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

Amina Abbou [a,b,*], Nabil Kadri [b,c,**], Nadjet Debbache [d], Sofiane Dairi [c,e], Hocine Remini [b,c], Farid Dahmoune [b,c], Farida Berkani [a,b], Khadidja Adel [a,b], Amine Belbahi [c,f], Khodir Madani [c]

[a] Laboratoire de Gestion et Valorisation des Ressources Naturelles et Assurance Qualité (LGVRNAQ), Faculté des Sciences de la Nature et de la Vie et des Sciences de la Terre, Université de Bouira, 10000 Bouira, Algeria
[b] Département des Sciences Biologiques, Faculté des Sciences de la Nature et de la Vie et des Sciences de la Terre, Université de Bouira, 10000 Bouira, Algeria
[c] Laboratoire de Biochimie, Biophysique, Biomathématiques et Scientométrie (L3BS), Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria
[d] Laboratoire de Biochimie Appliquée, Faculté des Sciences de la Nature et de la vie, Université de Bejaia, 06000 Bejaia, Algeria
[e] Département de Microbiologie Appliquée et Sciences Alimentaires, Faculté des Sciences de la Nature et de la Vie, Université de Jijel, 18000 Jijel, Algeria
[f] Département de Microbiologie et Biochimie, Faculté des Sciences, Université de M'sila, 28000 M'sila, Algeria

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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.

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

* Correspondence to: A. Abbou, Laboratoire de Gestion et Valorisation des Ressources Naturelles et Assurance Qualité (LGVRNAQ), Faculté des Sciences de la Nature et de la Vie et des Sciences de la Terre, Université de Bouira, 10000 Bouira, Algeria.
** Correspondence to: N. Kadri, Laboratoire de Biochimie, Biophysique, Biomathématiques et Scientométrie (L3BS), Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algeria.
E-mail addresses: minabbou20@gmail.com (A. Abbou), kadri.montp2@gmail.com (N. Kadri).

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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.

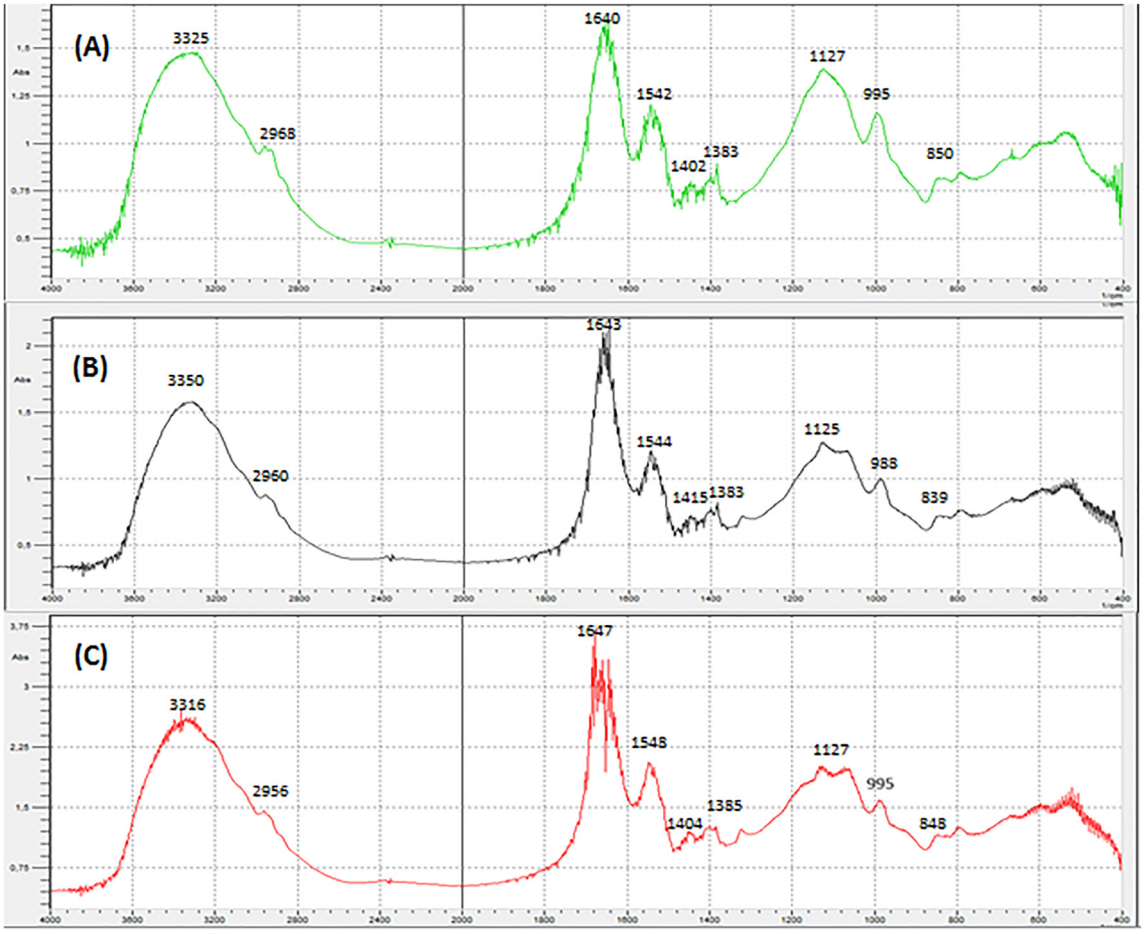

Fig. 1. FT-IR spectrometry of polysaccharides extract (AP, EP, PP) from *Pinus halepensis* Mill. seeds. (A): Acetone polysaccharides extract, (B): ethanol polysaccharides extract, (C): propanol polysaccharides extract.

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 ± 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 ± 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 ± 0.88 μg/mL) and PP samples (IC_{50} = 28.10 ± 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 ± 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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