract was higher (13.636 ± 0.20%) than EP (12.333 ± 0.08%) and PP (12.31 ± 0.09%) extracts. However, deproteinization by calcium chloride eliminated the same amount of protein (about 88%) from all extracts, in close agreement with previous observations (Huang, 2008).

3.2. FT-IR analysis of polysaccharide extracts

The three infrared spectra (A, B and C) of Fig. 1 show almost identical gaits. The most marked peaks for the extract AP, EP and PP respectively are: peaks 3325, 3350 and 3316 cm^{-1} represent hydroxyl (OH) groups [31], peaks 2968, 2960 and 2956 cm^{-1} design C—H groups [31,32], peaks 1647, 1643 and 1640 cm^{-1} express the presence of uronic acid by its COO or C═O groups [17,33], peaks 1542, 1544 and 1548 cm^{-1} are only impure (water, proteins or some polyphenols) [32,34]. The presence of OCH_3 groups (pectin methyl ester) is demonstrated by peaks 1402, 1415 and 1404 cm^{-1} [17,33]. Sulphate esters (SO) are also found (peaks 995, 988 and 995 cm^{-1}) [33] and finally the presence of α-glycosidic bonds was demonstrated by peaks 850, 839 and 848 cm^{-1} [17]. The OH, CH, COO, COO, OCH_3 groups as well as sulphate groups, uronic acid and glycosidic bonds are characteristic groups of polysaccharides and are present in all three extracts, the difference may therefore lie in the quantity and location of these groups.

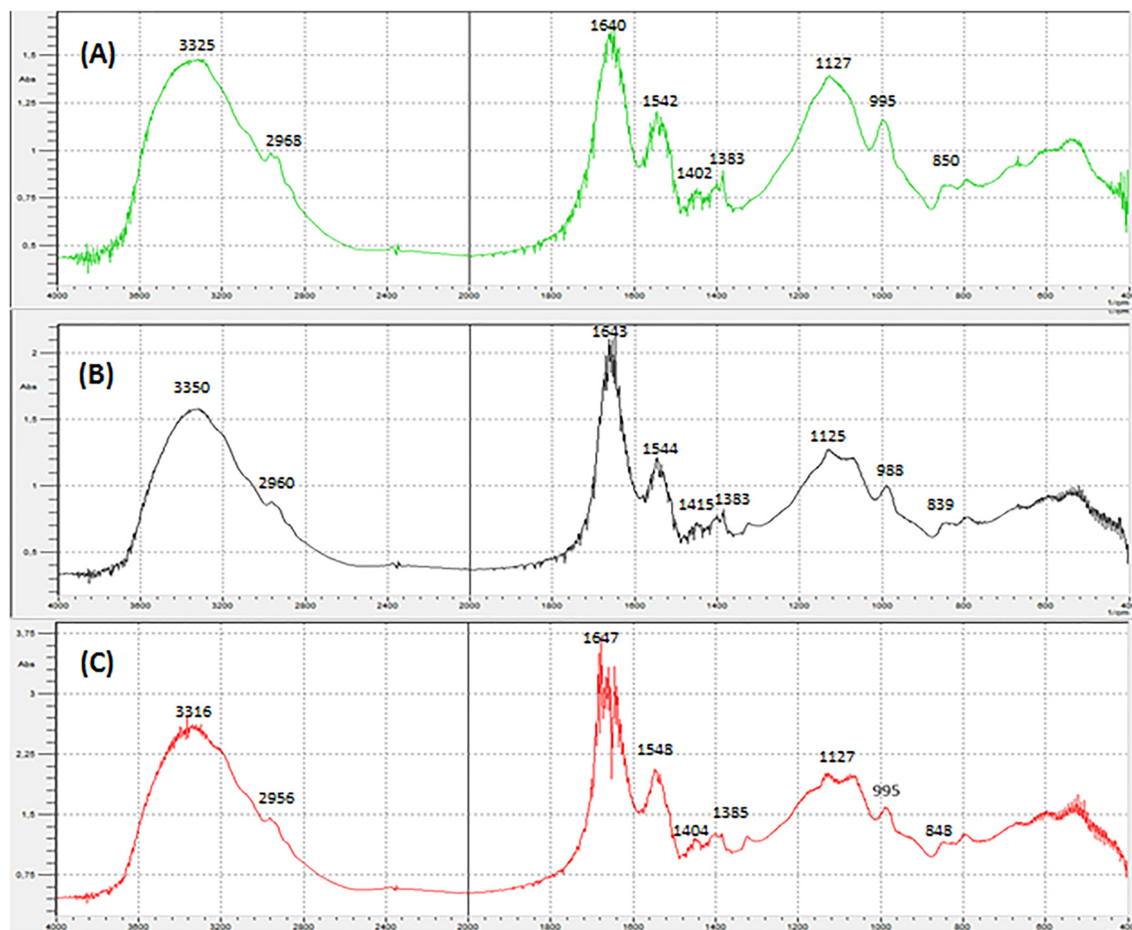

Fig. 1. FT-IR spectrometry of polysaccharides extract (AP, EP, PP) from *Pinus halepensis* Mill. seeds. (A): Acetone polysaccharides extract, (B): ethanol polysaccharides extract, (C): propanol polysaccharides extract.

3.3. Cyto-toxicity essay

Red blood cells are one of the most widely used models for the study of *in vitro* toxicity [18].

The results showed that no hemolysis was induced by any of the three polysaccharide extracts at all concentrations tested (no significant difference between the hemolysis percentages of the negative control and those obtained with the different concentrations of the different polysaccharide extracts). This implies that the polysaccharide extracts of Aleppo pine seeds do not present any toxicity even at high concentrations (4 mg/ml). The same results were obtained by Ktari et al. [35] for the polysaccharides of fenugreek seeds.

3.4. Antioxidant activities

To investigate the antioxidant activity of polysaccharide extracts of Aleppo pine seeds, six assays have been selected. The results of the total antioxidant activity estimated from the evaluation of DPPH and ABTS radical scavenging, metal chelation and lipid antiperoxidation activities, they were expressed as an $IC_{50.}$ The reducing power was expressed as an EC_{50} (represents the absorbance of 0.5) and the oxygen radical absorbance capacity is expressed in ORAC value (equivalent in μM trolox equivalent).

3.4.1. Free radical scavenging activity (DPPH and ABTS)

The ABTS and DPPH tests were widely used to evaluate the ability of molecules to scavenge free radicals. As shown in Table 2, the highest DPPH scavenging activity was displayed by the AP extract with $IC_{50} = 79.90 \pm 1.26$ μg/mL, comparatively to other extracts.

Obtained results showed that *Pinus halepensis* Mill. seeds are a rich source of polysaccharides with antioxidant activity compared to other studies. Recently, Ktari et al. [35] reported an antioxidant activity of 73.0 % at 10 mg/mL of fenugreek seeds. In addition, Trigui et al. [36] obtained a DPPH inhibition percentage of 63.25% at concentration of 1 mg/mL polysaccharides from black cumin seeds. Similarly, Sila et al. [37] found an $IC_{50} = 2.81$ and 2.59 mg/mL for polysaccharides from almonds and pistachios respectively.

In the case of ABTS scavenging activity, the AP extract was the most effective compared to other extracts ($IC_{50} = 57.29 \pm 0.46$ μg/mL), which highlights the presence of potential antioxidants in this extract. These results revealed that the polysaccharides of AP extract contain many hydroxyl groups, with high hydrogen donating capacity.

However, obtained results were more potent to those of other tested polysaccharides extracts such as *Plantago asiatica* seeds polysaccharides ($IC_{50} = 0{,}7$ mg/mL) [38], quinoa seeds polysaccharides ($IC_{50} = 1.108$ mg/mL) [39] and of *Sorghum bicolor* seeds polysaccharides ($IC_{50} = 20$ mg/mL) [40]. Many authors (Floegel et al. [41]; Thaipong et al. [42]) reported a good correlations between the results of the antioxidant activities using ABTS or DPPH radicals, which is in agreement with our studies. The difference revealed in the antioxidant activities of the three extracts may be due to the fact that ABTS test was more specific than the DPPH test, being exclusively an electron donor antioxidant. Whereas DPPH test combines the evaluation of both the hydrogen-donating capacity and reducing abilities. In addition, several studies reported that the antioxidant activity of polysaccharides can be influenced by several parameters (molecular weight, sulphate content, uronic acid content and the glycosidic bonds they contain) [43] and the presence of carboxyl groups [44]. Thus, it may be supposed that the three extracts may present different composition or different amount.

3.4.2. Metal chelating activity

Iron is another element which can induce oxidative damage to living tissues by the generation of OH•. Therefore, effective Fe^{2+} chelators afford protection against lipid peroxidation [35].

Different results were obtained regarding ferrous ion chelating activity, where PP was slightly less active than AP ($IC_{50} = 27.20 \pm 0.88$ μg/mL) and PP samples ($IC_{50} = 28.10 \pm 0.41$ μg/mL). Sila et al. [37] reported a metal chelating activity with an IC_{50} of 3.39 mg/mL for pistachio polysaccharides and 0.22 mg/mL for almonds and pistachio polysaccharides respectively and Trigui et al. [36] showed that black cumin seeds exhibited an IC_{50} of 0.78 mg/mL. Indeed, the metal chelating activity test showed less difference between extracts than what was observed in ABTS test. This may supposed the structure-antioxidant activity relationship, which is reported in several studies.

Also, according to the literature, compounds chelating metal ions generally have functional groups such as SH, COOH, OH, PO_3H_2, CO, NR_2, O and S [45]. These groups do not act in the same way and not with the same efficacy [46].

3.4.3. Lipid peroxidation

Unlike the ABTS and DPPH activities, AP and EP extracts demonstrated a weak lipid peroxidation inhibition activity. PP extract was effectively inhibited the lipid peroxidation initiated by iron-oxygen complexes with an IC_{50} of 4.88 ± 6.04 μg/mL, EP extract showed moderate activity against inhibition of lipid peroxidation with an IC_{50} of 39.51 ± 6.13 μg/mL. Although the lowest activity was obtained with AP extract ($IC_{50} = 142.3 \pm 5.52$ μg/mL). The statistical analysis revealed a significant difference between the IC_{50}s of the three extracts. Similarly, based on the findings of Sila et al. [37], the polysaccharide of almonds and pistachio may prevent the lipid peroxidation performed by the beta carotene bleaching test with the IC_{50} of 4.46 mg/mL and 3.39 mg/mL respectively.

3.4.4. Ferric reducing power

The reducing capacity of natural compounds is a good indicator of antioxidant potential, resulting from their ability to hydrogen atoms and/or electron transfer [35]. The ability of polysaccharide extracts to reduce the Fe^{3+}/ferricyanide complex was evaluated by following the formation of the Perl's index of the blue complex which absorbs at 700 nm [47].

AP marked a significant reductive power with an EC_{50} of 46.40 ± 5.21 μg/mL (Table 2). On the other hand, a lower reducing activity of EP and PP was recorded (EC_{50} of 164.5 ± 18.15 μg/mL and 173.7 ± 0.47 μg/mL respectively), which are also very interesting compared to other results found by other authors. In fact, EC_{50} of 7.76 mg/mL was found for polysaccharides from *Plantago asiatica* seeds [38] and 4–5 mg/mL for peony seed dreg polysaccharides [32].

Table 2
Antioxidant activity of crude polysaccharide extracts of *Pinus halepensis* Mill. seeds.

Antioxidant activity	AP	EP	PP
DPPH (IC_{50} μg/mL)	79.90 ± 1.26^b	82.02 ± 1.16^b	85.97 ± 1.71^a
ABTS (IC_{50} μg/mL)	57.29 ± 0.46^c	99.54 ± 0.58^b	676 ± 1.01^a
Metal chelation (IC_{50} μg/mL)	27.20 ± 0.88^b	$28.10 \pm 0.41^{a,b}$	28.75 ± 0.33^a
Lipid peroxidation (IC_{50} μg/mL)	142.3 ± 5.52^a	39.51 ± 6.13^b	4.88 ± 6.04^c
Ferric reducing power (EC_{50} μg/mL)	46.40 ± 5.21^b	164.5 ± 18.15^a	$173{,}7 \pm 0.47^a$
ORAC value (μM trolox equivalent) (30 μg/mL d'extrait)	1.93 ± 0.0^a	1.09 ± 0.39^b	1.19 ± 0.31^b

Values are means ± S.D. ($n = 3$). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract. The results with the same letter are not significantly different (P=0.05) according toTukey's test.

The reducing properties are associated with the reductones possessed by the extract, which on the one hand carry out their activity by giving a hydrogen atom which breaks the chain of free radicals [48]. On the other hand, they prevent the formation of peroxides by reacting with some peroxide precursors [46].

3.4.5. ORAC value

The oxygen radical absorbance capacity is the only antioxidant activity that combines and evaluates both degree and time of inhibition in a single amount [49] and where the operating mechanism is H-atom transfer reactions from the phenols to AAPH-derived peroxyl radicals [50]. In fact, it allows detecting the trapping of physiological radicals (OH and ROO°) which are most involved in lipid oxidation [51].

ORAC of the three extracts (AP, EP and PP) were tested and a very good results have been shown with the AP extract having the best ORAC value (1.93 ± 0.0 μM trolox equivalent) followed by the two extracts (EP and PP) which showed no significant difference with the ORAC values of 1.09 ± 0.39 μM trolox equivalent and 1.19 ± 0.31 μM trolox equivalent respectively. Knowing that for this activity, the concentration of 30 μg/mL has been tested, so this value is very important. Lu et al. [52] reported an ORAC value of 132.14 μM equivalent trolox for *Laminaria japonica* but which has been evaluated at 1 g/mL.

All these observations confirmed that the chemical assays showed only the ability of polysaccharide extracts to neutralize free radicals by electron or hydrogen atom transfer, and lack a biological significance because the peroxidation occur in a complex system where different mechanism of antioxidant implication may be explored [50]. This could explain the difference observed sometimes between chemical assay results and lipid model oxidation results. The lipid systems used revealed different mechanism by which antioxidants may act against lipid peroxidation. Indeed, the result of this study confirmed the difference in kind and structure of polysaccharide present in the tested samples and a relation between the hydro-solubility of the precipitant agent used and the antioxidant activity observed.

3.5. Antiinflammatory activities

3.5.1. Inhibition of BSA denaturation

Inflammatory and anti-inflammatory processes involve many molecules, most of which are proteins. The denaturation of these proteins causes them to lose their biological properties, which can trigger or accentuate inflammation [53]. This denaturation often involves the alteration of the bonds they constitute (hydrogen, electrostatics, hydrophobic and disulfide) [54].

The polysaccharide extracts had a good anti-denaturation activity of the BSA, in first position the AP extract with an IC_{50} of 153 ± 0.2 μg/mL, then the EP extract with an IC_{50} of 335.2 ± 2.14 μg/mL and finally the PP extract with an IC_{50} of 1159 ± 0.06 μg/mL with significant differences.

The exact mechanism of protein denaturation inhibition is still not very well known [55]. Chandra et al. [56] reported that protein denaturation increases the viscosity of the medium, so the denaturation protective effect of BSA can be supported by the viscosity change. On the other hand, for the global biological pathway, the BSA NMR analysis performed by Rösner et al. [57]. Williams et al. [58] showed that the latter contained two active sites with the amino acids threonine, lysine and tyrosine to which the bioactive molecules could bind to activate and regulate signal transduction. Duganath et al. [59] report that this effect could also be due to the binding of the bioactive molecules to plasma proteins and thus protect them from any aggression.

3.5.2. Anti-hemolytic activity

The erythrocyte hemolysis test has long been used for the strong analogy between the erythrocyte and lysosome membranes; both membranes have a lipid bilayer rich in protein (50%) and oligosaccharides. The stabilization or destabilization of one necessarily results in the stabilization or destabilization of the other [60].

Table 3 shows that the three extracts (AP, EP and PP) present very high antihemolytic activity with IC_{50}s of 8.01 ± 1.097, 31.25 ± 2.42 and 103.4 ± 2.82 μg/mL respectively. This mean that extracts are very effective with decreasing degrees (AP ˃ EP ˃ PP).

The exact mechanism of erythrocyte membrane stabilization is not yet well known. However, the literature reports several hypothetical mechanisms, for example, the extract could influence the surface volume ratio by increasing the erythrocyte membrane or reducing cell volume by interacting with membrane proteins. Therefore, this protective effect may be due to the ability of the extracts to modify the flow of calcium in the erythrocyte [61] or by binding to membrane components, in particular membrane proteins, thus contributing either to the regulation of the intracellular water volume by controlling the movement of sodium and potassium ions through protein channels [62] or by inducing a subsequent modification of the charges on the membrane surface, which may prevent physical interaction with the aggregating agents or promote charge repulsion [9].

3.5.3. Antiproteinase activity

Leelaprakash and Dass [63] reported that during the inflammatory reaction, leukocyte proteinases are very important in the development of tissue lesions and proteinase inhibitors would ensure a reduction of these lesions and would therefore be considered as an anti-inflammatory.

The EP extract exerted the best anti-proteinase activity with an IC_{50} of 24.19 ± 3.17 μg/mL, then the PP extract with an IC_{50} of 348.2 ± 3.42 μg/mL and finally in the last position the AP extract with an IC_{50} of 2032 ± 1.78 μg/mL. This effect could be explained by the fact that polysaccharide extracts compete with casein by binding to the active sites of trypsin and the difference observed between the three extracts could be due to the difference in affinity of their compounds to the active sites.

Ibrahim et al. [64] also found good anti-inflammatory activity *in vitro* against COX-1 and COX-2 of polysaccharide from *Adansonia digitata*.

3.6. Anticoagulant activities

The anticoagulant activity of polysaccharide extracts of Aleppo pine seeds was measured by two *in vitro* tests. One of which is the activated partial thromboplastin time that makes it possible to explore the activity of factors II, V, VII, IX, X, XI, XII of the endogenous pathway and the common coagulation pathway and the other which explores factors II, V, VII and X of the extrinsic pathway and the common coagulation pathway [65].

Table 4 shows that the three extracts exhibited high partial activated thromboplastin times compared to the negative control (41 ± 2 s) with a remarkable dependency dose. At the three concentrations used, the

Table 3
In vitro anti-inflammatory activities of crude polysaccharide extract of *Pinus halepensis* Mill. seeds.

Anti-inflammatory activities	AP	EP	PP
Inhibition of BSA denaturation (IC_{50} μg/mL)	153 ± 0.2^{c}	335.2 ± 2.14^{b}	1159 ± 0.06^{a}
Anti-hemolytic activity (IC_{50} μg/mL)	8.01 ± 1.097^{c}	31.35 ± 2.42^{b}	103.4 ± 2.82^{a}
Antiproteinase activity (IC_{50} μg/mL)	2032 ± 1.78^{a}	24.19 ± 3.17^{c}	348.2 ± 3.42^{b}

Values are means ± S.D. ($n = 3$). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract. The results with the same letter are not significantly different (P = 0.05) according to Tukey's test.

Table 4
anticoagulant activities (APTT and PT) of crude polysaccharide extracts (AP, EP and PP) of *Pinus halepensis* Mill. seeds.

	AP	EP	PP	Positive control (heparin)
APTT (s)				
100 μg/mL	210 ± 5^b	170 ± 7^c	150 ± 9^d	1700 ± 12^a
50 μg/mL	156 ± 4.5^b	110 ± 2^c	90 ± 8^d	854 ± 9^a
25 μg/mL	98 ± 8^b	66 ± 5.5^c	57 ± 3^d	509 ± 6^a
PT(s)				
100 μg/mL	90 ± 6^b	82 ± 5^c	67 ± 2.5^d	1300 ± 19^a
50 μg/mL	66 ± 4^b	58 ± 4^c	49 ± 3^d	699 ± 16^a
25 μg/mL	54 ± 3.5^b	47 ± 2^c	39 ± 1.5^d	402 ± 10^a

Values are means ± S.D. ($n = 3$). APTT (s) activated partial thromboplastin time (seconds) PT(s): prothrombin time (seconds). AP: acetone polysaccharide extract; EP: ethanol polysaccharide extract; PP: propanol polysaccharide extract. The results with the same letter are not significantly different ($P = 0.05$) according to theTukey's test.

difference is significant between the three extracts. In fact, the acetone extract has a higher activity than the ethanol extract and the latter is more active than the propanol extract. For PT activity, the extracts exhibited less activity but in the same order of effectiveness with the same dose of dependence. However, their activity was less than that of heparin, which is a reference molecule for both APTT and PT. These results are better than those found by Arivuselvan et al. [66] with the polysaccharides of brown algae (*Turbinaria ornata*) which showed 170 and 6 s for APTT and PT respectively at a concentration of 125 μg/mL.

Several authors (Nishino et al. [67]; Pereira et al. [68]; Zhang, et al. [69]) reported that anticoagulant activity was highly dependent on sulphate content, the binding sites of some sugars and their molecular size.

In addition, Fonseca et al. [70] confirm that the change in sulphation proportions and positions in polysaccharide chains could be critical importance to coagulation system activators and inhibitors.

4. Conclusion

This study was designed to evaluate, the effect of solvent precipitation (acetone, ethanol, propanol) of the polysaccharides extract from Aleppo pine seed on different biological activities (antioxidant, anti-inflammatory and anticoagulant activities). The results recorded showed that Aleppo pine seed polysaccharides are very active without any toxicity. However, the polarity of precipitation solvents plays a crucial role in yields, purity levels as well as activities. The AP extract was most effective in almost activities. This supposes that acetone is the best precipitating agent giving the most active polysaccharides with perhaps more functional groups. The polysaccharides of Aleppo pine seeds can be a nutraceutical agent for the very interesting activities they have presented but the precipitation solvent of polysaccharides play a crucial role on their bioactivity.

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Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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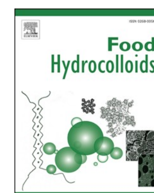

Optimising functional properties and chemical composition of *Pinus halepensis* Mill. Seeds protein concentrates

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ABSTRACT

Vegetable proteins are widely used in many food formulations due to their physico-chemical properties, low cost and availability. The main objective of this work is to study the chemical composition and properties of a protein concentrate of *Pinus halepensis*. Mill seeds (PHPC) and mainly to optimize the effect of pH, NaCl concentration and phosphate buffer (PB) molarity on functional properties (solubility, emulsifying activity index (EAI) and foaming capacity (FC)) of this concentrate by response surface methodology (RSM). The chemical composition was determined in terms of proteins, sugars, lipids, ash and moisture. The physico-chemical characteristics were studied by their water and oil holding capacity (OHC, WHC) and their surface hydrophobicity (SH). Finally, the functional properties of PHPC were studied in terms of solubility, EAI, FC, minimum gelling concentration (MGC) and finally heat coagulability (HC). A PHPC yield of 36.66 ± 0.7% was obtained. The WHC and OHC was 3.89 g water/g PHPC and 3.54 g oil/g PHPC respectively and a SH of 87.09 ± 0.78 was obtained. The optimization results showed that the optimal conditions for solubility, EAI and FC were: pH:10.88, NaCl:0 g/l, PB:0.078 M; pH:12, NaCl:0.55 g/l, PB:0.1M and pH:2, NaCl:0, PB:0 M respectively, having given a solubility of 87.13 ± 0.14%, an EAI of 36.82 ± 0.34 and a FC of 182.72. Then, the desirability of the three responses (solubility, EAI and FC) which was pH:12, NaCl: 0.55 g/l and PB of 0.1M was used to assess the stability of EAI and FC, to determine the MGC and HC. This study shows that Aleppo pine seeds are a good source of functional proteins, potentially applicable in the food industry and that pH, NaCl concentration and PB molarity have a major influence on functional properties.

1. Introduction

Vegetable proteins are a very good alternative to animal proteins whether for food or cosmetic application, because of their low cost, abundance and diversity of their sources (legumes, cereals and oilseeds), their adequate quality and nutritional value, their ease of digestion, their non-toxicity and finally for their functionality (S. Damodaran, 2000, p. 384; Rodrigues, Coelho, & Carvalho, 2012; Soria-Hernández, Serna-Saldívar, & Chuck-Hernández, 2015).

In America, 60% of the population relies heavily on the protein content of food when choosing their product, because among the three primary metabolites (carbohydrates, proteins and fats), proteins are the most beneficial for their health. Most adults perceive proteins as the most energy-efficient ingredient that is very healthy and improves muscle tone. They are macronutrients most considered in weight management diets (Cheatham, 2014).

In recent years, oilseed proteins have made a very significant contribution to protein intake in the diet. In 2004/2005, 380 million tonnes of oleaginous plants were produced and 207 million tonnes of protein meals were produced worldwide (Ash, Dohlman, & Davis, 2006).

The most commonly used proteins of oleaginous origin are that of soybean, peanut and rapeseed for their functionalities in food processing (additives and the protein film industry). With the awareness of their usefulness and therefore the increase in needs, new sources have been developed, such as cashew nut (Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009), milk weed (Hojilla-Evangelista, Evangelista, & Victor Wu, 2009) and almost all oilseeds. *Pinus halepensis* Mill seeds, come from

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a very abundant tree belonging to the Pinaceae family which can found on all the Mediterranean relief especially in Algeria and Tunisia (Maestre, Cortina, Bautista, & Bellot, 2003). The bible's manual of medicinal plants reports that they have been used extensively in pastry making, especially in Tunisia, and are also used to cure diabetes and sexual weakness in the eastern region of the Mediterranean (Schiller, 2014). Kadri et al. (2015) studied the chemical composition of its seeds and found a protein percentage of 26.62 ± 0.129 which is a very high percentage especially compared to other species *Pinus pinea* L., *Pinus pinaster* and *Pinus canariensis*.

Functional proteins are those that when added to food, confer nutritional, sensory, physico-chemical and organoleptic properties (color, texture, flavor …). Functional properties could be classified according to their physico-chemical mechanisms as follows: hydration properties (water/oil retention and solubility) rheological properties (viscosity, elasticity, aggregation and gelation), and protein surface properties (emulsifying and foaming activities, surface hydrophobicity and whipping) (Moure, Sineiro, & Domínguez, 2001).

Food applications of proteins is limited by their low solubility (Moure, Sineiro, & Domínguez, 2001), it is known that the pH, presence or absence of salts and its concentration and thus the ionic strength of the medium, as well as electrostatic repulsions influence the functional properties of proteins (Soria-Hernández et al., 2015). For this purpose and taking into account that according to the databases consulted, no studies were carried out on the functional properties of Aleppo pine seed proteins, the physico-chemical characteristics of *P. halepensis* Mill. Seed concentrated proteins (PHPC) (approximate composition, water and oil holding capacity (WHC and OHC), surface hydrophobicity (SH)) were determined and the solubility, emulsifying activity index (EAI) and foaming capacity (FC) conditions were optimized using the Box Behnken Design (BBD) by the response surface methodology, to study the effect of the parameters considered (pH, NaCl concentration and phosphate buffer molarity) on each of the responses and the relationship between solubility and functional activities. Then the heat coagulability (HC) and the minimum gelling concentration (MGC) were determined under optimal conditions.

2. Material and methods

2.1. Plant material

The seeds of Aleppo pine (*Pinus halepensis* Mill.) were obtained from the Collo forest located in Skikda province of Algéria in May 2018. They were cleaned with bidistilled water; dried in an oven at 40 °C for 2 days and then finely crushed using an electric grinder (KIKA Labortech-nik M20, Germany) until it became a fine powder (<250 μm) which was delipidated by the Soxhlet method with petroleum ether.

2.2. Preparation of the protein concentrate

A mass of 10 g of delipidated powder was macerated under stirring for 20 min at room temperature. After filtration and centrifugation at 4 °C for 20 min at 6000 rpm, the supernatant was filtered again and its pH was adjusted to 6 (with 0.1 M HCl), $CaCl_2$ was added gradually until a concentration of 1 M, then centrifuged at 6000 rpm for 20 min. Finally, the recovered pellet was washed with distilled water and freeze-dried (Rotimi E Aluko, McIntosh, & Katepa-Mupondwa, 2005).

2.3. Chemical composition and yield

The extraction yield was expressed by the ratio of the amount of extract to the amount of defatted powder used. Ash, moisture and fat were determined according to AOAC (1998) methods, the protein content was determined by the Bradford (1976) method and the carbohydrates content was carried out by the Dubois, Gilles, Hamilton, Rebers, and Smith (1956) method using BSA and glucose for calibration curves respectively.

2.4. Water and oil holding capacity (WHC/OHC)

The Tan, Ying-Yuan, and Gan (2014) method was used to determine the capacity of the extract to retain water or oil. For this purpose, 100 mg of extract was suspended with the same amount of water or sunflower oil (1.5 ml), vortexed for 1 min, and then centrifuged at 3000 rpm for 20 min. The water and oil retention capacity was expressed in gram of water or oil retained per gram of extract.

2.5. Surface hydrophobicity (SH)

The bromophenol blue (BPB) binding method was used to study the surface hydrophobicity of the protein concentrate. A volume of 1 ml of protein concentrate suspension (5 mg/ml in 20 mM PB at pH 7) was added to 200 μl of BPB solution (1 mg/ml in distilled water). The mixture was vortexed for 10 min and directly centrifuged at 3000 rpm for 15 min. Finally, the supernatant absorbance was read at 595 nm. A control containing 1 ml of PB (20 mM, pH 7) and 200 μl of BPB solution was used (Mune & Sogi, 2016). The surface hydrophobicity is according to the following formula:

$$\text{SH}\ (\%) = \text{BPB bound}\ (\%) = (\text{Absorbance control} - \text{Absorbance sample})/\text{Absorbance control} \times 100$$

2.6. Optimization of solubility conditions of PHPC and its functional properties

Before optimization of solubility conditions, three parameters (pH, NaCl concentration and PB molarity) were studied separately in the single-factor experiment, keeping the variables that were not studied constant each time in order to limite overall experimental work. After statistical analysis of the results of this preliminary study, three variables were selected as significant factors and three levels were used for each one. The study intervals were also determined for each parameter and then the response surface based on the Box Behnken Design was designed to obtain the conditions giving the best solubility, EAI and FC.

2.6.1. Protein solubility

A mass of 100 mg of PHPC was dispersed in 10 ml of different solutions prepared at the pH, NaCl concentration and molarity of PB determined according to the design of experiment. The dispersions were vortexed well for 15 min then centrifuged at 3000 g for 20 min. The protein content of the supernatant was determined by the Lowry method (Peterson, 1977) and the solubility was calculated as follows:

$$\text{Solubility}\ (\%) = \text{Protein content of supernatant} \times 100\ /\text{Total protein content}$$

Total protein content represents 100% solubility and is determined in 3% NaOH (Chao, Jung, & Aluko, 2018).

2.6.2. Emulsifying properties

The emulsifying properties of the PHPC were determined using the method reported by Boye et al. (2010). A volume of 45 ml of protein solution (0.5% in different solution of pH, NaCl and PB) was added into 15 ml of sunflower oil. After homogenization of the emulsion with an ultra turrax (IKA T25, Staufen, Germany) for 1 min at 20,000 rpm, 50 μL of the prepared solution were diluted in 5 ml of sodium dodecyl sulfate (SDS) at 0.1%. Finally, the absorbance was recorded at 500 nm. The EAI was calculated using following equation:

$$\text{EAI}\ (m^2/g) = 2 \times 2303 \times A_0 \times \text{DF} / C \times \phi \times 10{,}000$$

where A_0 is the absorbance of the emulsion after emulsification, DF is the dilution factor, C is the weight of the protein per volume (g/mL), ϕ is

the volume fraction of the oil in the emulsion.

2.6.3. Foaming properties

Foaming capacity of PHPC was determined according to the method of Shahidi, Han, and Synowiecki (1995). A volume of 20 ml of protein concentrate solution at 0.1% (W/V) was homogenized using a Moulinex_R62 homogenizer to incorporate the air for 1 min at room temperature (25 ± 1 °C). The FC was expressed as percentage of volume increase after homogenization, which was calculated according to the following equation:

FC (%) = ((volume after whipping − volume before whipping) / volume before whipping) × 100

2.6.3.1. Experimental design.

To optimize the factors affecting solubilization, foam capacity and emulsifying activity, the response surface methodology (RSM) with Box Behnken Design was studied using Minatab 17 (statistical analysis system Inc., SAS) software and the experimental values obtained for solubility, EAI and FC were compared to their values predicted based on the *t*-test ($p < 0.05$) (Table 8). In this study, fifteen tests were performed with the different values of pH (2, 7, 12), NaCl concentration (0, 0.275, 0.55 g/ml), and PB concentration (0, 0.05, 0.1 M) as shown in Table 2. The values were coded as follows: (+1) maximum value, (0) central value and (−1) minimum value. The experimental data were adjusted to a second order polynomial model and expressed by following equation:

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

B_0 (constant coefficient); Bi, Bii, Bij (regression coefficients for intercepting, linear, quadratic and interaction terms, respectively); xi and xj (independent variables); k (number of optimized factors).

2.6.3.2. Validation of model.

In order to draw conclusions from the validation of the model, the Minitab software provides the optimal conditions of the three factors (pH, NaCl, PB) from the three responses designed. The optimums responses obtained were used to test solubility, FC and EAI. Finally, the experimental optimums of each obtained response were verified by comparing them with the predicted values.

After optimization and validation of the experimental design, a compromise solution was obtained by using the desirability function. The desirability is an important function when multiple response optimization was carried out because it's not possible to optimize each one in separate way. For that, the overall solution must be included in optimal region leading to a certain degree of compliance with the proposed criteria for each variable of the system; namely, a compromise solution must be found. Desirability (d) always takes values between 0 and 1, where D = 0 for an undesirable response, and d = 1 represents a completely desirable value (Candioti, De Zan, Camara, & Goicoechea, 2014). The stability of the functional properties studied (FC and EAI) after 15, 30, 45 and 60 min and other functional properties (MGC and HC) were tested at the optimal conditions obtained by desirability.

Table 1
Solubility, emulsifying activity index and foaming capacity of PHPC on distilled water.

Functional properties	
Solubility	27.02 ± 0.52
EAI	20.89 ± 0.24
Foaming capacity	61.66 ± 0.66

Data are the mean ± SD of three analyses.

2.7. Stabilisation of foaming and emulsifying properties

To study the kinetic of the foaming and emulsifying activities depending on the time (15, 30, 45 and 60 min), the PHPC solution was prepared with the optimum of pH, NaCl and PB concentrations. The stability is expressed as a percentage of remaining of these two properties (Boye et al., 2010; Shahidi et al., 1995).

2.8. Minimum gelling concentration (MGC)

The method of O'Kane, Vereijken, Gruppen, and Van Boekel (2005) was used to determine the MGC with a slight modification. A volume of 5 ml of PHPC solution was prepared at the concentrations of 4–18% (w/v) and then heated in water bath at 95 °C for 10 min (in sealed tubes to avoid evaporation). After cooling, the tubes were placed at 4 °C for 12 h and then inverted. The MGC is the smallest concentration from which the contents of the inverted tube do not flow.

2.9. Heat coagulability (HC)

For Heat Coagulability (HC), the solubility method described above was used, the suspension of PHPC under optimal conditions was vortexed and the proteins of the supernatant were measured by the Lowry method. An aliquot of the supernatant was heated in a water bath at 100 °C for 30 min. After cooling and centrifugation at 3000 rpm for 15 min, a filtration was carried out on Whatman No. 2 filter paper, and concentration of proteins in the filtrate were again determined by the same method (Voutsinas, Nakai, & Harwalkar, 1983). The HC of the sample was calculated from the following equation:

% Heat Coagulability = Ps – Pf / Ps × 100

where:

Ps = % protein in supernatant
Pf = % protein in filtrate

3. Results and discussion

3.1. Proximate composition

As shown in Table 3, the extraction yield of PHPC was 36.66 ± 0.7%, of which approximately 69.33 ± 0.3% are proteins. Among the impurities, we found sugars representing 2 ± 0.2%, which can be justified by the presence of glycoproteins also reported by Kadri et al. (2015) and minerals (ash) found in the proportion of 4.9%. However, the lipids were found only in trace form, which confirms the good delipidation of the powder before extraction. The moisture test revealed a level of 2.4 ± 0.2% which is comparable to lyophilized extract dried by other methods such as Bambara concentrate in which the moisture content is of the order of 4% (Adeleke, Adiamo, & Fawale, 2018).

3.2. Water and oil holding capacities (WHC/OHC) and surface hydrophobicity

3.2.1. Water and oil holding capacities (WHC/OHC)

The Water and oil holding capacities of PHPC were evaluated and the results are represented in Table 4. The amount of water and oil that binds BPB depends on the polar and non-polar, ionized or deionized groups of proteins (Ghribi et al., 2015) and these properties mean that these proteins can be used as an additive to improve food quality (Tontul, Kasimoglu, Asik, Atbakan, & Topuz, 2018).

In our study, the WHC of PHPC was found at 3.89 g water/g PHPC (Table 4). This is in agreement with the WHC range of products with water retention capacity (1.49–4.71) (Kaur & Singh, 2007). This capacity can be explained by the large particle size of the extract as well as

Table 2
Box–Behnken design matrix and experimental and predicted data.

Run						pattern				
Run	pattern	Variables			Solubility	Pred Formula solubility	EAI	Pred Formula EAI	FC	Pred Formula FC
1	0−−	pH (X1)	C NaCl (X2)	PB M(X3)						
2	−0−	7	0	0	28	33.9310905	21.37	21.9767438	61.66	60.40375
3	+0−	2	0.275	0	44.11	49.4575682	24.177	24.515224	91.66	91.6725
4	0+−	12	0.275	0	26.14	24.5587616	18.731	19.3399277	50	62.485
5	−−0	7	0.55	0	21.4	13.3703086	23.84	22.1893426	16.66	2.08875
6	+−0	2	0	0.05	87.04	77.4362757	26.6	25.4061574	123.33	122.91375
7	000	12	0	0.05	81.6	77.4337243	27.71	26.7413426	106.66	102.03125
8	000	7	0.275	0.05	27.54	29.5491319	19.13	19.018366	41.66	41.66
9	000	7	0.275	0.05	29.12	29.5491319	18.84	19.018366	41.66	41.66
10	−+0	7	0.275	0.05	29.52	29.5491319	19.79	19.018366	41.66	41.66
11	++0	2	0.55	0.05	43.08	46.7025309	34.48	35.4261389	58.33	64.56875
12	0−+	12	0.55	0.05	57.4	67.5474691	24.14	25.3563611	93.33	93.74625
13	−0+	7	0	0.1	36.11	43.9489095	20.27	21.8257562	31.66	39.57125
14	+0+	2	0.275	0.1	21.18	24.406169	28.74	27.6611376	75	62.505
15	0++	12	0.275	0.1	73.85	70.1473624	24.91	24.1018413	100	99.9875

C NaCl: concentration of NaCl; PBM: Phosphate buffer molarity; EAI: emulsifying activity index; FC: foaming capacity. The codded values were (+): maximum value, (0): central value and (−): minimum value.

Table 3
Proximate chemical composition of PHPC.

	Rate (%)
yield	36.66 ± 0.7
Ash	4.9 ± 0.3
moisture	2.4 ± 0.2
proteins	69.33 ± 0.3
carbohydrates	2 ± 0.02
fats	–

Data are the mean ± SD of three analyses.

Table 4
Water and oil holding capacities (WHC/OHC) and surface hydrophobicity of PHPC.

Parameters	
Water holding capacity (g of water/g of PHPC)	3.89 ± 0.06
Oil holding capacity (g of oil/g of PHPC)	3.54 ± 0.02
Surface hydrophobicity (%)	87.09 ± 0.78

Data are the mean ± SD of three analyses.

the capacity of the sugar and fibers found in the concentrate as impurities which are known for this capacity (Zhao et al., 2012). The capacity of this extract is greater than that of most protein extracts reported by the bibliography, for example, WHC chickpea protein concentrates were found at 3.65 for freeze-dried extract and in agreement with that found for rapeseed protein isolate (3.85 g water/g extract) (Yoshie-Stark, Wada, Schott, & Wäsche, 2006). Therefore, this extract can be used as an additive for viscous foods (Aletor, Oshodi, & Ipinmoroti, 2002).

The oil retention capacity was found in 3.54 g oil/g extract (Table 4), This is in the range of literature values (1.1–4.1) (Kaur et al., 2007). This good capacity can be explained by the hydrophobic properties of PHPC and the non-polarity of the side chains of its amino acids. This extract can therefore be used as an additive to confer an organoleptic quality to a fatty food such as dairy products (Ghribi et al., 2015).

3.2.2. Surface hydrophobicity (SH)

The SH informs us about the surface-active properties of the extract. Table 4 shows that SH of PHPC studied by binding to the BPB has been found in order of 87.09 ± 0.78%. It is higher than that found by Tontul et al. (2018) (60.98%), this means that our PHPC can have promising surface-active properties.

3.3. Optimization by RSM

3.3.1. Model analysis

Combination of the three studied factors (pH, concentration of NaCl and PB molarity) and the value of the corresponding response obtained in different experiments were presented in Table 2. It indicated that solubility was ranged from 21.18 to 87.04, the EAI from 18.84 to 34.48, while FC varied from 16.66 to 123.33. The values of the experimental results are consistent with the predicted values for the three responses.

3.3.2. Analysis of response surface

RSM based on BBD was applied to disclose optimal levels for the studied parameters (pH, NaCl concentration and PB molarity). Surface response models were the best method which illustrates the effects of independent variables and their interactions on the solubility of PHPC, their emulsifying activity and their foaming capacity. Experimental data were fitted to second order polynomial model.

Table 5
Analyze of variance (ANOVA) for the experimental results obtained by solubility.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	7	0,018057	0,002580	27,04	0,0002
Linear	3	0,002770	0,000923	9,68	0,0075
X1	1	0,000662	0,000662	6,93	0,0344
X2	1	0,001539	0,001539	16,13	0,0053
X3	1	0,000570	0,000570	5,98	0,0440
Square	3	0,009962	0,003321	34,81	0,0001
X1X1	1	0,005900	0,005900	61,84	0,0006
X2X2	1	0,001645	0,001645	17,24	0,0044
X3X3	1	0,002002	0,002002	20,99	0,0032
Interaction	1	0,005325	0,005325	55,82	0,0003
X1X3	1	0,000668	0,005325	55,82	0,0006
Error	7	0,000644	0,000095		
Lack of fit	5	0,000024	0,000129	10,83	0,0873
Pure error	2	0,018725	0,000012		
Total	14				
S	**0.0097674**				
R-sq	**0.9643**				
R-sq (adj)	**0.9287**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

3.4. Solubility

3.4.1. Analyze of the model of solubility

In Table 5, it has been shown that for solubility, all linear parameters have been significant; X1 and X3 ($p < 0.05$) and X2 ($p < 0.01$) therefore highly significant. Their quadratic parameters are also very highly significant ($p < 0.01$), as well as for the quadratic parameter X1, X3. However, all other parameters are not significant ($p > 0.05$) and the only significant interaction parameter is the X1X3 ($p < 0.01$). Taking into account only the significant parameters with $p < 0.05$, the predictive equation has been deduced.

$$\text{Solubility} = -0.18664 + 0.00909\ X1 - 0.01387\ X2 + 0.00844\ X3 + 0.03997 \times 1 \times 1 + 0.02111 \times 2 \times 2 - 0.02329 \times 3 \times 3 + 0.03649 \times 1 \times 3$$

Table 5 shows also, the variance analysis of the experimental results. The F value of the model was 27.04, this being said that the model is significant. The determination coefficient (R^2) was 0.9643 which means that only 3.57% of the variations could not be explained, and that 96.43% were attributed to the independent variables of solubility of PHPC.

However, the value of R^2 is not always synonymous with a good regression model, it must be comparable to the adjusted R^2, which is verified in our case as shown in Table 5 (adjusted $R^2 = 0.9287$). In addition, the value of lack of fit was 0.087 (whose value must be insignificant ($p > 0.05$) compared to the pure error). Finally, the low value of S which is the standard variation error (0.009) implies that the values obtained are close to the adjusted line. All these values and significance indicate that this model is well and truly validated and that it could work for the prediction of the solubility of PHPC.

3.4.2. Response surface of solubility

Fig. 1 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of PHPC solubility.

The solubility depends mainly on pH because the linear and quadratic effect are significant and highly significant with ($p < 0.001$ and $p < 0.05$) respectively. As mentioned above, the three parameters studied (pH, NaCl and PB) are factors that significantly influence solubility and the interaction effect between pH and PB is also significant. A slight increasing of solubility was noted with increasing of pH value and decreasing then subsequently increasing of the NaCl concentration value. However, the solubility reaches its maximum when NaCl concentration decreases slightly and PB M increases significantly. On the other hand, it is result from the decreasing of pH value and very significant increasing of PBM, the increasing and then decreasing of solubility (Fig. 1 A, B, C). This is perfectly in agreement with the results of the preliminary study. The solubility of PHPC can be improved by varying the pH values; it increases as pH approaches the extreme pH values of 2 and 12. In general, as the pH increases, solubility decreases until it reaches the isoelectric domain, then increases. Because at pHs close to isoelectric pH, electrostatic repulsive forces favour the aggregation of proteins. The large volume of the aggregate and the bulk density therefore lead to the precipitation of these proteins and prevent their solubilization. However, at extreme pHs (far from the isoelectric domain) Electrostatic repellent forces help to separate positively charged proteins and increase interactions between them and the solvent (Mao & Hua, 2012). Protein solubility profile as a function of pH is used as an indicator of protein functionality, since functional properties are directly related to solubility (Ortiz & Wagner, 2002). Our results are similar to those found for the protein extracts from other plant matrices, as demonstrated by Hu et al. (2017) for walnut protein concentrate (Tontul et al., 2018), for chickpea protein isolate and Chao et al. (2018) for pea isolate.

Solubility also depends strongly on NaCl concentration but with a negative effect and very significant linear and quadratic effects ($p < 0.01$). The preliminary study showed that the salt concentration increases the solubility of the protein extract to the concentration of about 0.280 g/l and then decreases it to the minimum at 0.55 g/l, this result is very logical and can be explained by the phenomenon of salting in and salting out found by several authors such as Deng et al. (2011) for the protein isolate of *Ginkgo biloba* seeds. The optimum occurred without NaCl can be explained by the fact that at this pH and this molarity in phosphate buffer, the solubility reaches its maximum and the addition of NaCl does not affect it and this is confirmed by the very high significance of these two parameters interaction. These phenomena depend also on the conformational differences characteristic of proteins (Hu et al., 2017) and effect of NaCl on the ionic strength of the medium (Inyang & Iduh, 1996). Further, salts play an important role in a protein medium on the solubility of the protein, they reduce the charge of counter ions and therefore both those of attractions and electrostatic repulses (Bau, Mohtadi-Nia, Lorient, & Debry, 1985).

It was found that the molarity in PB has also a positive influence on solubility because its linear effect is significant ($p < 0.05$) and its quadratic effect is highly significant. This confirms the results of the preliminary study where we found that more the molarity of the phosphate buffer increases, more soluble the PHPC are at lower pH in the range studied. This may be due to the fact that the phosphate buffer on the stability of the protein (Pikal-Cleland, Rodríguez-Hornedo, Amidon, & Carpenter, 2000). Therefore the higher the molarity the more stable the protein is and therefore more soluble.

For this model, the optimal solubility conditions are: pH: 10.88; NaCl: 0; PB: 0.078. The protein extract was solubilized under these conditions and the actual solubility obtained was 87.13% ± 0.14 against a predicted value of 87.113% whose difference is not significant ($p < 0.05$). This solubility value is much better than the solubility obtained by dispersing these proteins in distilled water which was 27.02 ± 0.52 (Table 1).

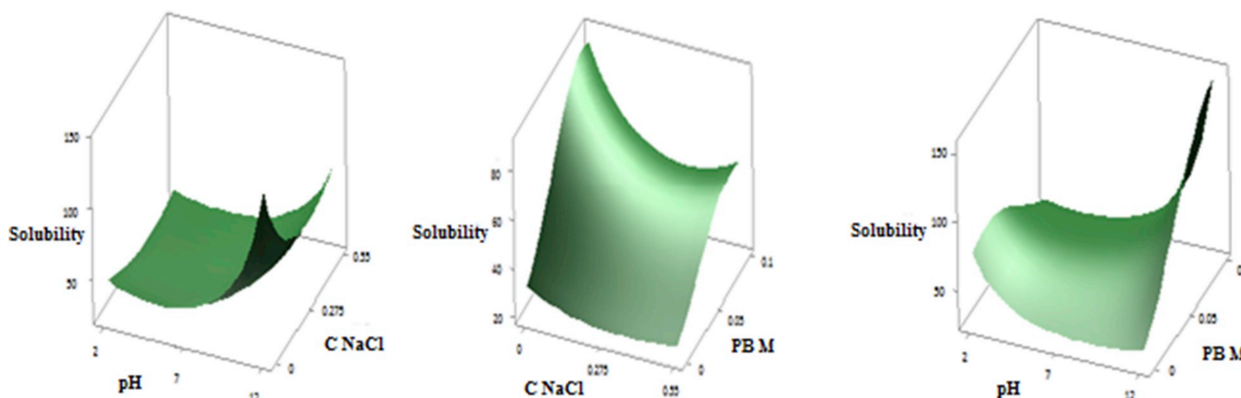

Fig. 1. Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on solubility of PHPC.

3.5. Emulsifying activity index

3.5.1. Analyze of the model of emulsifying activity index

For the emulsifying activity index, it has been shown that all linear parameters, their quadratic and their interactions were very highly significant ($p < 0.001$). The final predictive equation was obtained as follows:

(EAI^λ-1)/(λ × g^(λ-1)) = 4.058–2.961 X1 + 2.903 X2 + 2.395 X3 + 4.845 × 1 × 1+4.969 × 2 × 2 – 4.316 × 1 × 2 + 2.495 × 2 × 3

(λ = 3; g = 23,8189 is the geometric mean of EAI).

Table 6 shows the variance analysis of the experimental results of EAI. The F value of the model was 53.18, this being said that the model is significant. The determination coefficient (R^2) was 0.9815 which means that only 1.85% of the variations could not be explained, and that 98.15% were attributed to the independent variables of emulsifying activity index of PHPC and the value of adjusted R^2 (0.9631) is quite close to R^2.

In addition, the value of lack of fit was 0.06 which is not significant. Finally, the standard variation error was 1,09919. All these values and significance indicate that this model is well and truly validated and that it could work for the prediction of the emulsifying activity of PHPC.

3.5.2. Response surface of emulsifying activity index

Fig. 2 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of emulsifying activity index of PHPC. The parameters pH, NaCl and PB have significant effects ($p < 0.05$) on emulsifying activity, and the interaction effects between pH and PB, pH and NaCl as well as PB and NaCl are also significant ($p < 0.05$). For this activity, the EAI is maximal when NaCl concentration increases significantly and pH decreases and then increases. While the EAI increases slightly despite the significant increasing of PBM and NaCl concentration. Finally, the EAI increases very significantly when pH decreases significantly and PBM increases significantly (Fig. 2 A, B, C). Such as solubility, the emulsifying activity is also pH dependent because the linear and quadratic effects are very highly significant ($p < 0.001$) and it affects it in the same way as solubility (high activities at extreme pH and lower activities at neutral pH levels), as demonstrated by several authors such as Hu et al. (2017), Inyang, et al. (1996) and Tontul et al. (2018). The pH has also greatly influenced the emulsifying activity, this activity depends on the hydrophilic-lipophilic balance (Wu, Wang, Ma, & Ren, 2009) which in turn depends on the pH. At the oil-water interface, the lipophilic protein molecules are directed towards the lipid phase (oil) and the hydrophilic molecules towards the aqueous phase. The surface tension is thus reduced. At pH levels close to pHi (where protein solubility is reduced), protein adsorption is controlled by diffusion. Which is not the case at extreme pH levels (better protein solubility), the activation energy barrier does not allow protein migration to give way to diffusion, so protein solubility allows for improved interactions between the oil phase and the aqueous phase (Mao et al., 2012).

Although, most authors reported a more pronounced effect at basic pHs than at Acid pHs, contrary to the results obtained, whose optimum has been found at acid pH which can be explained by the interaction effect between pH and NaCl which is very highly significant ($p < 0.05$).

The same observation for the salt concentration, which also affects emulsifying activity with a very high significance ($p < 0.001$) and with a positive effect, the higher the salt concentration the higher the emulsifying activity increases. Our results are in agreement with those reported by other authors such as Deng et al. (2011), Hu et al. (2017) and (Inyang et al., 1996). The effect of salt on emulsifying activity could be due to its ability to form charged layers around the oil drips, which would promote repulsion between the droplets dispersed in the emulsion (Hu et al., 2017). The phosphate buffer affects positively the emulsifying activity with a very high significance.

The optimal emulsifying activity conditions for this model are: pH: 2; C NaCl: 0.55 g/l; PB: 0.1 M the EAI of the protein extract has been investigated under these conditions and the actual EAI obtained was 36.82 ± 0.34 against a predicted value of 36.65 whose difference is not significant. These optimal conditions have significantly improved the emulsifying activity of PHPC compared to its activity in distilled water (20.89 ± 0.24) (Table 1).

Table 6
Analyze of variance (ANOVA) for the experimental results obtained by Emulsifying activity.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	7	449.795	64.2564	53.18	0.0001
Linear	3	183.440	61.1468	50.61	0.0004
X1	1	70.119	70.1186	58.03	0.0001
X2	1	67.419	67.4188	55.80	0.0003
X3	1	45.903	45.9031	37.99	0.0005
Square	2	166.959	83.4796	69.09	0.0001
X1X1	1	87.187	87.1870	72.16	0.0002
X2X2	1	91.694	91.6936	75.89	0.0005
Interaction	2	99.395	49.6976	41.13	0.0001
X1X2	1	74.496	74.4961	61.66	0.0002
X2X3	1	24.899	24.8990	20.61	0.0033
Error	7	8.457	1.2082		
Lack of fit	5	8.251	1.2082	16.01	0.0600
Pure error	2	0.206	0.1031		
Total	14	458.252			
S	**1.09919**				
R-sq	**0.9815**				
R-sq (adj)	**0.9631**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

3.6. Foaming capacity

3.6.1. Analyze of the model of foaming capacity

The results of the analysis of variance of the pH effect, NaCl and PB on foaming capacity are represented in Table 7.

The results show that for linear parameters, only X2 is significant ($p < 0.01$). For quadratic parameters, X1X1 is very highly significant and X3X3 is significant, and finally the significant interaction parameters are X1X3 and X2X3. The equation of prediction was as follows:

FC = 3.7295 + 0.0008 X1 – 0.2806 X2 + 0.0518 X3 + 0.8413 × 1 × 1 + 0.0492 × 2 × 2 - 0.2327 × 3 × 3 + 0.1538 × 1 × 2 + 0.2234 × 1 × 3 + 0.3137 × 2 × 3

The model of FC as shown in Table 7 is significant at F value of 19.763. The R-sq is at 0.9726 which means that only 2.74% of the variation could not be explained by the model and then 97.26% were attributed to the independent variables used. This value of R-sq is very comparable to the value of R-sq adjusted (0.9234). Finally, the value of S is also very low (0.1555). However, for this response (FC) the value of lack of fit was significant ($0.012 < 0.05$) but this does not prevent the validation of the design given the validity of the other R^2 and adjusted R^2 parameters as well as S. Moreover, several authors have demonstrated that the significance of the value of lack of fit does not necessarily invalidate the design because it can be due to the value of the pure error which can be very small or zero due to the accuracy of the repeat measurements (Ahmad, Yusup, Bokhari, & Kamil, 2014; Bashir, Aziz, Yusoff, & Adlan, 2010; Marković et al., 2018). According to the obtained results, we can say that this model can be used for prediction of the effect of pH, NaCl and PB molarity on FC.

3.6.2. Response surface of foaming capacity

Fig. 2 (A, B, C) shows the three dimensional response surface profiles of multiple non-linear regressions of Foaming capacity of PHPC. The NaCl parameters have a significant effect on foaming activity, and the interaction effects between pH and PB and PB and NaCl are also

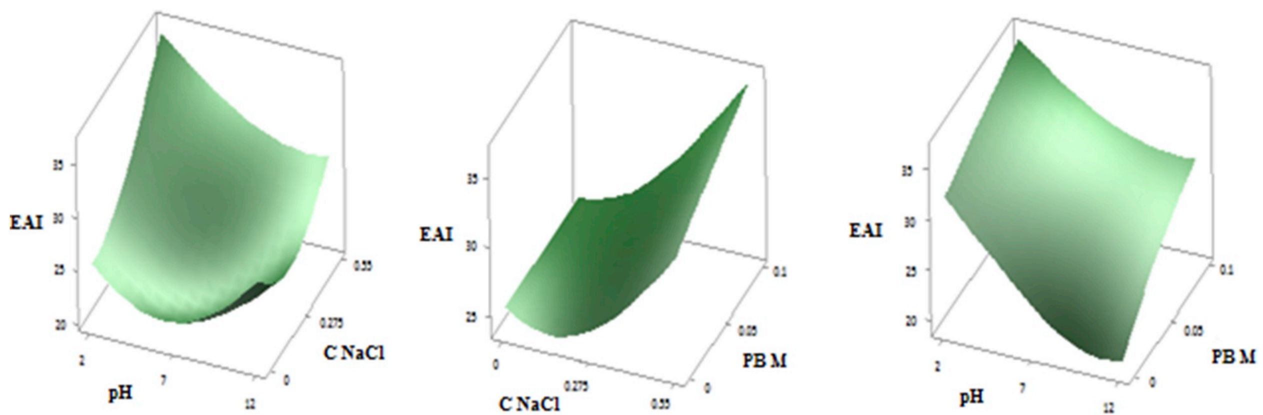

Fig. 2. Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on emulsifying activity (EAI) of PHPC.

Table 7
Analyze of variance (ANOVA) for the experimental results obtained by foaming capacity of PHPC.

Source	DF	Adj SS	Adj MS	F- Value	P-Value
Model	9	4.31315	047924	19.76	0.0023
Linear	3	0.65130	0.21710	8.95	0.0192
X1	1	0.00001	0.00001	0.00	0.9895
X2	1	0.62985	0.62985	25.97	0.0048
X3	1	0.02144	0.02144	0.88	0.3904
Square	3	2.97391	0.99130	40.87	0.0015
X1X1	1	2.97391	2.61342	107.74	0.0004
X2X2	1	0.00892	0.00892	0.37	0.5715
X3X3	1	0.19986	0.19986	8.24	0.0358
Interaction	3	0.68794	0.22931	9.45	0.0177
X1X2	1	0.09463	0.09463	3.90	0.1055
X1X3	1	0.19970	0.19970	8.23	0.0353
X2X3	1	0.39361	0.39361	16.23	0.0105
Error	5	0.12129	0.02426		
Lack of fit	3	0.00000	0.04043		0.0120
Pure error	2	4.43444	0.00000		
Total	14				
S	**0.1555747**				
R-sq	**0.9726**				
R-sq (adj)	**0.9234**				

Note: S – standard error of the regression; R-sq – regression coefficient and R-sq (adj)- adjusted regression coefficient, Adj SS: adjusted sum of square and Adj MS: Adjusted means square.

significant. The foaming capacity depends also on the pH with a very highly significant quadratic effect. On the concentration of NaCl with a very highly significant linear effect, and on the phosphate buffer with a significant quadratic effect, the interaction effects are significant for the pH-PBM and NaCl-PBM parameters. The value of FC decreases after significant decreasing of pH and slightly increasing of NaCl concentration. When NaCl concentration decreases very significantly and PBM increases, the FC increases slightly then quickly decreases. At last, the FC is maximal with significant decreasing of pH value and decreases with increasing of pH at the same time that PBM (Fig. 3 A, B, C). The effect of pH and NaCl on FC has also been reported by several authors (Hu et al., 2017; Inyang et al., 1996). Foaming capacity is highly related to solubility and Kinsella (1979) reported that only soluble proteins contribute to the formation of foams. This explains the better capacity at extreme pH (pH = 2) but ionic forces could depress the foams by reducing the coulombic forces of the polypeptides of protein molecules (Altschul & Wilcks, 1985).

The optimal FC conditions for this model are: pH: 2; NaCl: 0 g/l; PBM: 0 M the FC of the protein extract has been investigated under these conditions and the actual FC obtained was 183.55 ± 2.03 against a predicted value of 182.72 with no significant differences ($p < 0.05$). The

Table 8
Regression coefficient, standard error, and *t*-test results of response surface for solubility, EAI and FC.

	Regression coefficients	Standard error	t- value	P- value
Solubility				
Constant	−0,18664	0,00564	−33,10	0,0002
X1	0,00909	0,00345	2,63	0,0344
X2	−0,01387	0,00345	−4,02	0,0053
X3	0,00844	0,00345	2,44	0,0440
X1X1	0,03997	0,00508	7,86	0,0001
X2X2	0,02111	0,00508	4,15	0,0044
X3X3	−0,02329	0,00508	−4,58	0,0032
X1X3	0,03649	0,00488	7,47	0,0003
EAI				
Constant	4058	0,528	7,68	0,0001
X1	−2961	0,389	−7,62	0,0001
X2	2903	0,389	7,47	0,0003
X3	2395	0,389	6,16	0,0005
X1X1	4845	0,570	8,49	0,0002
X2X2	4969	0,570	8,71	0,0005
X1X2	−4316	0,550	−7,85	0,0002
X2X3	2495	0,550	4,54	0,0033
FC				
constant	3,7295	0,0899	41,48	0,0023
X1	0,0008	0,0551	0,01	0,9895
X2	−0,2806	0,0551	−5,10	0,0048
X3	0,0518	0,0551	0,94	0,3904
X1X1	0,8413	0,0811	10,38	0,0004
X2X2	−0,0492	0,0811	0,61	0,5715
X3X3	−0,2327	0,0811	−2,87	0,0358
X1X2	0,1538	0,0779	1,98	0,1055
X1X3	0,2234	0,0779	2,87	0,0353
X2X3	0,3137	0,0779	4,03	0,0105

EAI: emulsifying activity index; FC: foaming capacity.

foaming capacity obtained under these optimal conditions is much higher than that obtained in distilled water (61.66 ± 0.66) (Table 1).

The optimal conditions were determined by maximizing desirability using the Minitab prediction profiler. In order to verify the predictive capacity of the model, the results of the maximized conditions were used for a solubility test of the PHPC, their EAI and their FC. The optimal conditions obtained were: pH 12, NaCl concentration 0.55 g/l. molarity in PB 0.1 M. The experimental values for solubility, EAI and FC were 78.07 ± 0.98, 30 ± 0.52, 110 ± 2 respectively with composite desirability value of 0.77, intermediate values of desirability between (0–1) indicate more or less desirable response (Candioti et al., 2014). These experimental results were in agreement with the predicted values corresponding to 77.4, 30.05 and 111 respectively (no significant difference).

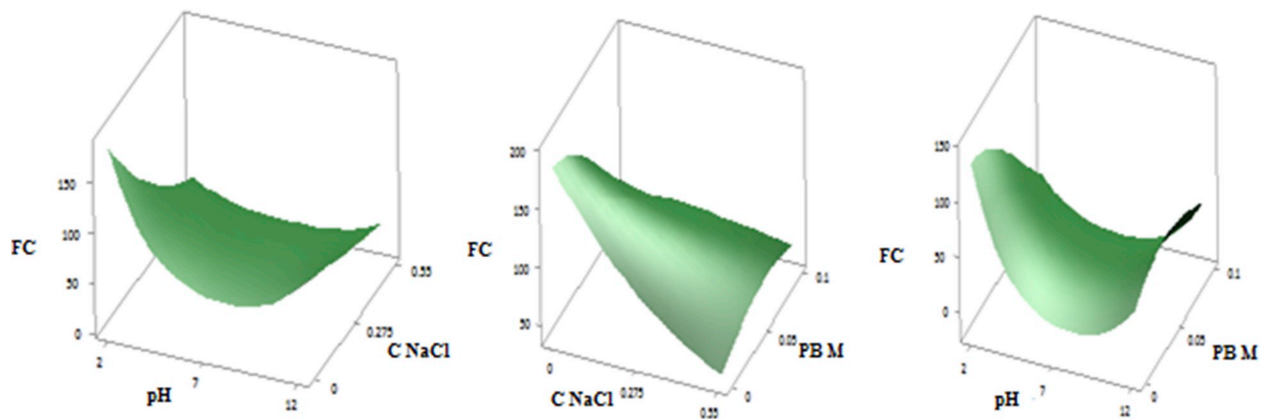

Fig. 3. Responses surfaces showing the effect of pH; NaCl (A), NaCl; PB (B) and pH; PB (C) on foaming capacity (FC) of PHPC.

3.7. Stabilisation of foaming and emulsifying properties

The optimal desirability conditions of the three responses (solubility, EAI and FC) were used to study the stability of the emulsion and that of the foam formed. The results of stability percentages are represented in Table 9.

A very good stability was exhibited for both the emulsion and the foam compared to that of sesame (Inyang et al., 1996). The stability of these two activities depends primarily on the solubility of the extract but also on the ionic strength of the medium.

Table 9
Percentages of foam and emulsion stability of PHPC.

	15 min	30 min	45 min	60min
Emulsion Stability index (%)	75 ± 2.3	68.5 ± 1.5	53 ± 2	51 ± 0.8
Foam stability (%)	90 ± 4.6	87 ± 2.2	83 ± 1.9	73 ± 4.3

Data are the mean ± SD of three analyses.

3.8. Minimum gelling concentration (MGC)

The temperature of 95 °C was used for the study of the gelation of *Pinus halepensis* Mill. proteins because for the formation of a protein gel, a denaturation of the proteins is necessary (Srinivasan Damodaran, 1988). According to Arntfield and Murray (1981), the denaturation temperatures of oat, fababean, field pea and soybean were 112, 88, 86 and 93 °C respectively.

The MGC of PHPC under optimal conditions of functional properties was around 6% of PHPC, it's showing the very good gelling capacity of these proteins under these conditions. The gelation phenomenon is a phenomenon much more associated with temperature. Heat treatment allows the denaturation of proteins by cleavage of the structure of the disulfide bonds and thus the deflagration of proteins or an activation of the sulfide groups buried inside the molecule. these sulfide groups can give intermolecular disulfide bonds (exchange of the disulfide season) which causes a deployment of the protein molecules followed by an aggregation and association step thus forming the gel (Bau et al., 1985), the optical and rheological properties of thermally irreversible gels are therefore obtained (Ziegler & Foegeding, 1990). The parameters that can improve gelling are the increase in time, temperature, pH and protein concentration (Coffmann & Garciaj, 1977). Sun and Arntfield (2010) reported also that the salt concentration improves significantly the gelling properties of proteins. In our study, the high pH and high salt concentration may have influenced this capacity. In addition to the solubility of its proteins which is 77.39%, the minimum concentration obtained is more interesting than that reported by most other protein extracts. O'Kane et al. (2005), Coffmann and Garciaj (1977) and (A. M. Altschul, 1958) obtained MGC of 16% for pea protein, 10% for mung bean protein and 8% for soy protein respectively.

3.9. Heat coagulability (HC)

A relatively high HC was demonstrated by PHPC which was 24% compared to concentrate of *Brassica juncea* mustard seeds and *Sinapis alba* (Rotimi E. Aluko, McIntosh, & Katepa-Mupondwa, 2005) and canola isolate seeds (Rotimi E Aluko & McIntosh, 2001) as well as soybean isolate and pea isolate (Voutsinas et al., 1983) which have not shown any coabulability to heat. Our result is comparable to the HC of sunflower isolate (22.5%). Voutsinas et al. (1983); Rotimi E Aluko et al. (2001) and Rotimi E. Aluko et al. (2005) explain that HC depends mainly on the solubility as well as the surface hydrophobicity of the protein extract, which justifies the heat coagulation of our PHPC under the conditions used which solubilize them at 77.39%.

4. Conclusion

In conclusion, this study confirmed that the functional properties are strongly related to the pH of the medium, its NaCl concentration and its PB molarity. In addition, the results show that Aleppo pine seeds are a good source of protein whose functional activities have been improved by the three parameters influencing them (pH, NaCl concentration and PB molarity). These proteins can therefore be used as food ingredients and the variation in pH, NaCl concentration and PB molarity can be employed as an effective processing method to improve the use of proteins as functional ingredients in food product formulations.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2019.105416.

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Abstract

Aleppo pine seeds are oilseeds whose tree is very abundant and unfortunately very little value. and yet, these seeds are of high nutritional and taste quality. The main goal of this work was to find some avenues of recovery for possible industrial uses. First of all, the polysaccharide fraction was valorized by studying some biological activities (antioxidant, anti-inflammatory and anti-coagulant in vitro) while studying the effect of three extraction solvents (acetone, ethanol, propanol) on the yield and these biological activities. The yield of the seeds in polysaccharides was about 2% and the extracts showed very good activities but it was the acetone which gave the best yield and most of the activities. Secondly, since these seeds are rich in proteins, the study of their functional properties was carried out by optimizing their pH, Nacl and phosphate buffer settings using a complete experimental design (box behnken). These proteins exhibited good functional properties and a better desirability was obtained at an extreme basic pH without Nacl and phosphate buffer. Finally, since no food product based on these seeds is industrialized, their potential in the production of a vegetable drink has been elucidated and as it is a new product, it was necessary to study in parallel all the unit operations of a standard manufacturing diagram of a vegetable drink; to try to understand and know the conditions of conception of this drink of good nutritional and physical quality. For this purpose the extraction (ratio, time and temperature), formulation, homogenization and packaging were studied using a fractional factorial design 2 (7-4). The potential and feasibility of this product was confirmed and the conditions were as follows: ratio 2/10, grinding time 120 s, temperature 60°C, addition of xanthan gum is not necessary but it can increase viscosity and stability, homogenization pressure as well as pasteurization play a very important role in the stabilization of the product.

Key words: Aleppo pine seeds, Polysaccharides, Proteins, functional properties, vegetable milk

Résumé

Les graines de pin d'Alep sont des graines oléagineuse dont l'arbre est très abondant et malheureusement très peu valorisées et pourtant, ces graines sont de hautes qualité nutritionnelles et gustatives. Le but principal de ce travail était de de trouver quelques pistes de valorisation pour d'éventuelles utilisations industrielles. En premier lieu, la fraction polysaccharidique a été valorisée en étudiant quelques activités biologiques (antioxydant, anti-inflammatoire et anti-coagulant *in vitro*) tout en étudiant l'effet de trois solvants d'extraction (acétone, éthanol, propanol) sur le rendement et ces activités biologiques. Le rendement des graines en polysaccharides était d'environ 2% et les extraits ont exhibé de très bonnes activités mais c'était l'acétone qui donnait le meilleur rendement et la plupart des activités. En second lieu, étant donné que ces graines sont riches en protéines, l'étude de leurs propriétés fonctionnelles a été réalisée, et cela en optimisant leurs conditions en pH, Nacl et tampon phosphate en utilisant un plan d'expérience complet (box behnken). Ces protéines ont exhibé de bonnes propriétés fonctionnelles et une meilleure désirabilité a été obtenue à un pH basique extrême sans Nacl et sans tampon phosphate. Enfin, vu qu'aucun produit alimentaire à base de ces graines n'est industrialisé, leur potentiel en production d'une boisson végétale a été élucidé et comme c'est un nouveau produit, il a fallu étudier en parallèle toutes les opérations unitaires d'un diagramme de fabrication standard d'une boisson végétale; pour essayer de comprendre et de connaitre les conditions de conception de cette boisson de bonnes qualité nutritionnelles et physique. Pour cela l'extraction (ratio, temps et température), formulation, homogénéisation et conditionnement ont été étudié en utilisant un plan factoriel fractionnaire 2 (7-4). le potentiel et la faisabilité de ce produit a été confirmé et les conditions étaient comme suit: le ratio 2/10, Le temps de broyage 120 s, La température 60°C, l'ajout de gomme de xanthane n'est pas nécessaire mais il peut augmenter la viscosité et la stabilité, la pression d'homogénéisation ainsi que la pasteurisation jouent un rôle très important dans la stabilisation du produit.

Mots clés: grains de pin d'Alep, Polysaccharides, Protéines, propriétés fonctionnelles, lait végétale.