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Phytochemical analysis, and antioxidant, anti-hemolytic and genoprotective effects of *Quercus ilex* L. and *Pinus halepensis* Mill. methanolic extracts

[Análisis fitoquímico y efectos antioxidante, antihemolítico y genoprotector de extractos metanólicos de *Quercus ilex* L. y *Pinus halepensis* Mill.]

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Abstract

Context: *Quercus ilex* and *Pinus halepensis* are largely used in Algerian folk medicine as a remedy for different health problems.

Aims: To determine the phytochemical composition as well as to evaluate the antioxidant, anti-hemolytic and genoprotective effects of *Quercus ilex* root bark extract (QIE) and *Pinus halepensis* young ovulate cones extract (PHE).

Methods: Methanolic extracts were prepared by maceration. Phenolic compounds and flavonoids were quantified spectrophotometrically and identified by HPLC analysis. *In vitro* antioxidant activity of the extracts was assessed by determining the DPPH and ABTS radicals scavenging activities and ferric reducing/antioxidant power (FRAP). Protective effect of the extracts against AAPH-induced erythrocyte hemolysis was assessed. pBluescript M13 (+) plasmid DNA was used as oxidation target to evaluate DNA protective effect of the extracts.

Results: Considerable phenolic compounds and flavonoids contents were found in the studied extracts. Catechin and phenolic acids (4-hydoxybenzoic, caffeic, coumaric, ferulic and gentisic acids) were identified in QIE, while catechin and cinnamic acid were identified in PHE. Both extracts scavenge DPPH radical with IC_{50} values of 5.67 µg/mL and 18.87 µg/mL, respectively. Antioxidant capacity against ABTS radical was 4.13 and 1.15 mM Trolox equivalent/mg extract. The two extracts also showed a considerable ferric reducing ability with 1.98 and 8.55 mM $FeSO_4$/mg extract, respectively. On the other hand, both extracts exerted a significant protective effect against AAPH-induced erythrocyte hemolysis and DNA protective activities were noticed.

Conclusions: *Quercus ilex* and *Pinus halepensis* extracts are important sources of bioactive compounds possessing important antioxidants, antihemolytic and genoprotective effects.

Keywords: anti-hemolytic; antioxidant; genoprotection; *Pinus halepensis*; *Quercus ilex*.

Resumen

Contexto: *Quercus ilex* y *Pinus halepensis* se usan en la medicina popular argelina como un remedio para diferentes problemas de salud.

Objetivos: Determinar la composición fitoquímica, así como evaluar los efectos antioxidantes, antihemolíticos y genoprotectores del extracto de corteza de raíz de *Quercus ilex* (QIE) y el extracto de conos ovulares de *Pinus halepensis* (PHE).

Métodos: Los extractos metanólicos se prepararon por maceración. Los compuestos fenólicos y los flavonoides se cuantificaron espectrofotométricamente y se identificaron mediante análisis de HPLC. La actividad antioxidante *in vitro* se evaluó mediante la determinación de las actividades de captación de radicales DPPH y ABTS y la potencia férrica reductora/antioxidante (FRAP). Se evaluó el efecto protector de los extractos contra la hemólisis eritrocítica inducida por AAPH. El ADN plasmídico pBluescript M13 (+) se usó como diana de oxidación para evaluar el efecto protector de los extractos al ADN.

Resultados: En los extractos estudiados se encontraron considerables contenidos de compuestos fenólicos y de flavonoides. La catequina y los ácidos fenólicos (4-hidroxibenzoico, cafeico, cumárico, ferúlico y gentísico) se identificaron en QIE, mientras que la catequina y el ácido cinámico se identificaron en PHE. Ambos extractos eliminaron el radical DPPH con valores de CI_{50} de 5,67 µg/mL y 18,87 µg/mL, respectivamente. La capacidad antioxidante contra el radical ABTS fue 4,13 y 1,15 mM de equivalente de Trolox/mg de extracto. Los dos extractos también mostraron una considerable capacidad de reducción férrica con 1,98 y 8,55 mM $FeSO_4$/mg, respectivamente. Por otro lado, ambos extractos ejercieron un efecto protector significativo contra la hemólisis eritrocítica inducida por AAPH y se observaron actividades protectoras al ADN.

Conclusiones: Los extractos de *Quercus ilex* y *Pinus halepensis* son fuentes importantes de compuestos bioactivos que poseen efectos antioxidantes, antihemolíticos y genoprotectores.

Palabras Clave: antihemolítico; antioxidante; genoprotección; *Pinus halepensis*; *Quercus ilex*.

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INTRODUCTION

It is commonly recognized that reactive oxygen species (ROS) are involved in a variety of physiological processes, including cellular signal transduction, cell proliferation, differentiation and apoptosis (Bland et al.,1995; Sánchez et al., 2015). In healthy individuals, ROS production is continuously balanced by natural antioxidative defense systems. However, oxidative stress or oxidant/antioxidant imbalance occurs when ROS overwhelm antioxidative defense systems. ROS can react with polyunsaturated fatty acids of cell membranes and induce lipid peroxidation leading to the generation of secondary free radicals, which can directly react with other biomolecules, enhancing biochemical lesions (Phaniendra et al., 2015). Increased formation of oxidized products contributes to several pathologies such as atherosclerosis, ischemia-reperfusion, heart failure, Alzheimer's disease, rheumatic arthritis, cancer, and other immunological disorders (Pincemail et al., 2002).

DNA is also an important target for ROS attacks resulting usually in the induction of oxidatively induced DNA damage and a variety of cell lesions. The accumulation of DNA damage through misrepair or incomplete repair may lead to mutagenesis and consequently transformation, particularly if combined with a deficient apoptotic pathway (Kryston et al., 2011).

An increasing number of studies highlighted the importance of medicinal plants as an easily available and potent sources of safe antioxidants, healthcare supplements and biomolecules that help in preserving good health and combating diseases (Bhatt et al., 2013).

Quercus ilex L. (*Fagaceae*), is largely used in Mediterranean countries in folk medicine to treat diarrhea, gastritis ulcer and skin infections (Kennouf et al., 2003; Berahou et al., 2007). *Pinus* species, including *Pinus halepensis* Mill. (*Pinaceae*) are used against rheumatic pain, common cold, bronchitis, cough and wound healing (Süntar et al., 2012; Kizilarslan and Sevgi, 2013).

The present study was designed to evaluate the antioxidant, antihemolytic and DNA protective effect of *Quercus ilex* root bark extract and *Pinus halepensis* young ovulate cones extract.

MATERIAL AND METHODS

Chemicals

All the chemicals used in this study were of analytical/HPLC grade and obtained from Sigma-Aldrich Co. (St. Louis, USA). Solvents and salts of the greatest available chemical purity were purchased from Panreac (Panreac Química SLU, Barcelona, Spain) and VWR/Prolabo (VWR International, Radnor, Pennsylvania, USA).

Plant material

Quercus ilex (root bark) and *Pinus halepensis* (young ovulate cones) were collected from the region of Medjana, in eastern Algeria (GPS coordinates: 36°11'19.2"N 4°40'25.4"E). The plants were identified by Pr. Houssine Laouar, University of Sétif. Voucher specimens (No R. F. 2016/39 and No Z. V. 2016/41) respectively, were deposited at the laboratory of botany in the University of Sétif. Algeria. Plant material was washed and air-dried at room temperature and then reduced to powder.

Preparation of plant extracts

One hundred grams of the powdered plant material were macerated in 80% aqueous methanol (1:10, w/v) and left overnight under continuous agitation. After filtration, the filtrate was concentrated under reduced pressure in a rotary evaporator (Büchi, Flawil, Switzerland). The resulting methanolic extracts were then lyophilized (Christ Martin, Osterode am Harz, Germany) and kept at -20°C until use.

Measurement of total phenolic content

The total phenolic contents of *Quercus ilex* Lam and *Pinus halepensis* extracts were measured according to the method described by Li et al. (2007) and Meziti et al. (2017). A volume of 200 µL of diluted sample was added to 1 mL of 1:10 diluted

Folin–Ciocalteu reagent. After 4 min, 800 µL of saturated sodium carbonate solution were added. After 2 hours of incubation at room temperature, the absorbance was measured at 765 nm using a UV-Visible spectrometer (Specord 200 Plus, Analytik Jena, Jena, Germany). Gallic acid was used for the standard calibration curve. The results were expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g extract).

Measurement of flavonoid content

Aluminum-chloride colorimetric assay was used to determine the total flavonoid contents in the extracts as previously reported by Meziti et al. (2017). Briefly, 1 mL of extracts (0.25-0.5 mg/mL) was mixed with the same volume of 2% aluminum trichloride ($AlCl_3$) solution and allowed to stand for 15 min. The absorbance of the mixture was then determined at 430 nm with a UV-Visible spectrometer (Specord 200 Plus, Analytik Jena, Jena, Germany). Quercetin was used for the standard calibration curve. The data were expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g extract).

HPLC analysis

HPLC analysis was performed as described by Abay et al. (2015) and Meziti et al. (2017) The extracts were dissolved in HPLC grade MeOH and then filtered through a PTFE (0.45 µm) filter by an injector to remove particulates. The 1260 Infinity HPLC System (Agilent Technologies, Santa Clara, U.S.A.) was coupled with 6210 Time of Flight (TOF) LC/MS detector (Agilent Technologies, Santa Clara, U.S.A.). Injection volume was 10 µL. Separation was carried out on ZORBAX SB-C18 (4.6 x 100 mm, 3.5 µm) column (Agilent Technologies, Santa Clara, U.S.A.). Mobile phases A and B were ultra-pure water with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.6 mL min^{-1} and column temperature was 35°C. The solvent program was as follow: 0-1 min 10% B, 1-20 min 50% B, 20-23 min 80% B, 23-25 min 0% B; 25-30 min 10% B. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325°C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 4000 V and finally, fragmentation voltage of 175 V. Phenolic compound were identified by the retention time of sample chromatographic peaks compared with those of authentic standards using the same HPLC operating condition.

DPPH radical scavenging activity

Radical scavenging activity of the studied extracts against the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was evaluated as described by Saija et al. (1998). Aliquots of 37.5 µL of the extracts at different concentrations (01 – 70 µg/mL) were added to 1.5 mL DPPH 100 mM in methanol. The mixture was shaken and left to stand at room temperature for 20 min. The absorbance was measured at 517 nm using a UV-Visible spectrometer (Specord 200 Plus, Analytik Jena, Jena, Germany), and the percentage of radical scavenging activity was calculated as follow:

$$\text{DPPH scavenging activity (\%)} = \left[\frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}}\right] \times 100 \quad [1]$$

Where Abs control is the absorbance of the control reaction mixture without the test compounds, and Abs sample is the absorbance of the test compounds.

ABTS radical scavenging activity

The ability of the studied extracts to scavenge the ABTS radical (2,2'-azino-bis(3-ethylbenzothiazo-line-6-sulphonic acid) was evaluated according to the method of Kutlu et al. (2014). ABTS+ radical was generated by a reaction between ABTS (1.7 mM) and 4.3 mM potassium persulfate. The mixture was allowed to stand in the dark at room temperature for 12-16 h before use, and then the ABTS solution was diluted with phosphate buffered saline at pH 7.4 to give an absorbance of 0.7 ± 0.02 at 734 nm. To 2 mL of the ABTS+ solution, 50 µL of different concentrations (0.02 – 1.00 mg/mL) of the extracts were added and the decay in absorbance was followed in a UV-Visible spectrometer (Specord 200 Plus, Analytik Jena, Jena, Germany) for 6 min. at 734 nm. The unit of total antioxidant activity was defined as the concentration of Trolox exerting an equivalent antioxidant activity

and expressed as mmol Trolox equivalent/mg dry extract.

Ferric Reducing Antioxidant Power assay

The ferric reducing antioxidant power **(**FRAP**)** of the extracts was evaluated following the method of Benzie and Strain (1996). Briefly, the FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v/v), respectively. Fifty microliters of methanolic solution with different concentrations (0.02 – 0.5 mg/mL) of the extracts were added to 1 mL of the FRAP reagent. The absorbance of the reaction mixtures was then measured using a UV-Visible spectrometer (Specord 200 Plus, Analytik Jena, Jena, Germany) at 593 nm after 4 min of incubation at room temperature. The FRAP reagent alone was used as a blank and an iron (II) sulfate solution (100 – 1000 µM) was used for the preparation of the standard curve. Results were expressed as mmol Fe (II)/mg dry extract.

Animals

Swiss albino mice weighing 25–30 g were brought from the Pasteur Institute of Algeria. They were kept in a climate-controlled environment with a 12 h light/dark cycle (7:00 am to 7:00 pm) at 22°C, with free access to food and water *ad libitum*. Animals were treated in accordance with European Union Guidelines for Animals Experimentation (2007/526/EC). The approval to perform the part of this study that involves animal experiments, respecting ethical consideration, was obtained from the Scientific Council of the Faculty of Natural Sciences and Life, University Setif-1 (Algeria) under the reference: CSF/SNV/18/06.

AAPH-induced erythrocytes hemolysis

Anti-hemolytic effect of Quercus *ilex* Lam and *Pinus halepensis* extracts was assessed according to the procedure described by Girard et al. (2006). Blood was collected through direct heart puncture from anesthetized mice and then diluted to 2% in physiological buffer (NaCl 125 mM, sodium phosphate 10 mM, pH 7.4). In a 96 well plate, 120 µL of mice erythrocytes suspension (2%) were preincubated with 60 µL of different concentrations of the extracts (12.5, 25, 50 and 75 µg/mL) or Trolox (50, 75 µg/mL) for 30 min. at 37°C. To each well, 120 µL of AAPH (100 mM) were then added and the mixture was incubated at 37°C. Free radical chain oxidation was the result of aqueous peroxyl radicals generated by thermal decomposition of AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) at 37°C. The kinetic of hemolysis was monitored by measuring optical density decrease at 630 nm, using a 96-well microplate reader (ELX 800, Bio-TEK instruments, Winooski, VT, USA). Trolox was used as reference while physiological buffer served as the control.

DNA oxidation induced by the photolysis of H_2O_2

The protective effect of the studied extracts against DNA damage generated by hydrogen peroxide (H_2O_2) and ultraviolet (UV) radiations, known as reactive oxygen species generating system was checked on pBluescript M13 (+) plasmid DNA as oxidation target (Kizil et al., 2010). The reaction mixture contained 200 ng of plasmid DNA in phosphate buffer (7.14 mM phosphate and 14.29 mM NaCl, pH 7.4). A final concentration of 2.5 mmol/L of H_2O_2 was added with or without 1 µL of (100, 250, 350, 500 µg/mL) of the extracts. The reaction was initiated by UV irradiation at 300 nm with an intensity of 8000 µW cm−1 and continued for 5 min at room temperature on the surface of a UV transilluminator (BioRad, Hercules, CA, USA). After irradiation, the reaction mixture (10 µL) with gel loading dye was placed on 1% agarose gel for electrophoresis (BioRad, Hercules, CA, USA). Electrophoresis was performed at 40 V for 3 h in the presence of ethidium bromide (10 mg/mL). Untreated pBluescript M13+plasmid DNA was used as a control in each run of gel electrophoresis along with partial treatment, i.e., only UV treatment and only H_2O_2. Gels were stained with ethidium bromide and scanned with the Gel-Doc-XR Gel documentation system (BioRad, Hercules, CA, USA). Densitometric analysis was used to quantify the intensity of each DNA band.

Statistical analysis

Results are expressed as mean ± SD. The hemolysis sigmoids were fitted by computer analysis Software Graph Pad. Prism (V5.00). Statistical significance of the difference was assessed by a one-way analysis of variance followed by Dunnet's test or by Student's t-test as appropriate. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Total phenolic compounds

Polyphenols are one of the most important groups of secondary metabolites of plants. A number of studies focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical-scavengers (Zhang and Tsao, 2016).

Quercus ilex extract showed a high phenolic content (490.81 ± 29.62 µg GAE/mg dry extract), which was two folds higher than that found in *Pinus halepensis* extract (251.40 ± 7.07 µg GAE/mg dry extract). There are a very few studies in the literature that focused on phenolic content of *Quercus ilex* root and no previous study was carried out on phenolic content of *Pinus halepensis* young ovulate cones. Vinha et al. (2016) showed that the total phenolic contents in the acorn tissues of different *Quercus* species including *Quercus ilex* ranged from 18 to 32 µg GAE/mg extract, which were much lower than the present results. These differences could be related to the divergence in repartition of phenolic compound between the different part of the plant or different climatic conditions (Males et al., 2010; Tolić et al., 2017).

Flavonoid content

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties (Djeridane et al., 2010). The results of the present study showed that unlike phenolic content, flavonoids content of *Pinus halepensis* extract (3.69 ± 0.19 µg QE/mg dry extract) was significantly higher ($p<0.05$) than that of *Quercus ilex* extract (3.11 ± 0.04 µg QE/mg dry extract).This result could be due to the proportions of different classes of polyphenols present in the plant extracts.

HPLC analysis

Results obtained for the polyphenolic composition of the extracts are presented in Table 1. According to the retention time of standards compounds, *Quercus* extracts presented a chemical profile (Fig. 1) composed of six identified phenolic compounds. Catechin was the most important phenolic compound identified followed by caffeic acid. Gentisic and hydroxybenzoic acids were minor compounds in *Quercus ilex* extract.

Table 1. Phenolic compounds determined by HPLC-TOF/MS in *Quercus ilex* and *Pinus halepensis* extracts.

Phenolic compounds	Retention time (min)	*Quercus ilex* (µg/g extract)	*Pinus halepensis* (µg/g extract)
Ferulic acid	1.076	606.16	-
Gentisic acid	4.4	59.12	-
Catechin	6.16	7359.44	15545.52
4-Hidroxybenzoic acid	6.606	335.26	-
Caffeic acid	7.69	2374.82	-
Coumaric acid	9.97	861.348	-
Cinnamic acid	16.04	-	380.94

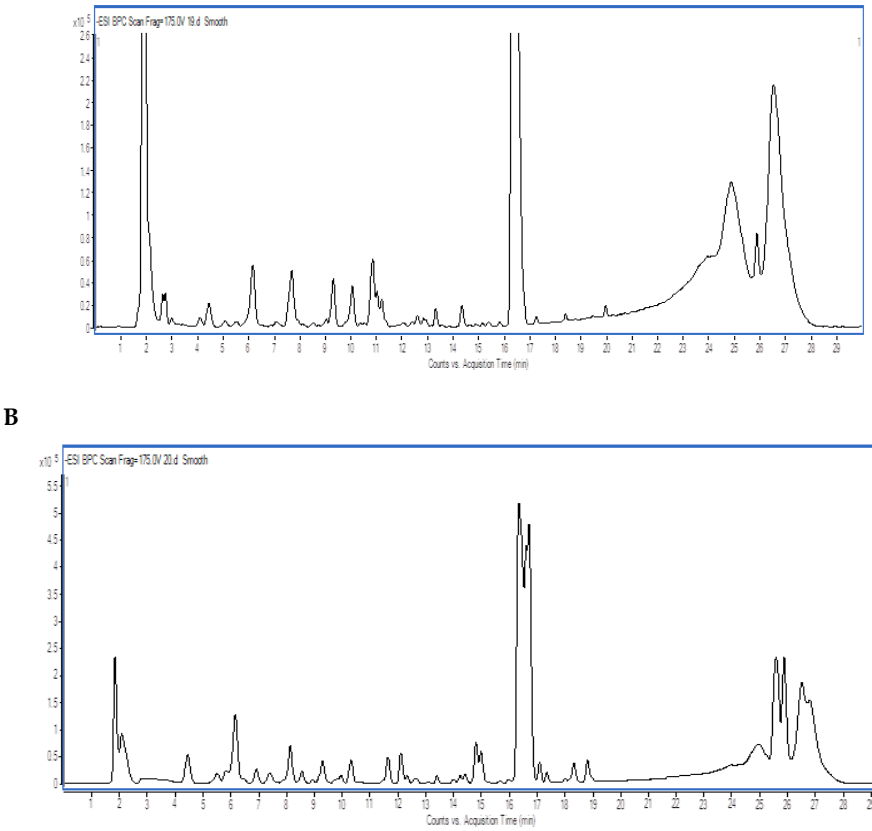

Figure 1. HPLC chromatograms of *Quercus ilex* **(A)** and *Pinus halepensis* **(B)** extracts.

These results are partially in concordance with the phytochemical investigation of García-Villalba et al. (2017) who studied the leaf composition of different *Quercus* species and identified, nine phenolic acids (gallic acid, 3,4-dihydroxybenzoic acid, chlorogenic acid, 4-hydroxybenzoic acid, 4-O-caffeoylquinic acid, caffeic acid, coumaric acid, ferulic acid and salicylic acid). They also reported that the predominant flavonoids in all *Quercus* species were catechin and gallocatechin.

On the other hand, only two phenolic compounds were identified in *Pinus halepensis* extract, catechin which represents the major phenolic compound of the extract and cinnamic acid. In comparison with *Quercus ilex* extract, the concentration of catechin in *Pinus halepensis* extract was twofold higher than in *Quercus ilex* extract. This result could be the explanation of the high flavonoid content obtained by $AlCl_3$ method.

DPPH radical scavenging activity

Substances which are able to perform reduction by either hydrogen or electron-donation can be considered as radical scavengers and therefore antioxidants. The color change degree of DPPH radicals from violet to yellow upon reduction indicates the radical scavenging potential of the antioxidant (Moghaddam et al., 2012).

Results showed that both extracts exerted considerable dose-dependent scavenging activity on DPPH radical (Fig. 2). However, *Quercus ilex* extract with IC_{50} = 5.67 µg/mL, was significantly more potent ($p<0.05$) than *Pinus halepensis* extract that gave an IC_{50} value of 18.87 µg/mL.

The obtained results are aligned with published data that report high DPPH scavenging activity for *Quercus* species (Vinha et al., 2016; Amessis-Ouchemoukh, 2017). Santos et al. (2010) tested the

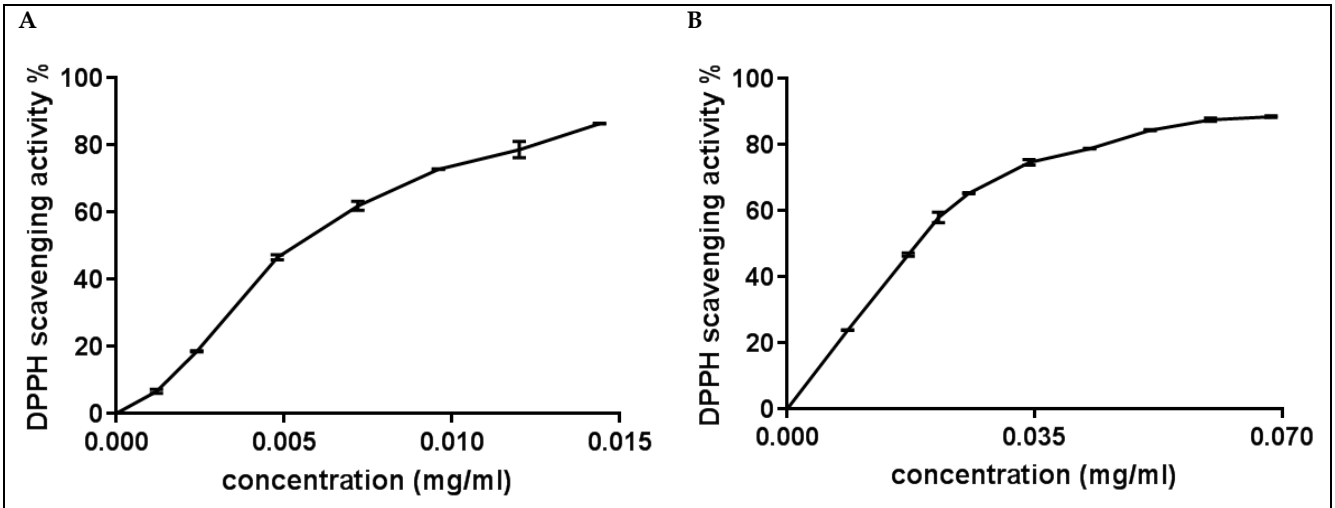

Figure 2. DPPH radical-scavenging activity of *Quercus ilex* and *Pinus halepensis* extracts.

In the plots is represented % DPPH scavenging activity vs. the concentration (mg/mL). **(A)** *Quercus ilex* extract (IC_{50}: 5.67 ± 0.09 µg/mL); **(B)** *Pinus halepensis* extract (IC_{50}: 18.87 ± 0.20 µg/mL). The values are means ± SD (n=3).

scavenging activity of different extracts prepared from cork of *Quercus suber* using DPPH radicals and reported an IC_{50} values ranging from 2.79 to 5.84 µg/mL. Nevertheless, direct quantitative comparisons cannot be made due to differences in the experimental conditions.

The radical scavenging activity of *Quercus ilex* and *Pinus halepensis* extracts is probably attributed to the presence of phenolic compounds. Indeed, phenolic compounds are recognized as potentially antioxidant substances with the ability to scavenge free radical species, and reactive forms of oxygen (Aboul-Enein et al., 2007; Villano et al., 2007). The scavenging effect of phenolic compounds is attributed to their low potential redox making them thermodynamically able of reducing free radicals by a transfer of hydrogen or electron from hydroxyl groups and delocalization of unpaired electron leading to the formation of a stable phenoxyl radical (Dai and Mumper, 2010).

ABTS radical scavenging activity

The ABTS assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements. It is based on the ability of antioxidants to scavenge the stable radical cation ABTS•+ [2,2′-azinobis(3- ethylbenzothiazoline-6-sulphonic acid)], a blue-green chromophore with maximum absorption at 734 nm, which decreases in its intensity in the presence of antioxidants (Kumar et al, 2017). Results of ABTS radical cation scavenging ability were expressed as Trolox equivalent antioxidant capacity (TEAC) as shown in Table 2.

Similarly, to the DPPH assay, ABTS assay revealed that Quercus *ilex* extract exerted a significantly ($p<0.05$) higher antioxidant capacity. In fact, *Quercus ilex* extract was shown to be fourfold more potent than *Pinus halepensis* extract. In contrast, Wang et al. (1998) found that some compounds, which have ABTS scavenging activity did not show DPPH scavenging activity. The present study further showed the capability of the extracts to scavenge different free radicals (DPPH and ABTS) in different systems.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay is simple, fast and cost-effective. It was originally employed to measure reducing power in plasma, but its use has been extended for assessing antioxidant activity in other

biological fluids, foods, and plant extracts (Huang et al., 2005).

This assay is a typical electron transfer-based method that measures the reduction of ferric ion complex (Fe^{3+}–tripyridyltriazine) to the intensely blue colored ferrous complex (Fe^{2+}–tripyridyltriazine) by antioxidants in acidic media (Antolovich et al., 2002).

Compared with *Pinus halepensis* extract, *Quercus ilex* extract showed a significantly higher ferric reducing antioxidant capacity ($p<0.05$) as it has been recorded in DPPH and ABTS assays (Table 2). These results are in close agreement with those reported by Belkacem et al. (2014) and Parikh and Patel (2017) who found a strong relationship between phenolic contents and antioxidant activity assessed by DPPH, ABTS and FRAP assays.

Anti-hemolytic activity

Erythrocyte membrane is mostly susceptible to free radicals attack due to its high content of polyunsaturated fatty acids, as well as molecular oxygen transport by hemoglobin (García-Becerra et al., 2016). Lipid peroxidation in human erythrocyte membrane mediated by AAPH, induces membrane damage and subsequent hemolysis (Aman et al., 2013). The principle of this essay is to submit erythrocyte to a thermo-controlled free radical aggression. All families of antioxidant present in the extract are mobilized to fight off the oxidant attack and to protect the integrity of erythrocytes resulting in the delay of hemolysis.

The incubation of red blood cells (RBC) with the tested extracts did not induce hemolysis, indicating that the extracts are nontoxic and harmless for the cells. The preincubation of erythrocytes with different concentrations of the extract for 30 min followed by AAPH challenge resulted in a potent protective effect against AAPH-induced RBC lysis. This protective effect increased in concentration-dependent manner (Fig. 3). *Pinus halepensis* extract showed a significant ($p<0.01$) antihemolytic activity at the concentrations of 50 and 75 µg/mL, with half hemolysis times (HT_{50}) of 73.18 and 93.63 min, respectively. *Quercus ilex* extract at the same concentrations exhibited a stronger effect (HT_{50} = 116.46 and 143.6 min, respectively). These values were higher than those obtained with the control (Trolox) that has an HT_{50} of 102.97 and 100.33 min at 50 and 75 µg/mL, respectively.

Tables 2. Total phenols, total flavonoid, DPPH scavenging activity, ABTS scavenging activity and ferric reducing-antioxidant power of *Quercus ilex* and *Pinus halepensis* extracts.

Extract	Total phenols (µg GAE/g DE)	Total flavonoids (µg QE/g DE)	DPPH (IC_{50} µg/mL)	ABTS (mM TE/mg DE)	FRAP (mM $FesO_4$/mg DE)
Quercus ilex	490.81 ± 29.62[a]	3.11 ±0.04[b]	5.67 ± 0.09[b]	4.12 ± 0.90[a]	8.55 ± 1.52[a]
Pinus halepensis	251.40 ± 7.07[b]	3.69 ± 0.19[a]	18.87± 0.2[a]	1.15 ± 0.27[b]	1.98 ± 0.22[b]

The values are expressed as mean ± SD (n = 3). [a]Significantly higher than the [b]value of the same column ($p<0.05$). Total phenols are expressed micrograms of gallic acid equivalent per gram of dry extract (µg GAE/g DE). Total flavonoids are expressed as micrograms of quercetin equivalents per gram of dry extract (µg QE/g DE). DPPH scavenging activity is expressed as the concentration inhibiting 50% radical (IC_{50}). ABTS scavenging activity is expressed as millimole of Trolox equivalent per milligram of dry extract (mM TE/mg DE). Ferric reducing-antioxidant power is expressed as millimole $FeSO_4$ equivalent per mg dry extract (mM $FesO_4$/mg DE).

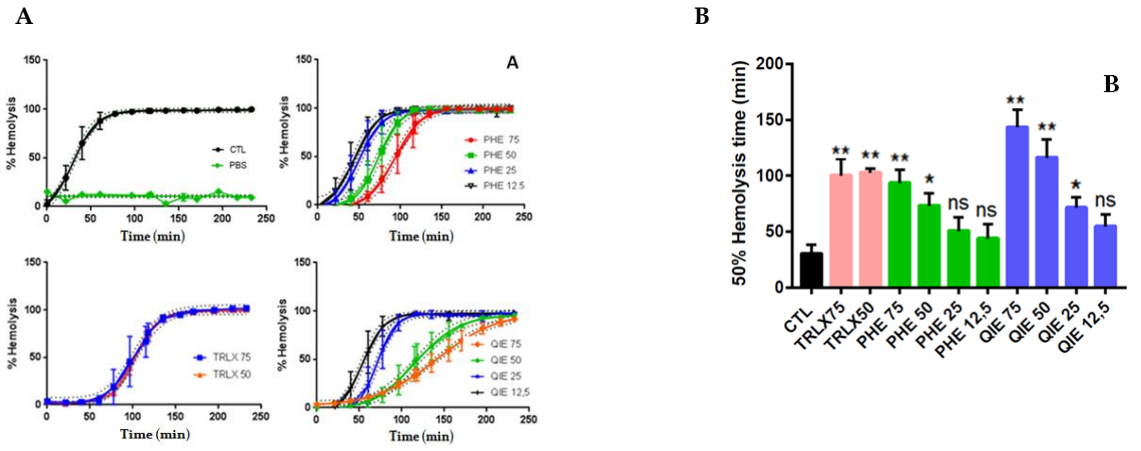

Figure 3. Inhibitory effect of *Pinus halepensis* and *Quercus ilex* on AAPH induced erythrocyte hemolysis.

Erythrocyte hemolysis was induced by peroxyl radical generated by AAPH thermal decomposition at 37°C. Mice RBC suspension (2%) was pre-incubated with different concentrations of PHE, QIE extracts (12.5, 25, 50, 75 µg/mL) or Trolox (TRLX) (50, 75 µg/mL) for 30 min. at 37°C.

(A) Kinetic of hemolysis was followed by measuring the decrease in absorbance at 630 nm. (**B)** Half hemolysis time resulting from different treatments. Control (CTL): RBC suspension with AAPH, without extracts or Trolox; PBS: RBC suspension with PBS instead of AAPH. Significance *vs.* control: p<0. 01 (**), p<0.05 (*), not significant (ns).

These results were in concordance with those obtained with DPPH and ABTS radical scavenging activity and ferric reducing antioxidant power. This concordance could be partially explained by the presence of flavonoids in both extracts and the higher polyphenolic content found in *Quercus ilex* extract that was twofold the polyphenolic content of *Pinus halepensis* extract. The protective effect of flavonoids and polyphenols on erythrocyte membrane was previously reported (Chaudhuri et al., 2007; De Freitas et al., 2008). Sohretoglu et al. (2007) reported in their study conducted on the protective effect of three different *Quercus* species, *Quercus cerris*, *Quercus macranthera* and *Quercus aucheri* against H_2O_2 induced cytotoxicity, a significant protective effect of the plants extracts at 10 and 25 µg/mL concentrations against hydrogen peroxide induced hemoglobin release from erythrocytes.

Catechin, the major phenolic compound in both studied extracts, was shown by Grzesik et al. (2018) to be a potent antihemolytic compound. It prolonged the time of hemolysis by more than 100% at a concentration of 25 µM in AAPH induced hemolysis and at concentration of 1 µM in hypochlorite induced hemolysis.

Effect of *Quercus ilex* and *Pinus halepensis* extracts on DNA damage

UV-photolysis of H_2O_2 leads to the formation of highly reactive and strong oxidizing hydroxyl radical. The OH radicals can damage all components of DNA molecules such as purine bases, pyrimidine bases and the deoxyribose backbone (Cadet et al., 2015). Hence, the native supercoiled configuration of plasmid DNA changes to open circular and nicked linear forms, which cause a change in their electrophoretic mobility properties on the gel (Yasmeen and Gupta, 2016).

Fig. 4A shows the electrophoretic pattern of DNA in the presence or absence of different concentrations (100, 250, 350 and 500 µg/mL) of *Quercus ilex* and *Pinus halepensis* extracts. The plasmid DNA showed two bands on agarose gel electrophoresis (lane 1), the faster moving prominent band corresponded to the native supercoiled circular DNA (sc DNA) and the slower moving very

faint band was the open circular form (oc DNA). The UV irradiation of DNA in the presence of H_2O_2 (lane 2) induced the cleavage of sc DNA to give prominent oc DNA and a faint linear (Lin) DNA, indicating that OH generated from UV-photolysis of H_2O_2 produced DNA strand scission. It was noted that only UV treatment and only H_2O_2 treatment (lanes 3 and 4, respectively) could not induce damage as noted in combined treatment (lane 2). The addition of the extracts (lanes 6-9) to the reaction mixture suppressed the formation of lin DNA and induced a significant protection of native supercoiled circular DNA in a dose-dependent manner. In fact, in the presence of 100, 250, 350 and 500 µg/mL of *Quercus ilex* extract, the intensity of sc DNA bands (Fig. 4B) scanned from the agarose gel electrophoretic patterns was 61.82, 69.04, 83.55 and 86.33%, respectively. The presence of 100, 250, 350 and 500 µg/mL of *Pinus halepensis* extract gave an intensity values of 89.64, 91.26, 92.53 and 94.41%, respectively as compared with the untreated plasmid DNA.

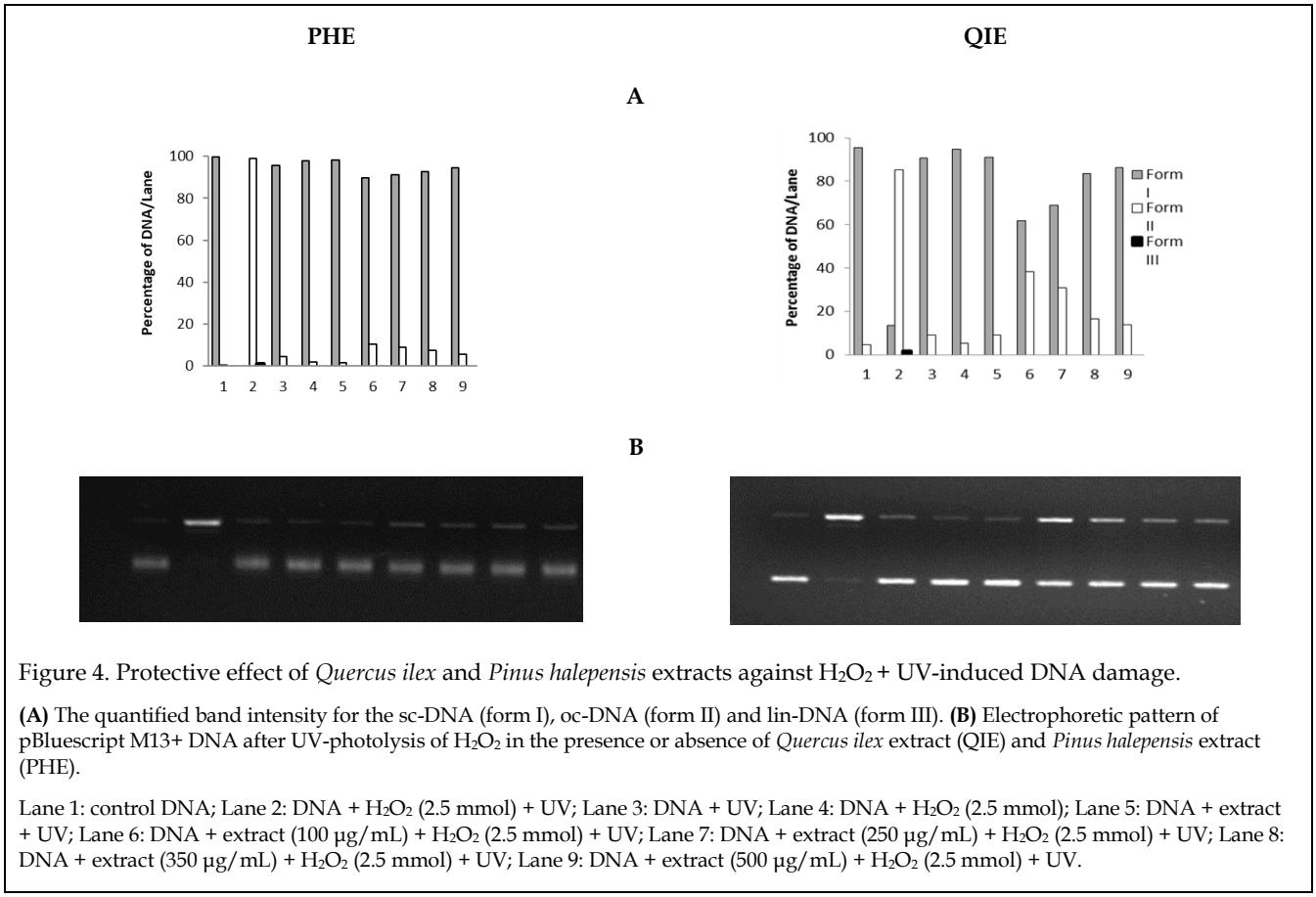

Figure 4. Protective effect of *Quercus ilex* and *Pinus halepensis* extracts against H_2O_2 + UV-induced DNA damage.

(A) The quantified band intensity for the sc-DNA (form I), oc-DNA (form II) and lin-DNA (form III). **(B)** Electrophoretic pattern of pBluescript M13+ DNA after UV-photolysis of H_2O_2 in the presence or absence of *Quercus ilex* extract (QIE) and *Pinus halepensis* extract (PHE).

Lane 1: control DNA; Lane 2: DNA + H_2O_2 (2.5 mmol) + UV; Lane 3: DNA + UV; Lane 4: DNA + H_2O_2 (2.5 mmol); Lane 5: DNA + extract + UV; Lane 6: DNA + extract (100 µg/mL) + H_2O_2 (2.5 mmol) + UV; Lane 7: DNA + extract (250 µg/mL) + H_2O_2 (2.5 mmol) + UV; Lane 8: DNA + extract (350 µg/mL) + H_2O_2 (2.5 mmol) + UV; Lane 9: DNA + extract (500 µg/mL) + H_2O_2 (2.5 mmol) + UV.

This protective activity against DNA damage could be assigned to the presence of potent antioxidants in the extracts. Phenolic compound seems to be the best candidates for this effect. Indeed, Sevgi et al. (2015) reported that phenolic acids possess protective effects on pBR322 plasmid DNA against the mutagenic and toxic effects of UV and H_2O_2. The studies of Piao et al. (2013) and George et al. (2017) affirmed that flavonoids protect DNA against oxidative damage induced by carcinogenic agent such as γ irradiation and benzo(a)pyrene in human leukemia cells (HL-60) and Chinese hamster lung fibroblasts (V79-4).

CONCLUSIONS

The results obtained from this study showed that *Quercus ilex* root bark and *Pinus halepensis*

young ovulate cone methanolic extracts exhibit significant antioxidant, antihemolytic and DNA protective effects. These effects may be due to their high content of phenolic and flavonoid contents. These results provide evidence that these plants could be potential sources of natural antioxidant agents and good candidates for future biomedical applications to promote human health with limited side effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Concepts or ideas						x
Design						x
Definition of intellectual content						x
Literature search	x					
Experimental studies	x		x	x	x	
Data acquisition	x		x	x	x	
Data analysis	x	x				
Statistical analysis	x					
Manuscript preparation	x					
Manuscript editing	x					
Manuscript review	x	x	x	x	x	x

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