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BIOTHÉRAPIE

Phytochemical Analysis, Antioxidant, and Acetylcholinesterase Inhibitory Activity of Propolis from Northeastern Algeria

Analyse phytochimique, activité antioxydante et activité inhibitrice de l'acétylcholinestérase de la propolis du Nord-Est algérien

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Abstract Propolis is a bee resinous substance consisting mainly of phenolic compounds having nutritional and therapeutic properties and formed by the mixing of the tree and plant secretions collected by honey bees. Herein, the present study was aimed to assess the antioxidant and anticholinesterase activity of ethanolic and acetone propolis extracts from two sampling sites in Souk Ahras city (northeast Algeria). The antioxidant activity of the extracts was determined by using the common antioxidant assays (1,1-diphenyl-2-picrylhydrazyl [DPPH], acide 2-2'-azino-bis (3-ethylbenzothiazoline-6-sulphonique) [ABTS], galvinoxyl radical [GOR], and cupric reducing antioxidant capacity [CUPRAC]), and the anticholinesterase activity was determined against acetylcholinesterase (AChE). Moreover, the total phenolic (TPC), flavonoid (TFC), and condensed tannins (CTC) contents were quantified. The propolis extracts showed a potent antioxidant/inhibitory activity which almost met that of synthetic antioxidants used as standards (butylated hydroxytoluene [BHT] and butylated hydroxyanisole [BHA]). In addition, the AChE activity was highly strongly inhibited in a dose-dependent manner by the propolis extracts. The propolis extracts proved their richness in bioactive molecules able to enhance various biological activities and processes.

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Keywords Antioxidant and anticholinesterase activity · Chemical composition · Propolis · Souk Ahras city (Algeria)

Résumé La propolis est une substance résineuse des abeilles, composée principalement de composés phénoliques ayant des propriétés nutritionnelles et thérapeutiques, et formée par le mélange des sécrétions des arbres et des plantes recueillies par les abeilles. Ici, la présente étude visait à évaluer l'activité antioxydante et anticholinestérase des extraits de propolis éthanolique et acétonique de deux sites d'échantillonnage dans la ville de Souk Ahras (nord-est de l'Algérie). L'activité antioxydante des extraits a été déterminée en utilisant les dosages antioxydants courants (DPPH, ABTS, GOR, et CUPRAC), et l'activité anticholinestérase a été déterminée par rapport à l'acétylcholinestérase (AChE). De plus, les teneurs totales en composés phénoliques (TPC), en flavonoïdes (TFC) et en tanins condensés (CTC) ont été quantifiées. Les extraits de propolis ont montré une puissante activité antioxydante/inhibitrice qui a presque atteint celle des antioxydants synthétiques utilisés comme standard (butylhydroxytoluène (BHT) et butylhydroxyanisole (BHA). De plus, l'activité AChE a été très fortement inhibée de manière dose-dépendante par les extraits de propolis. Les extraits de propolis ont prouvé leur richesse en molécules bioactives capables d'améliorer diverses activités et processus biologiques.

Mots clés Activité antioxydante et anticholinestérasique · Composition chimique · Propolis · Ville de Souk Ahras (Algérie)

Introduction

Recently, the developed industries producing cosmetic and pharmaceutical products derived from nature are of great interest to the human population to maintain safely their

health and beauty. Hence, alternative medicine, a treatment form, as well as cosmetic natural products are widely and frequently used by patients and the general population due to their less hazards and side effects. Furthermore, ethnopharmacology provides a valuable source of the new determined natural bioactive molecules, and subsequently, the natural products can be classified following their therapeutic values [1]. In this regard, the major therapeutic agents approved and developed between 1981 and 2010 were of natural origin [2]. The natural products that have been traditionally applied for therapeutic benefits have potential biological and antioxidant activities owed to the presence of bioactive molecules, in particular secondary metabolites [3,4]. Interestingly, the natural compounds derived from herbs, plants, and essential oils have been, in recent years, largely used as antimicrobial additives in food production and preservation [5]. Furthermore, bees have been commonly cultivated by the human for centuries due to their crucial role in agriculture production and plant pollination, resulting in crop diversity [6], in addition to their therapeutic and nutritional products, including mainly honey, royal jelly, wax, and venom [6,7]. Accordingly, the use of bee products in treating various health conditions is known as apitherapy, where worldwide researchers, in the last few years, have paid great attention to developing these natural compounds as effective alternative drugs [8]. On the contrary, propolis is a resinous natural bee product and whose name is derived from the Greek words ("Pro" means "in front of" and "Polis" means "community" or "city") [9]. It can be built by bees as a clingy substance by mixing the sap with their salivary releases and beeswax, which consequently serves as a strong adhesive material [10,11] used to block holes and cracks, embalm dead invaders, and cover the internal walls of the hive [12], as well as the propolis antimicrobial activity ensure the protection of the honey bee colony against infections and parasites [10]. Propolis is composed of a wide range of active natural molecules, including primarily and especially phenolic acids and flavonoids, followed by esters, terpenes, aromatic aldehydes, alcohols, fatty acids, steroids, vitamins, and minerals, and noticeably, the propolis chemical composition varies depending on the collection sites [13]. Generally, raw propolis typically consists of nearly 50% resinous substances, 30% waxes, 10% essential oils, 5% pollen, and 5% minor substances (impurities, amino acids, soils, and dead bees) [14]. As a result, the therapeutic potential of propolis effectively depends on the synergetic effects of its bioactive molecules [15], and hence, it was applied as a worldwide remedy in folk medicine to treat principally wounds, skin burns, sore throat, and stomach ulcer [16]. Recent previous studies proved the efficient antioxidant, antimicrobial, anti-inflammatory, antitumor, and anti-obesity properties of propolis (bee glue) [17]. Accordingly, propolis was involved in producing many commercialized products, such as medical devices, healthy foods and beverages, and cosmetics intended for consumers [18]. Importantly, chemicals induced oxidative stress leading to the generation of reactive oxygen species (ROS) damaging the cell membrane and DNA [19], and consequently causing various pathologies, including cancers [20] and cardiovascular and neurological diseases [21] can be effectively attenuated by exogenous synthetic and natural antioxidants [22]. In addition, Alzheimer's disease, the common ROS consequence, can be treated by using acetylcholinesterase (AChE) inhibitor drugs (an abundant neurotransmitter in the brain) [23]. Also, Alzheimer's development can be inhibited by using some synthetic active substances, like galanthamine (alkaloid) [24], and because of their serious side effects, the development of safe drugs would be a priority target for future drug discovery [23]. Thus, several researchers over the world paid attention to optimizing pharmaceutical production by using natural antioxidants instead of synthetic antioxidants to ensure less side effects on human health. Among the most natural bioactive molecules, phenolic compounds have a significant ability to acquire resistance to environmental stress [25], and interestingly, flavonoids and phenolic acids are the most important groups of secondary metabolites, and sources of natural antioxidants and anticholinesterase activities in human diets [24]. Taken together, the present work was the first to analyze the phytochemical composition and to evaluate in vitro the antioxidant and anticholinesterase activity of bee propolis from the Souk Ahras region (northeast Algeria).

Materials and methods

Choice of area and harvest

The raw propolis of *Apis mellifera* was collected by the grid (propolis collectors) method between July and September 2019 from beehives in different sites (Bendada and Lakhdara) in Souk Ahras city (northeast Algeria). The raw propolis sample of each sampling site was gathered, cleaned, and packed into hermetically closed plastic bags against light, and subsequently stored in the freezer at −18°C until use.

Preparation of propolis extracts

The raw propolis was beforehand ground into a granulated powder using a food grinder and then the extracts were prepared by simple maceration. Here, 10 g of two powder samples was extracted with 100 ml of two organic solvents (ethanol and acetone 70%) for 72 h with stirring in a dark bottle at room temperature. The process was performed a minimum of three times. The extracts were afterward filtrated using Whatman No. 1 filter paper, and the filtrate was concentrated and evaporated under pressure in a rotary evaporator at 40°C

to result in four extracts (two ethanol extracts and two acetone extracts). Of note, the crude extracts were stored at 4°C until analysis.

Determination of the yield percentage of the propolis extracts

The yield of the extracted raw propolis material was determined as previously described [26] based on the percentage of the obtained extract mass and the initial propolis mass before extraction. The percentage yields of the obtained material after extraction and removal of solvent were determined according to the following equation:

Yield (%) = EM/IM × 100,

where EM stands for the extract mass (g) and IM for the initial mass (g).

Phytochemical analysis

The secondary metabolites, including steroids, flavonoids, saponins, tannins, alkaloids, quinones, and carotenoids, present in the crude propolis extracts were analyzed (E1E, E1A, E2E, and E2A) using diverse analytical methods.

Total phenolic content (TPC)

The TPC of the propolis samples was spectrophotometrically assessed as previously described [27] with some modifications [28,29] using the Folin–Ciocalteu reagent (FCR) based on the quantification of the total concentration of hydroxyl groups present in the extracts [30]. In brief, 10 mg of the extracts was dissolved in 10 ml of methanol to result in a concentration of 1 mg ml^{-1}, and then 20 µl of the extracts (1 mg ml^{-1}) was mixed with 100 µl of FCR (1:10 in distilled water) and 75 µl of sodium carbonate solution (7.5%) in 96-well microplates. After 2 h of incubation time in the dark at room temperature, the absorbance was measured using a microplate reader (EnSpire®, PerkinElmer, Inc., Massachusetts, USA) at 765 nm against blank. The tests were applied in triplicate. The optical density (OD) of a known gallic acid concentration as a standard served to draw the standard calibration curve (10–100 µg ml^{-1} [$R >$ 0.99]) promoting to determine the TPC in µg gallic acid equivalents mg^{-1} dry extract weight (µg GAE mg^{-1} extract).

Total flavonoid content (TFC)

The TFC was quantified according to a method described elsewhere [30]. Aluminum and the oxygen atoms present on carbons 4 and 5 of the flavonoids form a stable complex. In this method, a volume of 50 µl of the extracts (1 mg ml^{-1} in methanol) was mixed with 130 µl of MeOH, then 10 µl of potassium acetate and 10 µl aluminum nitrate were added to the mixture. After incubation for 40 min at room temperature, 96-well microplate readers (Perkin Elmer, EnSpire®) were used to measure the absorbance at 415 nm. All samples were performed in triplicate. The TFC was determined using the quercetin calibration curve with the linearity range of 25 to 200 µg ml^{-1} ($R > 0.99$). Results were expressed as µg quercetin equivalents per milligram dry extract weight (µg QE mg^{-1} extract) [29].

Condensed tannins content (CTC)

The CTC in the propolis extracts was quantified as previously reported [31,32] using the FCR method. In brief, a mixture composed of 10 µl of Folin solution (0.5 N) and 50 µl of each tested extract was agitated, incubated for 15 min, and then mixed with 250 µl of Na_2CO_3. After 30 min of incubation time in the dark at room temperature, the absorbance of each sample was measured at 760 nm using a DR 2800 HACH LANGE brand spectrophotometer with a tube-shaped tank of 5 ml. Of note, tannic acid was used as a standard to draw the calibration curve enabling to determine the content of condensed tannins in propolis extracts. Tannins content was expressed as micrograms of tannic acid equivalent per milligram of extract (µg TAE mg^{-1}).

Antioxidant activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay

The anti-free-radical activity was spectrophotometrically determined by assaying DPPH assay, as previously described [33]. In this assay, 40 µl of various concentrations of MeOH sample solution was mixed with 160 µl DPPH (0.1 mM) and then shaken vigorously. The absorbance of remaining DPPH was measured at 517 nm after 30 min of incubation time in the dark in a 96-well microplate reader (EnSpire® Multimode Plate Reader, PerkinElmer, Inc., Massachusetts, USA) at room temperature. The butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) were used as antioxidant comparative standards. The DPPH radical scavenging activity is calculated using the following equation, and the results are displayed as 50% inhibition concentration (IC50) value (µg ml^{-1}):

DPPH scavenging effect (%) = AControl − ASample/AControl × 100,

where AControl and ASample are the absorbances of the control and the test sample, respectively.

ABTS radical cation decolorization assay

The ABTS radical cation assay was applied, as described elsewhere [34], with slight modifications to evaluate the

free-radical-scavenging capacity of propolis samples. Briefly, the oxidation solution of ABTS was prepared by reacting with potassium persulfate (final concentration: 2.45 mM), kept in the dark at room temperature for 16 h before use, and the mixture was then diluted in water to get an absorbance of 0.700 ± 0.020 at 734 nm. After that, 40 µl of the sample solution in different concentrations of methanol (6.25, 12.5, 25, 50, 100, 200, and 400 µg ml^{-1}) was added to 160 µl of the diluted ABTS solution in 96-well microplates. The test was performed in triplicate. The mixture was homogenized, and the absorbance was read at 734 nm after 10 min using a 96-well microplate reader against blank (methanol). Of note, the percentage of inhibition (IC50, µg ml^{-1}) of each concentration can be determined using the below-mentioned equation, and the antioxidant activity of the extracts was compared with that of the positive controls (BHA and BHT):

Scavenging effect of ABTS (I) (%) = (AControl − ASample)/AControl × 100,

where AControl is the initial ABTS concentration, and ASample is the ABTS absorbance present in the sample.

Cupric reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay enabling to assess the reduction of Cu^{2+} ions was performed according to the method previously reported [35] with slight modifications. In this method, each well of a 96-well plate was filled with a mixture composed of 50 µl of copper (II) chloride (10 mM), 50 µl of neocuproine alcoholic solution (7.5 mM), and 60 µl of ammonium acetate buffer solution (1 M, pH = 7.0), whereas the initial mixture was formed by mixing 40 µl of different concentrations of extracts and standards, and then adjusted to the final volume of 200 µl. The resulting mixture was shielded from light at room temperature. After 60 min, the absorbance was recorded at 450 nm against a blank reagent by using a 96-well microplate reader. The results were displayed as A0.5 (µg ml^{-1}) corresponding to a concentration indicating 0.50 absorbance intensity, and the reduction capacity of the extracts was compared with BHA and BHT used as antioxidant standards [19].

Galvinoxyl free-radical-scavenging (GOR) activity

The galvinoxyl radical's trapping activity was evaluated as described elsewhere [36]. Briefly, a mixture composed of 40 µl of the different concentrations (extracts or standards) and 160 µl of methanolic galvinoxyl solution (0.1 mM; 4 mg of galvinoxyl in 100 ml of methanol) was added to each well of the 96-well microplates. After incubation time in light-free for 2 h at room temperature, the absorbance was measured at a wavelength of 428 nm. A galvinoxyl solution in methanol was used as a control. Experiments were performed in triplicate. The percentage of inhibition of galvinoxyl is determined by the following formula:

Galvinoxyl scavenging effect (%) = (Ac − As)/Ac × 100,

where Ac refers to the absorbance of the control, and As refers to the sample absorbance.

The results were expressed as IC50, which was determined by matching the inhibition percentages to the extract concentrations. The antioxidant capacity of the extract was compared with that of the BHT and BHA standards [37].

Determination of AChE inhibitory activity

The AChE inhibitory activity was determined by the spectrophotometric method, as previously reported [38], with slight modifications. In brief, 150 µl of 100 mM sodium phosphate buffer (pH 8.0), 10 µl of sample solution dissolved in methanol at various concentrations, and 20 µl of AChE (5.32×10^{-3} U) solution were mixed and incubated at 25°C for 15 min before adding 10 µl of 0.5 mM DTNB (5,5-dithio-bis(2-nitrobenzoic)) acid. The reaction was then initiated by the addition of 10 µl of acetylthiocholine iodide (0.71 mM). The hydrolysis of these substrates was monitored spectrophotometrically through the reaction of DTNB with thiocholine (hydrolyzed product of acetylthiocholine iodide) resulting in yellow 5-thio-2-nitrobenzoate anion. The absorbance was recorded at a wavelength of 412 nm every 5 min for 15 min using a 96-well microplate reader (Perkin Elmer Multimode Plate Reader, EnSpire®). Experiments were performed in triplicate experiments, and galantamine was used as a reference compound. The results were displayed as IC50, and the AChE inhibition percentage was determined by comparison of sample reaction rates relative to blank samples (methanol in phosphate buffer, pH 8) using the following formula:

Inhibition of AChE (%) = $E - S \times 100$,

where E is the activity of the enzyme without a test sample, and S is the activity of the enzyme with a test sample [24].

Results

The yield

The yield of propolis extracts was estimated using 10 g of propolis powder, and the results are displayed as a percentage. As indicated in Table 1, the different extract solvents revealed different yields. Also, the maximum dry matter yield of propolis after the extraction process was usually observed in Sample 2 (E2E, 31.29% and E2A, 34.54%). Meanwhile, the extraction solvents of increasing polarity revealed the highest yield of the propolis samples (20.07% and 34.54%, respectively) in acetone.

Content of the bioactive molecules

The chemical compositions varied differently in the propolis samples. The TPC of the extracts was estimated according to the calibration curve prepared from gallic acid ($y = 0.0034x + 0.1044$, $R^2 = 0.997$), and the TFC was estimated following the prepared standard curve of quercetin ($y = 0.0048x$, $R^2 = 0.997$). As seen in Table 1, the levels of phenolic compounds were higher in Sample 2 of propolis extracts (E2A = 211.06 ± 1.47 µg GAE mg^{-1}, E2E = 188.51 ± 1.03 µg GAE mg^{-1} of propolis extract) and slightly lower in Sample 1 (E1A = 152.23 ± 0.59 µg GAE mg^{-1}, E1E = 131.45 ± 0.68 µg GAE mg^{-1}) than those of the standards, but the acetone extract of the two samples has the highest content of phenolic compounds. In addition, flavonoids and condensed tannins were present in large quantities and at very close values in all propolis extracts, where the flavonoids content ranges from 85.83 ± 0.29 to 91.18 ± 0.12 µg QE mg^{-1} of extract, and the tannins content is between 79.34 ± 0.21 and 83.87 ± 0.09 µg TAE mg^{-1} of extract.

Antioxidant activities

IC50 and A0.5 were calculated by linear regression analysis and expressed as mean ± SD ($N = 3$). Analysis of variance (ANOVA) revealed a highly significant effect ($P < 0.001$) in the antioxidant activity of the propolis extracts assayed by DPPH, ABTS, GOR, and CUPRAC.

The antioxidant activity of propolis extract samples evaluated by DPPH, ABTS, GOR, and CUPRAC assays showed higher radical scavenging activity as evidenced by IC50 or lower A0.5. In addition, all extracts, in particular the ethanol fractions, revealed significant antioxidant activity to varying degrees, and hence IC50 and A0.5 values were found to be significantly ($P < 0.05$) varied in a function of the extract fraction. The used antioxidant assays showed the highest antioxidant activity in propolis extracts from Sample 2, especially the ethanolic extract E2E (Table 2). Furthermore, the DPPH free-radical-scavenging activity of the E2E and E2A extracts showed higher scavenging capacity than that of BHA (5.73 ± 0.41 µg ml^{-1}) and the other fractions with IC50 values equal, respectively, 3.49 ± 0.04 and 3.93 ± 0.03 µg ml^{-1} of extract. Here, the E1E and E1A extracts showed a greater inhibitory potential than that of BHT (22.32 ± 1.19 µg ml^{-1}) with IC50 = 10.61 ± 0.07 µg ml^{-1} and IC50 = 10.92 ± 0.07 µg ml^{-1}, respectively, but this activity is relatively lower compared to BHA. In parallel, the ABTS assay demonstrated high antioxidant power, with IC50 values close to those of the BHA (IC50 = 1.81 ± 0.10 µg ml^{-1}) and BHT (IC50 = 1.29 ± 0.30 µg ml^{-1}), primarily in the E2E and E2A fractions, with IC50: 3.11 ± 0.03 µg ml^{-1} and IC50: 3.30 ± 0.01 µg ml^{-1} respectively. Similarly, the antioxidant activity assayed by GOR showed remarkable antioxidant activity compared to the standards, where the highest IC50 value was noticed in the E2E (16.08 ± 0.03 µg ml^{-1}) and E2A (16.40 ± 0.01 µg ml^{-1}) extracts, and a moderate antioxidant activity whose IC50 values were, respectively, 32.89 ± 0.01 and 33.96 ± 0.04 µg ml^{-1} in E1E and E1A extracts; however, all these values remain higher than those of the BHA and BHT standards (IC50 = 5.38 ± 0.06 and 3.32 ± 0.18 µg ml^{-1}, respectively). Furthermore, the CUPRAC test showed that all four extract fractions effectively reduced copper ions, and so, E2E extract was found to be the best in Cu^{2+} reduction with A0.5 equals 4.63 ± 0.06 µg ml^{-1} and is similar to that of BHA (3.64 ± 0.19 µg ml^{-1}) followed by the E2A fraction with A0.5 = 7.97 ± 0.02 µg ml^{-1} which is very close to the BHT value (9.62 ± 0.87 µg ml^{-1}). None of the E1E and E1A extracts

Table 1 Physical characteristics, yields, and chemical composition of ethanol and acetone propolis extracts

Sample name	Extract	Solvent	Aspect	Color	Yield (%)	TPC (µg GAE mg^{-1})	TFC (µg QE mg^{-1})	CTC (µg TAE mg^{-1})
Sample 1	E1E	Ethanol	Solid sticky	Yellowish brown	18.32	131.45 ± 0.68	85.83 ± 0.29	79.34 ± 0.21
	E1A	Acetone	Solid sticky	Yellowish brown	20.07	152.23 ± 0.59	89.72 ± 0.12	80.34 ± 0.23
Sample 2	E2E	Ethanol	Solid sticky	Dark brown	31.29	188.51 ± 1.03	88.12 ± 0.14	82.25 ± 0.31
	E2A	Acetone	Solid sticky	Dark brown	34.54	211.06 ± 1.47	91.18 ± 0.12	83.87 ± 0.09

E1E: Ethanolic Extract 1; E1A: Acetonic Extract 1; E2E: Ethanolic Extract 2; E2A: Acetonic Extract 2; TPC: total phenolic content; TFC: total flavonoid content; CTC: condensed tannin content

Results are expressed as mean ± SD ($N = 3$)

The values of TPC, TFC, and CTC are not significantly different ($P > 0.05$)

(A0.5 = 11.86 ± 0.06 and 12.67 ± 0.02 µg ml^{-1}, respectively) showed higher antioxidant activity than that of the BHA and BHT standards (Table 2).

AChE inhibitory activity

The AChE inhibitory capacity of propolis extracts (expressed as percentages inhibition at different final concentrations and IC50 values), where galanthamine was used as a positive control, showed a high percentage inhibition, with values up to 84.28% inhibition at 100 and 200 µg ml^{-1}. Also, the E2E extract revealed higher AChE inhibitory activity followed by the E2A extract with IC50 values of 10.00 ± 0.07 and 11.38 ± 0.06 µg ml^{-1}, respectively. Conclusively, this activity is very interesting compared to galantamine, showing better inhibitory activity against AChE (IC50 = 6.27 ± 1.15 µg ml^{-1}), while the extracts of Sample 1 were inactive (Table 3).

Discussion

This study is the first to investigate the extraction yields, bioactive compounds, antioxidant potential, and effect on AChE inhibition of propolis extracts from Souk Ahras city (Northeastern Algeria). Researchers stated that the healing activities of propolis have been identified by Roman and Greek physicians as well as other scientists, such as Dioscorides, Galen, Aristotle, and Pliny [9]. Moreover, the chemical composition of propolis varies in function of phytogeographic characteristics [39] and the flora of the collection site [40]. The plant type of different habitats is strongly

Table 2 Antioxidant potential of the propolis extracts using different antioxidant assays

Extracts	IC50			A0.5
	DPPH assay (µg ml^{-1})	ABTS assay (µg ml^{-1})	GOR assay (µg ml^{-1})	CUPRAC assay (µg ml^{-1})
E1E	10.61 ± 0.07	7.05 ± 0.04	32.89 ± 0.01	11.86 ± 0.06
E1A	10.92 ± 0.07	8.87 ± 0.03	33.96 ± 0.04	12.67 ± 0.02
E2E	3.49 ± 0.04	3.11 ± 0.03	16.08 ± 0.03	4.63 ± 0.06
E2A	3.93 ± 0.03	3.30 ± 0.01	16.40 ± 0.01	7.97 ± 0.02
BHT	22.32 ± 1.19	1.29 ± 0.30	3.32 ± 0.18	9.62 ± 0.87
BHA	5.73 ± 0.41	1.81 ± 0.10	5.38 ± 0.06	3.64 ± 0.19

IC50: 50% inhibition concentration; DPPH: 1,1-diphenyl-2-picrylhydrazyl; CUPRAC: cupric reducing antioxidant capacity; ABTS: acide 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonique); GOR: galvinoxyl radical; E1E: Ethanolic Extract 1; E1A: Acetonic Extract 1; E2E: Ethanolic Extract 2; E2A: Acetonic Extract 2; ANOVA: analysis of variance. Reference compound: BHT: butylated hydroxytoluene; BHA: butylated hydroxyanisole

IC50 and A0.5 values are defined as the concentration of 50% inhibition percentages and the concentration at 0.50 absorbance, respectively. IC50 and A0.5 were calculated by linear regression analysis and expressed as mean ± SD ($N = 3$)

ANOVA revealed a highly significant effect ($P < 0.001$) in all antioxidant activities

Table 3 Inhibitory AChE activity of propolis extracts

Extracts	AChE (% inhibition)							IC50 µg ml^{-1}
	3.125 µg	6.25 µg	12.5 µg	25 µg	50 µg	100 µg	200 µg	
E1E	na	na	na	na	na	na	na	-
E1A	na	na	na	na	na	na	na	-
E2E	na	34.78 ± 0.84	49.94 ± 0.00	59.59 ± 0.04	69.66 ± 0.08	78.65 ± 0.27	84.28 ± 0.12	10.00 ± 0.07
E2A	33.12 ± 4.45	33.91 ± 1.59	49.78 ± 0.08	58.19 ± 0.00	69.55 ± 0.12	68.66 ± 0.20	76.61 ± 0.15	11.38 ± 0.06
Galantamine[a]	35.93 ± 2.28	43.77 ± 0.00	68.50 ± 0.31	80.69 ± 0.41	85.78 ± 1.63	91.80 ± 0.20	94.77 ± 0.34	6.27 ± 1.15

AChE: acetylcholinesterase; IC50: 50% inhibition concentration; E1E: Ethanolic Extract 1; E1A: Acetonic Extract 1; E2E: Ethanolic Extract 2; E2A: Acetonic Extract 2; na: not absorbance

Values are displayed as mean ± SD. Measurements were performed three times

[a] Reference compounds

responsible for the diversity of the chemical and biological properties of propolis synthesized by bees. In this regard, Algeria is characterized by diversified ecosystems including coastal, desert, and mountain zones resulting in a variety of propolis [39]. Thus, propolis samples from the Souk Ahras city may have different chemical compositions. As reported, propolis of this region consists mainly of lipophilic compounds [41]. As the solubility of propolis in water is low, it must be purified by extraction using organic solvents [42]. This is likely due to the hydroxyl group, which turns water into a poor solvent for many organic compounds [43]. Propolis can be effectively extracted by ethanol, methanol, chloroform, ether, acetone, and preferably ethanol providing low-wax propolis extracts rich in bioactive compounds [42]. Overall, propolis can be better extracted by polar solvents which consequently enhance the antioxidant properties of extracts compared to nonpolar solvents and lead to decreased extraction yields [41]. Therefore, in the present study on the chemical and biological characteristics of propolis from the city of Souk Ahras, ethanol and acetone (70%) were used as propolis extraction solvents. The obtained results referred to the different biological properties of each extract to the different components present in each. The two propolis extracts, compared to those from the Bendada region (downtown), revealed samples of the highest number of active components. In this study, the extraction yields of the propolis extracts vary between 18% and 35%, and this result differs from that in a previously reported study [44], where the yield was between 5.4% and 74.30%, and for the cold extraction of propolis collected from different regions of Algeria (Mountain, Plain, Sahara), the yield was found to be higher with 70% ethanol because the extraction rate was from 64.8% to 74.30%. This percentage is higher than that obtained in our case study in Algeria (18.32%–34.54%). These results prove that the extraction rate changes from one sample to another from the same region, which can be explained by the variations of the geographical origins. On the contrary, our yield values are lower than those reported in a previous study [45] conducted on French propolis, and reported a yield of 64.9% with EtOH (70%) and 66.4% with absolute methanol. Another previous study conducted on three Polish regions reported significant extraction rates of P1 = 33.44%, P2 = 57.5%, and P3 = 63.7% [46]. Our finding of the propolis yield was close to that reported in a previous study [39] conducted on propolis collected from four different regions of the Sétif city (northeast Algeria), where the propolis yields varied from 28.24% ± 0.22% to 40.73% ± 0.21% for the ethanolic extracts. Similar extraction yields (18.33% ± 1.82%) were previously reported in a study conducted on ethanolic extraction of Indonesian propolis [47,48], in addition to that conducted on three propolis extracts from the region of South Sulawesi city of Indonesia, showing, respectively, yield of 17.06% ± 0.23%, 18.03% ± 0.38%, and 20.21% ± 0.14% [49], whereas a lower yield (12.12%) value than ours was previously reported [43] and also another study conducted on propolis ethanolic extraction from Eastern Canada by Soxhlet (SE), microwave-assisted (MAE), and ultrasound-assisted (UAE) extraction methods showed different yields as ours (38.72%, 17.52%, and 14.74%) [41]. In the present study, the acetone extract produced higher yield values than other solvents (20.07% and 34.54% for samples N1 and N2, respectively), but this yield is lower than that of the Algerian propolis yield of 39.5% to 51.10% [44]. This could be due to the extraction methods and the differences in the propolis origin [50,51]. In addition, three different yield values to ours (32.28%, 16.5%, and 29.32%) were reported in propolis extracted, respectively, by SE, MAE, and UAE methods using acetone as the solvent. Furthermore, the TPC of the tested samples varied between 131.45 ± 0.68 and 211.06 ± 1.47 mg GAE/g extract. Based on the region of propolis, the highest TPC was observed in Sample 2 from the Bendada (downtown) region, while the lowest was observed in Sample 1 from the region of Lakhdara. Previous [44,52] and more recent studies [53] have reported phenol levels in propolis extract from Algeria as 11.53 ± 0.60 and 600 ± 0.017 mg GAE g^{-1}. Our results regarding the content of total phenolic and total flavonoid compounds are consistent with those previously reported [54,55] investigating the chemical composition of propolis from France, Turkey, Brazil, and Argentina, although the values differ slightly from the conclusions of Socha et al. [7] and Wezgowiec et al. [46] who measured the TPC of several Polish propolis samples. However, some previous studies [49,56] have found very high amounts of phenolic compounds in propolis samples from various regions of Guanajuato in Mexico and the region of the South Sulawesi city in Indonesia. An extremely high content of phenolic compounds was reported in a study conducted on Malaysian propolis (646.30 ± 30.44 mg g^{-1}) [43], and noteworthy this variation was higher than the amounts reported in other countries. Slight narrow variations in the content of polyphenols were found in propolis extracts from the Northeast Region of Brazil and Bangladesh [57–59], and also very low amounts of total phenol were reported in propolis from Eastern Canada, Lithuania, and Marechal Deodoro city, State of Alagoas, Brazil [41,57,60]. As the essential polyphenolic compounds inhibiting the antioxidant activity in propolis extracts are flavonoids, their content in the tested propolis samples was found between 85.83 and 91.18 mg QE g^{-1} (Table 1). The determined range of the TFC in propolis extracts was found to be relatively narrow and included in a very wide range reported in the literature. A similar range of TFC in red propolis from Brazil was previously determined [48]. Previous studies have reported that flavonoids content differed depending on the propolis origin, and in parallel, the study conducted on Algerian propolis

showed values ranging from 61 to 133 mg QE g^{-1} [39], and that conducted on propolis of Algeria and France [45,52] reported maximum values of 69 mg of flavonoids per gram. A relatively wide range of TFC (3.33–194 mg g^{-1}) in propolis from various regions of Algeria has also been investigated [44]. Our findings showed a higher amount of flavonoids than that reported in some previous studies [7,41,54,55,58] and very low values were recorded in another previous study [40]. The variation in flavonoids content found in the propolis from Indonesia was greater (791 mg QE g^{-1}) than that of propolis from Mexico (379 mg g^{-1}) and Turkey (292 mg QE g^{-1}) [10,49,56]. These results showed a high content of flavonoids in the propolis collected from the city of Souk Ahras. As the acetone (polar solvent) extraction proved to be the best extraction solvent in terms of overall yield and phenolic content, a great interest was paid to adoption as the benchmark and 100% extraction solvent of phenolic compounds. Furthermore, tannins, water-soluble phenolic compounds, have significant astringent, antimicrobial, and antioxidant properties [61]; provide specific color to propolis; and have higher content in dark-colored propolis [62]. Table 1 showed similar tannins content in both samples of propolis extracts because the highest tannins content was noticed in Sample 2. It was reported that the tannins content can be higher in dark-colored propolis [62]. Hence, the tendency to collect propolis which is likely to vary depending on the needs of the hive was reported to be effective on the difference in tannins content. Thus, the increase in tannins content can be related to the vegetation where during the post-honey production season, the propolis collection activity changes from lower herbaceous sources to higher woody sources. In this study, the propolis from the Souk Ahras city was proved to be a very good source of tannins (Table 1). In this context, previous studies conducted on different propolis extracts, including that of Brazil propolis [63], showed higher levels of tannins compared to our result, and tannins levels varying between 41.38 and 54.39 mg g^{-1} were previously reported [62]. Moreover, the antioxidant activity of propolis extracts was evaluated by four different methods based on different action mechanisms to evidence the composition of extracts acting by various mechanisms, such as the prevention of the chain initiation, the connection of the transition metal catalysts, the decomposition of peroxides, the prevention of continuous abstraction of hydrogen, reductive capacity, and trapping of radicals [64]. Our results showed potent and diverse antioxidant activity in all propolis extracts with significant variations ($P < 0.05$), indicating the highest antioxidant potential in propolis extract of Sample 2 assayed by DPPH, ABTS, GOR, and CUPRAC methods. The scavenging activity values toward DPPH ranged from 3.49 to 10.92 ± 0.07 μg ml^{-1}, while those toward ABTS were from 3.11 to 8.87 μg ml^{-1}. The radical scavenging activity of galvinoxyl was between 16.08 and 33.96 μg ml^{-1}, and the reducing antioxidant capacity of cupric was between 4.63 and 12.67 μg ml^{-1}. Sample 2 of propolis extract revealed the highest scavenging activity and reducing power, which was associated with the highest TPC and TFC (Table 1). In addition, ethanol propolis extracts have exhibited a strong antioxidant activity by scavenging free radicals, free-radical scavenging of cations, and reducing activity. Also, results indicated that the extract with the greatest antioxidant capacity was composed of the highest amounts of flavonoids and phenols (the acetone extract; Table 1), and this is in line with those previously reported. Although the antioxidant properties of propolis are related to the diversity of geographical areas, plant type, time of year, and type of bee, only some of them have excellent antioxidant quality [56]. Differences in free-radical-scavenging activity between samples may result from different free-radical-quenching mechanisms as well as differences in the chemical composition of propolis extracts. The various compounds present in propolis were found to exhibit various free-radical-scavenging activities [65], and this may be related to the method of analysis [66]. A previous study [58] reported low values of DPPH (4664 ± 68.01 μmol Trolox g^{-1}) in the ethanolic extract of Brazilian red propolis, and this value was lower than that found in propolis extracts grown in France (1650 μmol Trolox g^{-1}) [45] and Turkey (1370 μmol Trolox g^{-1}) [10]. Likewise, low values of the percentage of DPPH activity ranging from 39 to 186 μmol Trolox g^{-1} were reported in Venezuelan propolis [40] and 1.9 μmol Trolox g^{-1} in Argentinian propolis. Indeed, the antioxidant activity of the studied propolis extracts assayed by the DPPH test was superior to that of propolis from Eastern Canada, Poland, Indonesia, and Bangladesh [15,41,46,59]. It was reported that the western Algerian propolis whose IC50 = 19.95 mg ml^{-1} has lower scavenging antioxidant activity [67] because average activities were previously found in various propolis extracts [49,54,57]. Unlike these, our results are in line with those reported in the previous studies investigating the antioxidant activity of various propolis extracts, including the study reporting [56] similar results to those of BHA (standard) and that reporting inhibition percentages similar to those of BHT [68], in addition to the study conducted on Brazilian propolis and reported an IC50 value of 8.01 μg ml^{-1} [60]. Furthermore, the antioxidant activity of the propolis extracts assayed by the ABTS test revealed very potent antioxidant activity, similar to that previously reported [7]. It has been previously found that the antioxidant inhibition percentages of Brazilian propolis extracts using the ABTS test are superior to 50% [69] and the ABTS antioxidant activity equals 3.1 μmol Trolox g^{-1} of propolis extract [55], and conclusively, the ABTS antioxidant activity was found to be ranged from 52 to 420 μmol Trolox g^{-1} [40]. On top of that, CUPRAC and GOR assays showed significant antioxidant

activity in the studied propolis extracts, while a much higher activity was observed in the ethanolic extracts. The antioxidant activity of Turkish propolis assayed by CUPRAC was reported to be between 2461.6 ± 278 and 8580.3 ± 2.34 mg Trolox 100 g^{-1} of extract [10]. Also, it showed low reducing power activity in the propolis of Bendada (downtown; Table 2). Due to being strong electron or hydrogen donors, the phenolic compounds, flavonoids, and vitamins in propolis have the potential to neutralize free radicals, convert copper to copper forms, and consequently lead to greater reduced activity. Accordingly, this propolis may have great relevance in the prevention and control of disorders where reactive nitrogen species and ROS are believed to play a key role in their pathogenesis. Furthermore, the anticholinesterase activity of different propolis extracts against AChE was potential in propolis extracts of Sample 2, despite its low content of phenolic compounds. This interesting inhibitory effect against AChE explains the presence of inhibitory molecules. By comparison with some previous studies investigating the activity of propolis AChE, propolis from the Souk Ahras city seems to have the best ability to inhibit the target enzyme than that reported in a study conducted on propolis of Constantine city (northeast Algeria) [53], showing IC50 values = 81.21 ± 6.06 μg ml^{-1} for AChE; meanwhile, those conducted on Moroccan [70] and Turkish [71] propolis revealed IC50 values ranging from 0.085 ± 0.006 to 0.743 ± 0.006 mg ml^{-1} and 0.081 ± 0.009 to 1.053 ± 0.016 mg ml^{-1}, respectively.

Conclusion

In view of the results obtained, propolis extracts from the Souk Ahras city of Algeria showed antioxidant activity with strong anticholinesterase activity by inhibiting key enzymes involved in neurodegenerative diseases. These results proved that propolis is a promising valuable alternative natural source of synthetic antioxidants and a potential source of dual-action molecules involved in the development of new drugs or nutrients for the management of Alzheimer's disease. All these effects can be attributed to the high polyphenol content in the natural product. As the antioxidant activity effectiveness of propolis has been well investigated, the present study as well as the most recent studies were devoted to highlighting the neuroprotective properties and the physiological health benefits of propolis. Thus, it is highly recommended to isolate and identify the propolis bioactive compounds, using especially a chromatographic analysis. Also, propolis should be widely cultivated in Algeria due to its bioactive molecules that can be used as drugs and as a treatment for Alzheimer's disease, but their toxic effects must be tested to ensure the safety of their use.

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