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RESEARCH ARTICLE

Antioxidant, Anti-inflammatory and Acetylcholinesterase Inhibitory Activities of Propolis from Different Regions of Morocco

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Abstract Propolis is a bee product used since ancient times in folk medicine. Antioxidant activity is one of the properties attributed to this natural product, which varies depending on the botanical and geographic origin. The antioxidant capacities of propolis from different regions of Morocco were determined. Samples from the Khamissat and Immouzzer regions showed the highest antioxidant activities (ABTS assay), reducing power, and peroxyl and hydroxyl radical scavenging capacities. The best capacities to inhibit 5-lipoxygenase were found in propolis samples from Rabat (DPPH assay). A strong correlation was found between the amounts of phenols, flavonoids, and antioxidant and anti-inflammatory activities. These results support the hypothesis that phenols contribute greatly to the pharmacological properties of propolis and suggest that propolis could be important in prevention of diseases in which free radicals are implicated.

Keywords: Moroccan propolis, phenolic compounds, antioxydant activity, acetylcholinesterase

Introduction

Propolis is a resinous mixture collected by the *Apis mellifera* bee from bud and exudates of various plants and transformed in the presence of bee enzymes. Propolis is generally constituted of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other substances (1,2). Propolis has been used in traditional medicine, cosmetics, and the food industry from Europe to East Asia mainly due to the presence of phenolic compounds, which have reducing activities, are hydrogen-donors, and have metal chelating properties (3-5). Among the phenolic compounds, flavonoids are reported as having many biological and pharmacological activities, such as antimicrobial, anticancer, anti-inflammatory, and antioxidant (5-8).

Information about propolis from Morocco is scarce. One of the few studies concerning propolis extracts from this country was focused on evaluation of *in vitro* and *in vivo* anticancer properties (9). In the present study, the antioxidant (measured using several methods), anti-inflammatory, and anti-acetylcholinesterase activities were evaluated in 14 extracts obtained by maceration of propolis from different regions of Morocco, and related to the concentrations of phenols and flavonoids.

The principal aim of this study was to contribute to the knowledge of Moroccan propolis through analysis of antioxidant properties and the capacity to inhibit the acetylcholinesterase activity of extracts obtained by maceration.

Materials and Methods

Propolis samples The present study was carried out using propolis from several regions of Morocco (Fig. 1). The regions were, 1) Fez-Boulemane (Sefrou, Bhalil, Immouzzer), 2) Taza (Taza, Taounate), 3) East (Oujda), 4) Khenifra (Zaouiat cheikh), 5) Rabat (Rabat, Khemissat), 6) Gharb (Kenitra, Sidi Slimane, Moulay Bousselham), 7) Tanger (Larache), and 8) Agadir (Sidi ifni).

Samples were collected by scraping of walls, frames,

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Fig. 1. Collection sites of Moroccan propolis. Regions: 1) Fez-Boulemane: Sefrou, Bhalil, Immouzzer; 2) Taza: Taza, Taounate; 3) East: Oujda; 4) Khenifra: Zaouiat cheikh; 5) Rabat: Rabat, Khemissat; 6) Gharb: Kenitra, Sidi slimane, Moulay Bousselham; 7) Tanger: Larache; 8) Agadir: Sidi Ifni

and the entrance of bee hives by professional beekeepers and given directly to our laboratory. Samples were transported in tubes in the dark and at environmental temperatures from Morocco to Portugal (Table 1).

Propolis extract obtained by maceration Propolis (1.0 g) was chopped into small pieces and extracted using 30 mL of 70% ethanol and maintained for 96 h at 37ºC under agitation (200 rpm). After this step, the obtained solution was left to sediment and the supernatant was centrifuged for 10 min at 2,550×*g*. A clear solution, without further purification, was used for successive analyses.

Spectrometric characterization of flavonoids Samples were diluted in 70% ethanol and placed in quartz cuvettes. The absorbance was measured between 250 and 450 nm and the absorption spectra of samples were plotted to assess the compositional diversity of propolis from different regions of Morocco.

Total phenol content The total polyphenol content in propolis samples was determined using the method of Gulcin *et al*. (10). Hydro-alcoholic extracts (25 µL) were mixed with 125 µL of Folin-Ciocalteu reagent (0.2 N) and 100 µL of 7.5% Na_2CO_3, and the absorbance was measured at 760 nm after 2 h of incubation at room temperature. The total polyphenol content was expressed as mg per g of caffeic acid equivalents using a calibration curve.

Total flavones and flavonol content The amounts of flavones and flavonols in extracts were determined according to the method of Miguel *et al*. (5) with minor modification. An amount of 100 µL of Al_2Cl_3 (20%) was added to 100 µL of extract, and after 1 h at room temperature the absorbance was measured at 420 nm. Total flavones and the flavonols content were calculated as quercetin equivalents (mg per g) using a calibration curve.

Antioxidant activities

***2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity*:** Scavenging of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was assayed following the method of Hatano *et al*. (11) with some modification. Solutions with different extract concentrations were prepared and 25 µL of each solution was added to 150 µL of DPPH solution (63.4 µM) and 125 µL of 96% ethanol. The mixture was left to stand for 1 h in the dark at room temperature. Reduction of the amount of the DPPH radical

Table 1. Vegetation predominant in each region where propolis samples were collected

Region	Location	Predominant vegetation
Fez-Boulemane	Sefrou	*Olea*, *Pinus*, *Quercus*, *Juniperus*, *Rosmarinus*, *Cistus*, *Lavandula* and *Pistacia*
	Bhalil	*Olea*, *Pinus*, *Quercus*, *Juniperus*, *Rosmarinus*, *Cistus*, *Lavandula* and *Pistacia*
	Immouzzer	*Olea*, *Pinus*, *Quercus*, *Juniperus*, *Rosmarinus*, *Cistus*, *Lavandula* and *Pistacia*
Taza	Taza	*Olea*, *Pinus*, *Quercus*, *Juniperus*, *Rosmarinus*, *Cistus*, *Lavandula* and *Pistacia*
	Taounate	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
East	Oujda	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
Tanger	Larache	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
Rabat	Rabat	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
	Khemissat	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
Gharb	Kenitra	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
	Sidi Slimane	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
	Moulay Bousselham	*Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus*
Khenifra	Zaouiat Cheikh	*Quercus*, *Pinus*, *Ceratonia*, *Abies*; *Cedrus*, *Juniperus*, *Acer*, *Tilia*, *Euonymus*, and *Lavandula*
Agadir	Sidi Ifni	*Arganier*, *Acacia*, and *Euphorbia*

present was measured using a decrease in the absorption value at 517 nm. The extract concentration providing 50% inhibition (IC_{50}) was calculated using a graph of the scavenging effect percentage against the extract concentration. The scavenging effect percentage was calculated from the formula: $[(A_0-A_1/A_0)*100]$, where A_0 is the absorbance of a negative control (blank sample containing the same amount of solvent and DPPH solution) and A_1 is the absorbance of the sample. The percentage was plotted against the extract concentration, and IC_{50} values were determined (concentration of extract able to scavenger 50% of the DPPH free radical). Butylated hydroxytoluene (BHT) was used as a positive control.

***2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical-scavenging activity*:** Determination of the ABTS radical scavenging activity was carried out as reported by Miguel *et al*. (5). Briefly, the ABTS radical was generated by reaction of a 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature. Samples (25 μL) were added to 275 μL of ABTS and the absorbance at 734 nm was read after 6 min. Several extract concentrations were prepared and the percentage inhibition that was calculated from the formula: $[(A_0-A_1/A_0)*100]$ was plotted against the extract concentration, and IC_{50} values were determined (concentration of extract able to scavenger 50% of the ABTS free radical). A_0 is the absorbance of a negative control (blank sample containing the same amount of solvent and ABTS solution) and A_1 is the absorbance of the sample. Butylated hydroxytoluene (BHT) was used as a positive control.

Reducing Power The reducing power was determined according to the method described by Miguel *et al*. (5) The propolis extract (100 μL) was mixed with 500 μL of 0.2 M sodium phosphate buffer (pH 6.6) and 500 μL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After 500 μL of 10% trichloroacetic acid was added, the mixture was centrifuged for 10 min at 2,550×g. The upper layer (500 μL) was mixed with 500 μL of distilled water and 100 μL of 0.1% ferric chloride. The absorbance of the mixture was measured at 700 nm. Ascorbic acid was used as a positive control.

Hydroxyl radical scavenging activity A volume of 200 μL of hydrogen peroxide (10 mM) was added to a mixture containing 10 mM Fe_2SO_4,$7H_2O$, 10 mM EDTA, 10 mM 2-deoxyribose, 0.1 M phosphate buffer (pH 7.4), and the sample, and the resultant mixture was incubated for 1 h at 37°C, according to the method of Chung *et al*. (12). Trichloracetic acid (1 mL) and 1 mL of thiobarbituric acid were added before a new incubation at 100°C for 10 min. After cooling, the absorbance was measured at 520 nm. The IC_{50} value was calculated for each sample as reported above. Mannitol was used as a positive control.

Thiobarbituric acid reactive substances (TBARS) Analysis was based on a modified thiobarbituric acid reactive substances assay to measure the antioxidant ability of the sample (13). Egg yolk homogenate was used as a lipid-rich medium. An aliquot of yolk material was made up to a concentration of 10%(w/v) in KCl (1.15%, w/v). The yolk was then homogenized for 30 s, followed by ultrasonication for 5 min. An amount of 250 mL of 10% (w/v) homogenate and 50 μL of sample were added to a test tube and made up to 500 μL with distilled water, followed by addition of 750 μL of 20% acetic acid (pH 3.5) and 750 μL of 0.8%(w/v) 2-thiobabituric acid (TBA) in 1.1%(w/v) sodium dodecyl sulphate (SDS). The mixture was stirred using a vortex and then kept at 95°C for 1 h. After cooling to room temperature, 2.5 mL of *n*-butanol was added to each tube, followed by stirring and centrifugation at 2,550×*g* for 10 min. The absorbance of the supernatant was measured at 532 nm and IC_{50} values calculated as reported above. Butylated hydroxytoluene (BHT) was used as a positive control.

Oxygen radical absorbance capacity (ORAC) An ORAC assay is based on the capacity of antioxidants in a sample to quench peroxyl radicals generated from thermal decomposition of AAPH (2,2'-azobis-2-methyl-propan-imidamide, dihydrochloride). The ORAC method used fluorescein (FL) as the fluorescent probe following the method of Ou *et al*. (14). An amount of 0.414 g of AAPH was dissolved in 10 mL of phosphate buffer 75 mM (pH 7.4) and kept in an ice bath. A solution of fluorescein (0.00419 mM) was prepared in phosphate buffer and kept in the dark at 4°C. A new concentration (8.16×10^{-5} mM) was prepared before reactions. A trolox standard (0.02 M) was prepared in phosphate buffer and diluted to 50, 25, 12.5, and 6.25 μM. As the ORAC assay is extremely sensitive, the samples must be diluted (1/1,000; 1/10,000) before analysis to avoid interference.

An amount of 150 μL of a fluorescein working solution, 25 μL of sample, and either a blank (Milli-Q water) or a standard were mixed on a plate, which was then covered with a lid and incubated in a preheated (37°C) microplate reader for 10 min. An amount of 25 μL of AAPH solution was then added to each well. The microplate was shaken for 10 s and then the fluorescence was read every minute for 2 h at an excitation wavelength of 485 nm and an emission wavelength of 527 nm. The net area under the curve (AUC) of the standards and samples was calculated. A standard curve was obtained by plotting Trolox concentrations against the average net AUC of two measurements for each concentration. Final ORAC values

were calculated using a regression equation between the Trolox concentration and the net AUC, and are expressed as μmol of Trolox/g propolis.

Lipoxygenase inhibition activity Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13-hydroperoxy linoleic acid was followed spectrophotometrically at 234 nm due to the appearance of a conjugated diene, using a UV/visible spectrophotometer, according to the method of Frum and Viljoen (15).

The reaction was initiated by addition of 3 μL of lipoxygenase solution (0.054 g in 1 mL of 0.005% borate buffer, Tween 0.1 M, pH 9) to 937 μL of borate buffer, 10 μL of sample, and 50 μL of linoleic acid (0.001 M). The absorbance was read every minute for 5 min. The percentage inhibition of the enzyme was calculated and the IC_{50} values were compared. Nordihydroguaiaretic acid (NDGA) was used as a positive control.

Acetylcholinesterase inhibitory activity Acetylcholinesterase degrades the substrate acetylcholine in acetic acid and thiocholine, which interacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Accumulation of 5-thio-2-nitrobenzoic acid was measured at 405 nm. An acetylcholinesterase inhibition assay was adapted from the method of Mata *et al*. (16). An amount of 25 μL of sample, 425 μL of Tris-HCl buffer (0.1 M, pH 8), and 25 μL of enzyme (0.28 U/mL) were added, then the mixture was agitated and incubated for 15 min at room temperature. After this, 75 μL of substrate (0.005 g of iodine acetylcholine in 10 mL of buffer) and 475 μL of DTNB (0.059 g in 50 mL of buffer) were then added. The absorbance was read after 30 min and compared with the absorbance of the control. The percentage inhibition of the enzyme was calculated and the IC_{50} values were compared. Analyses were carried out in triplicate. Galantamine was used as a positive control.

Statistical Analysis Statistical analyses were performed using SPSS version 18.0 software (SPSS Inc.). Data correspond to triplicate experiments. The effect of the collection zone of propolis was evaluated using analysis of variance and means were compared using Tukey's multiple-range test ($p<0.05$). Correlations between the phenol and the flavonoid contents and antioxidant activity were analyzed using Pearson's correlation coefficient (r) at a significance level of 99% ($p<0.01$).

Hierarchical cluster analysis was used to investigate the similarities and dissimilarities among the propolis samples with respect to the total phenols and flavonoid content, and the capacity to scavenge DPPH free radicals. For classification, the Ward's minimum variance method was used. The squared Euclidean distance was used as the dissimilarity measure for Ward's method.

Results and Discussion

Total phenolics and flavonoid contents The amounts of total phenolics and flavonoids found in propolis extracts changed according to the place where samples were collected (Table 2). Different regions of Morocco produced propolis containing different concentrations ($p<0.05$) of phenols, ranging from a minimal value of 0.74 mg/g for propolis from Kenitra to a maximal value of 91.22 mg/g for propolis from Immouzzer (Table 2). The highest concentration of flavonoids was found in a sample from Oujda (34.27 mg/g) and the lowest was from a sample from Kenitra (0.20 mg/g).

The amounts of total phenols found in the propolis samples were within the range reported by Miguel *et al.* (*5*) and Silva *et al*. (17) for propolis from southern Portugal and Uruguay, respectively, but much different from other reported amounts (18). Distinct regions may be responsible for the diversity of results obtained in these studies. However, cluster analysis showed that the same cluster could include samples (Immouzzer) with higher phenol and flavonoid levels than other samples (Sefrou and Bhalil) collected in the same region (Fez-Boulemane). All samples from three regions (Taza, Tanger, and Gharb) had low levels of phenols and flavonoids and they were all grouped together in cluster 1. These results may indicate that the types of phenols and/or flavonoids are more important for grouping samples than their amounts and such is

Table 2. Phenol and flavonoid contents in macerated propolis samples from different areas of Morocco[1)]

Sample	Phenol (mg/g)	Flavonoid (mg/g)
Moulay Bouslham	5.98±1.12$^{f2)}$	1.75±1.39^{d}
Sidi Ifni	6.82±1.12^{f}	1.80±1.39^{d}
Bhalil	12.86±1.12^{e}	3.25±1.39^{d}
Zawiat Chikh	5.34±1.12^{fg}	1.87±1.39^{d}
Rabat	53.51±1.12^{c}	33.31±1.39^{a}
Sidi sliman	1.45±1.12^{gh}	0.30±1.39^{d}
Khamissat	65.67±1.12^{b}	12.78±1.39^{c}
Larache	6.00±1.12^{f}	2.05±1.39^{d}
Kenitra	0.74±1.12^{i}	0.20±1.39^{d}
Oujda	44.73±1.12^{d}	34.27±1.39^{a}
Immouzzer	91.22±1.12^{a}	26.30±1.39^{b}
Taza	7.83±1.12^{f}	1.68±1.39^{d}
Taounat	5.89±1.12^{f}	0.93±1.39^{d}
Sefrou	6.211±1.120^{f}	1.05±1.39^{d}

[1)]Results are shown as the mean±SD (n=3).
[2)]In the same column, values with the same superscript are not significantly different ($p<0.05$).

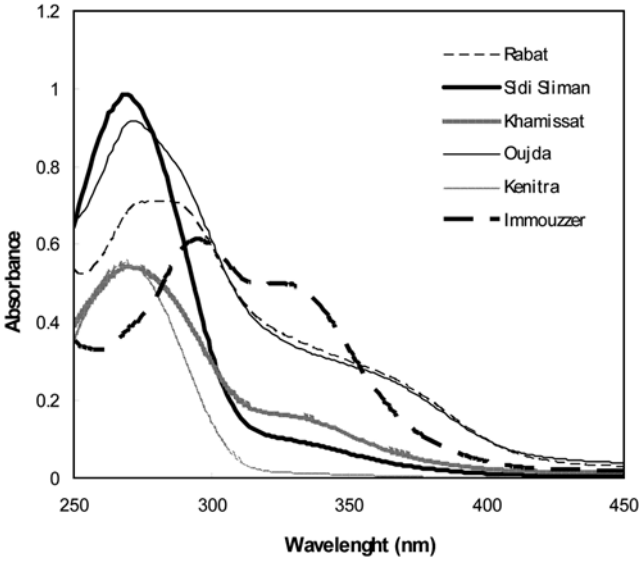

Fig. 2. Absorption spectra of ethanol propolis extracts from different areas in Morocco. Different dilutions were made: Sidi sliman (40), Kenitra (160), Immouzzer, Oujda and Rabat (320), Khamissat (640).

characteristic of each zone because is highly dependent on plants present in those regions.

UV-visible absorption spectroscopy The absorption spectra of the hydro-alcoholic extracts of propolis were dissimilar, particularly the spectrum of a sample from Immouzzer (Fig. 2) for which maximal absorption was observed at 290 and 330 nm, whereas samples from Rabat, Oujda, and Khamissat had maximal absorption at 270-350 nm.

The absorption profile between 240 and 550 nm is attributed to flavonoids and phenols (19). Two characteristic flavonoid UV/Vis bands have been reported. Band I is in the 300 to 550 nm range due to the B ring, and band II is in the 240 to 285 nm range due to the A ring. A band I ranging from 240-285 nm is characteristic of flavones and flavonols, whereas the characteristic band of flavanone, with no C ring instauration, is in the range of 270-295 nm. The absence of a 3-OH group in flavones and flavanones is responsible for a band at approximately 303-304 nm, whereas the characteristic band of 3-hydroxylated flavonols is approximately 352 nm (20). The diversity of absorption spectra from propolis ethanol extracts from different locations in Morocco suggests that diverse classes of flavonoids are present.

The absorption profiles of samples allows categorization into 3 groups: 1. Immouzzer from the region Fez-Boulemane, 2. Oujda, Rabat and Khamissat from East and Rabat regions, respectively; 3. Kenitra and Sidi sliman from the region Gharb. These profiles are a consequence of the compounds present in the samples and, therefore, may explain the differences in antioxidant activities found in the present work.

The chemical composition of propolis is complex and depends on the flora in the areas where it is produced. In the eastern regions (Rabat and Gharb), where our samples of propolis were collected, plants from the genera *Olea*, *Ceratonia*, *Chamaerops*, *Euphorbia*, *Juniperus*, *Quercus*, *Pinus*, *Pistacia*, *Thuya*, and *Eucalyptus* predominate, but the sample absorption profiles differed. A predominance of one plant species over others can determine this result. More detailed information about flora will be needed for further studies.

Moise *et al*. (9) recently reported for the first time the chemical composition of two extracts of propolis from Morocco revealing a complex mixture of molecules, including flavones, flavonols, and flavanones, among other components. Further study involving chromatographic and spectral studies are needed.

Antioxidant activity The antioxidant properties of extracts can be evaluated using diverse *in vitro* assays. Antioxidant assays of foods and biological systems can be divided into two groups. There are assays that evaluate lipid peroxidation and assays that measure the free radical scavenging ability (21). A lipoxygenase assay has been used as an indication of anti-inflammatory and antioxidant activities. Due to the production of peroxides, compounds that are able to inhibit the enzyme can be considered as antioxidants. At the same time, products are converted from other compounds that play key roles in inflammatory processes (22). Therefore, compounds that are able to inhibit the enzyme also possess anti-inflammatory properties. Values indicating the ability to prevent oxidation and/or inflammation are shown in Table 3 and 4.

ABTS radical scavenging capacity The capacity of propolis extracts to scavenge ABTS was different depending on the geographic origin. The capacity for scavenging these free radicals was evaluated in 14 propolis samples from diverse regions of Morocco. The IC_{50} values for all of these samples are shown in Table 3. The best antioxidant activities were obtained in samples from Immouzzer (IC_{50}=0.009 mg/mL), Oujda (IC_{50}=0.022 mg/mL), and Khamissat (IC_{50}=0.013 mg/mL). The worst antioxidant activities were found in samples from Sidi sliman and Kenitra (Table 3).

For a sample from Kenitra it was impossible to determine the IC_{50} value due to a weak antioxidant ability. A negative correlation between the phenol and flavonoid content *versus* IC_{50} values was found (p<0.01) (Table 5). IC_{50} values indicate the concentration of a sample able to scavenge 50% of free radicals. Therefore, the lower these values, the better the antioxidant activities.

Table 3. IC_{50} values (mg/mL) for macerated propolis extracts from different locations in Morocco. IC_{50} values for the ORAC method are given as μmol of Trolox Equivalents (TE)/g[1)]

Sample	ABTS	DPPH	Hydroxyl	ORAC
Moulay Bouslham	0.279±0.016$^{cde2)}$	0.526±0.030^{c}	1.398±0.008^{b}	1106.423±78.151^{e}
Sidi ifni	0.236±0.016^{de}	0.384±0.030^{de}	0.719±0.008^{e}	1570.866±78.151^{c}
Bhalil	0.125±0.016^{f}	0.239±0.030^{f}	1.111±0.008^{d}	1404.360±78.151^{bcd}
Zawiat chikh	0.366±0.016^{b}	0.409±0.030^{d}	1.441±0.008^{a}	1198.169±78.151^{cde}
Rabat	0.136±0.016^{f}	0.008±0.030^{g}	0.104±0.008^{j}	1455.039±78.151^{bc}
Sidi sliman	1.009±0.016^{a}	1.125±0.030^{b}	-	1353.256±78.151^{abcd}
Khamissat	0.013±0.016^{g}	0.025±0.030^{g}	0.059±0.008^{k}	2012.152±78.151^{a}
Larache	0.408±0.016^{b}	0.559±0.030^{c}	1.191±0.008^{c}	1278.603±78.151^{cde}
Kenitra	-	1.813±0.030^{a}	0.540±0.008^{h}	1291.157±78.151^{cde}
Oujda	0.022±0.016^{g}	0.025±0.030^{g}	0.398±0.008^{i}	1143.483±78.151^{de}
Immouzzer	0.009±0.016^{g}	0.019±0.030^{g}	0.078±0.008^{jk}	1865.506±78.151^{b}
Taza	0.209±0.016^{e}	0.294±0.030^{ef}	0.577±0.008^{g}	ND[3)]
Taounat	0.297±0.016^{c}	0.459±0.030^{cd}	0.651±0.008^{f}	ND
Sefrou	0.296±0.016^{c}	1.086±0.030^{b}	0.677±0.008^{f}	ND
BHT	0.004±0.016^{g}	0.089±0.030^{g}	-	ND
Mannitol	-	-	0.001±0.008^{l}	ND

[1)]Results are shown as the mean±standard error (n=3).
[2)]Values with the same superscript in the same column are not significantly different (p<0.05).
[3)]ND, not detected; -, the concentrations assayed did not permit determination of IC_{50} values.

Table 4. IC_{50} values (mg/mL) for macerated propolis extracts from different locations in Morocco[1)]

Sample	TBARS	Lipoxygenase	Acetylcholinesterase
Moulay Bouslham	0.685±0.023$^{ab2)}$	1.968±0.016^{e}	-[3)]
Sidi ifni	0.572±0.023^{d}	1.206±0.016^{f}	-
Bhalil	0.144±0.023^{e}	0.217±0.016^{h}	0.569±0.006^{b}
Zawiat chikh	0.312±0.023^{d}	2.182±0.016^{c}	0.743±0.006^{a}
Rabat	0.014±0.023^{f}	0.149±0.016^{i}	0.089±0.006^{c}
Sidi sliman	-	-	-
Khamissat	0.249±0.023^{d}	0.248±0.016^{h}	0.087±0.006^{c}
Larache	0.609±0.023^{bc}	1.995±0.016^{e}	0.559±0.006^{b}
Kenitra	-	-	-
Oujda	0.051±0.023^{f}	0.272±0.016^{h}	0.043±0.006^{d}
Immouzzer	0.241±0.023^{d}	0.380±0.016^{g}	0.085±0.006^{c}
Taza	-	2.092±0.016^{d}	-
Taounat	0.699±0.023^{a}	2.521±0.016^{a}	-
Sefrou	0.657±0.023^{ab}	2.461±0.016^{b}	-
BHT	0.097±0.023^{ef}	ND	ND
NDGA	ND	0.020±0.016^{j}	ND
Galantamine	ND	ND	0.003±0.006^{e}

[1)]Results are shown as the mean±SD (n=3).
[2)]Values with the same superscript in the same column are not significantly different (p<0.05).
[3)]-, the concentrations assayed did not permit determination of IC_{50} values. ND, Not detected

BHT, used in the ABTS assay, showed a better activity than almost all propolis samples. Nevertheless, the activities of samples from Immouzzer, Oujda, and Khamissat (Table 3) was not significantly different from the activity of BHT.

The capacity of propolis extracts to scavenge ABTS was previously reported (5) for other propolis samples. A correlation between the scavenging activity and the phenol and flavonoid contents was also reported by Miguel *et al.* (5). Propolis from Algeria, a neighbour country of Morocco, has also been reported as possessing the ability to scavenge free radicals measured using several methods. (23) This capacity, along with a reducing power, may be due to the presence of phenols and flavonoids (23).

Reducing power Samples with the best reducing power also had the best capacity for scavenging ABTS free

Table 5. Pearson correlation coefficients for compounds/antioxidant activities, compounds/acetylcholinesterase inhibition, compounds/lipoxygenase inhibition[1)]

	Phenol	Flavonoid
DPPH	-0.623**	-0.592**
ABTS	-0.609**	-0.535**
Hydroxyl	-0.743**	-0.647**
TBARS	-0.640**	-0.774**
ORAC	+0.676**	+0.218NS
Lipoxygenase	-0.752**	-0.743**
Acetylcholinesterase	-0.873**	-0.866**

[1)]Pearson correlation significance levels: NS, not significant; -, assay not performed; **significant at $p<0.01$

radicals. The reducing power of propolis samples is shown in Fig. 3. The reducing activities were dose-dependent. In this analysis, samples were distributed in the three groups of 1) samples with the highest absorbance values (Rabat, Immouzzer, Oujda, and Khamisset), 2) samples with the worst reducing power (Kenitra and Sidi sliman), and an intermediate group containing all remaining samples. This pattern was similar to the ABTS scavenging ability (data not shown). The similarity of profiles for free ABTS radical scavenging and reducing power was related to similar mechanisms of one electron-transfer involved in both analyses. Although the best reducing power was found for propolis extracts from Rabat, Immouzzer, Oujda, and Khamissat, the ascorbic acid sample (positive control) was markedly better than the propolis samples (Fig. 3).

The capacity of propolis to reduce ferric to ferrous ions was also previously reported (24) in samples from Slovenia. Propolis extracts prepared with 70% ethanol had a higher reducing power than extracts prepared with 96% ethanol.

DPPH radical scavenging capacity Samples with the best capacity for scavenging ABTS free radicals were not always the most effective for scavenging DPPH free radicals. The Rabat sample is an example (Table 3). Propolis from Kenitra had the poorest capacity for scavenging DPPH free radicals, followed by samples from Sefrou and Sidi sliman (Table 3). Samples from Rabat, Immouzzer, Oujda, and Khamissat had good capacities. Propolis from Rabat was among the best DPPH scavengers, but not for ABTS. The ability to scavenge DPPH radicals was dependent on the sample concentration (data not shown).

A negative correlation between the phenol and flavonoid content *versus* IC_{50} values was found (Table 5) ($p<0.01$).

A dendrogram (Fig. 4) based on total phenols and flavonoids and the capacity to scavenge DPPH free radicals obtained from hierarchical cluster analysis (using the average between groups with squared Euclidean distance measure clustering method) showed three main clusters. Cluster 1 included samples from Bhalil, Kenitra, Larache, Moulay Bouslham, Sefrou, Sidi ifni, Sidi sliman, Taounat, Taza, and Zaouiat cheikh (all belonging to the Fez-Boulemane, Taza, Gharb, Khenifra, and Agadir regions). Cluster 2 included samples from Immouzzer and Khamissat (Fez-Boulemane and Rabat regions, respectively). Cluster 3 included samples from Oujda and Rabat (regions East and Rabat regions, respectively). Samples from Oujda and Rabat contained more active antioxidants than other samples from the other regions of Morroco, except Immouzzer, which originates from the Fez-Boulemane region (cluster 2) (Fig. 4).

The IC_{50} values were similar to values obtained in the ABTS analysis, probably due to the similarity of both methods involving one electron-transfer.

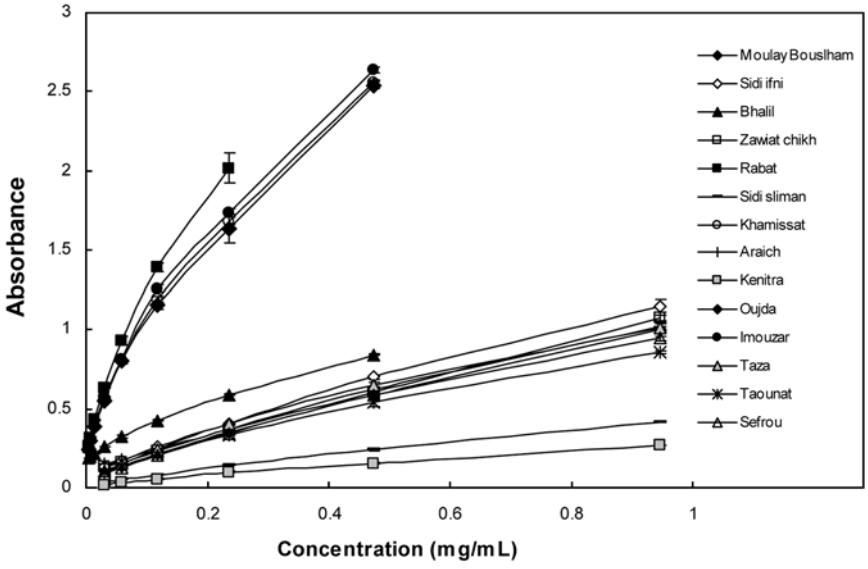

Fig. 3. The reducing power of macerated hydro-alcoholic extracts of propolis from different areas in Morocco.

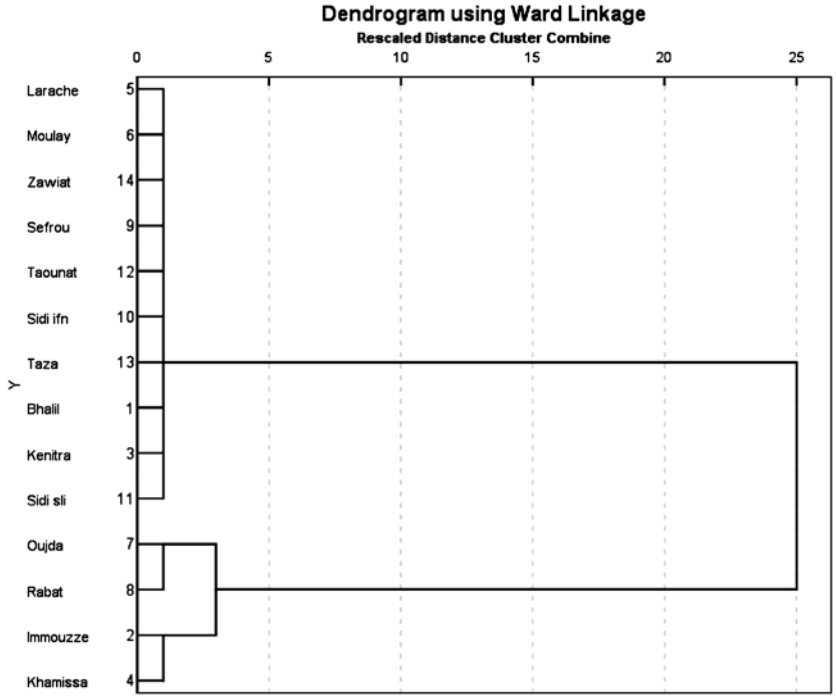

Fig. 4. Dendrogram for classification of propolis samples with respect to total phenols, total flavonoids, and the capacity to scavenge DPPH free radicals.

The highest capacity for scavenging the DPPH free radical was found in samples belonging to clusters 2 and 3, which also had higher levels of phenols and/or flavonoids. Nevertheless, there were other propolis samples from the same region and belonging to the same cluster that had low levels of phenols and, consequently, a lower activity. A more precise description of the flora surrounding the beehives from where propolis samples were collected is necessary for further studies.

Hydroxyl radical scavenging capacity The hydroxyl radical scavenging capacity of samples was dependent on the geographic origin of propolis. In some extracts, the activity was too low to determine IC_{50} values. Values are shown in Table 3. Samples from Khamissat and Immouzzer were the most effective for scavenging hydroxyl radicals, immediately followed by samples from Rabat. Propolis from Sidi sliman had the worst ability for scavenging this radical. Similar to other assays, values were too low to determine the IC_{50} value because none of the concentrations tested was able to scavenge 50% of the hydroxyl radical (Table 3). The radical capacity was also dose-dependent (data not shown).

Mannitol was significantly better as a hydroxyl radical scavenger than the remaining samples of propolis, except for the relatively high activity of a propolis sample from Immouzzer (Table 3).

A negative correlation was found between the phenol and flavonoid content and the capacity for scavenging hydroxyl radicals ($p<0.01$) (Table 5), similar to results for ABTS and DPPH.

Propolis from Oujda had a lower capacity for scavenging hydroxyl radicals than for scavenging both ABTS and DPPH. The number and position of the hydroxyl and methoxy groups in the aromatic flavonoid or phenolic acid rings are important factors for the hydroxyl scavenging activity, which may partially explain these results (25).

The importance of this group of compounds in the capacity for scavenging free radicals is shown by the negative correlation found between the phenol and flavonoid content and the capacity to scavenge hydroxyl radicals ($p<0.01$).

Peroxyl radical scavenging capacity The antioxidant activity, evaluated using the capacity for scavenging peroxyl radicals, was also evaluated in propolis collected in different places of Morocco. Results, expressed as equivalents of Trolox (μmol TE/g of propolis), showed that the best extracts for scavenging DPPH and ABTS free radicals were not always the best for scavenging peroxyl radicals. The most active propolis samples were from Khamissat and Immouzzer. The worst samples were from Oujda and Moulay Bouslham (Table 3). A positive and linear correlation was found between the ORAC values and the total polyphenol content (Table 5). The activity of propolis from Oujda with relatively high amounts of total phenols (>44 mg/mL) and flavonoids (>34 mg/mL) was weak, in contrast to the capacities for scavenging DPPH and ABTS

free radicals. The relatively good peroxyl radical scavenging capacity of samples from Sidi ifni also contrasts to results from the DPPH and ABTS assays for the same samples in which weak activities were found.

An ORAC assay measures the antioxidant scavenging activity against a biologically relevant radical source (peroxyl) induced using 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The activities found in the present study are within the range reported recently for Uruguayan propolis (17). Silva *et al*. (17) also concluded that the capacity for scavenging free peroxyl radicals of Uruguayan propolis could be related to the presence of specific mixtures of polyphenolic compounds and not to a particular compound. The absence of a correlation between flavonoids and ORAC assay results in the present study has also been previously reported (26) for samples of honey from Chile.

Capacity for preventing lipid peroxidation Propolis samples from Rabat and Oujda had the best capacity for preventing lipid peroxidation (Table 4).

The ability for preventing lipid peroxidation was dose-dependent, similar to results for the capacity to scavenge free radicals and the reducing power.

A negative correlation was observed between phenols and TBARS, as well as between flavonoids and TBARS (Table 5) ($p<0.01$).

The capacity to prevent lipid peroxidation has been reported recently for Italian propolis obtained by different harvesting methods (8). Propolis was able to inhibit lipid oxidation by preventing TBARS formation in lipids incorporated into an oil/water emulsion. The capacity was dependent on the harvesting method of propolis and the phenol extraction solvent used.

A wide variety of methods are used to assess antioxidant capacity and no single assay provides an accurate method to determine the capacity to prevent lipid oxidation and/or to scavenge free radicals, particularly in a mixed or complex system such as propolis. Therefore, it is essential to use diverse methods to assess different aspects of the oxidation process. In addition, there is no universal antioxidant. Different compounds can act as antioxidants through different mechanisms.

Capacity to inhibit 5-lipoxygenase A lipoxygenase assay was used as an indicator of anti-inflammatory and antioxidant activities. Samples from Rabat and Bhalil showed the lowest IC_{50} values (0.149 and 0.217 mg/mL, respectively) (Table 4). These samples possessed the best anti-inflammatory activities, as measured by this method. Extracts from Khamissat and Oujda had relative low IC_{50} values (0.248 and 0.272 mg/mL, respectively), but these were significantly higher than values of samples from Rabat (Table 4). Propolis from Sidi sliman and Kenitra did not show any activity and IC_{50} values could not be determined (Table 4). A negative correlation was found between the phenol content and lipoxygenase inhibition and between the flavonoid content and lipoxygenase inhibition (Table 5) ($p<0.01$).

Flavonoids and phenolic acids, along with terpenoids, inhibit 5-lipoxygenase (27). In the present study, identification of compounds was not performed and, therefore, the results may be attributed to different compounds and/or different amounts of the same compounds in samples from different areas of Morocco.

Inhibition of acetylcholinesterase Only few samples had the capacity to inhibit acetylcholinesterase activity (Table 4). Only 7 samples had enough activity to determine IC_{50} values. The activity was dose-dependent (data not shown). Propolis from Oujda had the best capacity for inhibiting acetylcholinesterase activity with the lowest IC_{50} value, nevertheless this value was much less than the positive control (galantamine) (Table 4). A negative correlation was found between the phenol and flavonoid content and IC_{50} values for inhibition of acetylcholinesterase ($p<0.01$) (Table 5).

Phenols and flavonoids inhibit acetylcholinesterase activity (28). Inhibition of acetylcholinesterase, the fundamental enzyme in the breakdown of acetylcholine by termination of the nerve impulse transmission at cholinergic synapses, is a possible strategy for treatment of Alzheimer's disease, which is characterized by a decline in cognitive function and mental atrophy (28,29). Therefore, propolis with a high phenol content may be an alternative for prevention and/or retardation of Alzheimer's disease symptoms. A water-soluble derivative of propolis prepared from fresh Chinese propolis significantly inhibited the acetylcholinesterase activity in the hippocampus of scopolamine-treated mice, suggesting that propolis has potential as a pharmaceutical for brain protection (30). The inverse correlation observed between IC_{50} values and the phenols and flavonoid content is representative of the importance of these groups of compounds for inhibition of acetylcholinesterase activity.

Samples from Khemisset and Imouzzer were good scavengers of active oxygen species, including peroxyl and hydroxyl radicals. These samples were also good scavengers of free radicals, like ABTS, and showed a strong reducing power. Propolis samples from Oujda and Rabat had the best capacity to prevent lipid peroxidation and Rabat samples also had an anti-inflammatory activity. Some of the propolis samples were also able to inhibit acetylcholinesterase. These properties can be considered useful to prevent various neurodegenerative diseases, cardiovascular disorders, diabetes, and Alzheimer disease.

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