Original article

Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis

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Summary Biological properties of Moroccan propolis have been scarcely studied. In the present work, the total phenols and flavonoids from 21 samples of propolis collected in different places of Morocco or 3 supplied in the market were determined, as well as the *in vitro* capacity for inhibiting the activities of acetylcholinesterase, α-glucosidase, α-amylase, lipoxygenase, tyrosinase, xanthine oxidase and hyaluronidase. The results showed that samples 1 (region Fez-Boulemane, Sefrou city) (IC_{50} = 0.065, 0.006, 0.020, 0.050, 0.014 mg mL^{-1}) and 23 (marketed) (IC_{50} = 0.018, 0.002, 0.046, 0.037, 0.008 mg mL^{-1}) had the best *in vitro* capacity for inhibiting the α-amylase, α-glucosidase, lipoxygenase, tyrosinase and xanthine oxidase activities, respectively. A negative correlation between IC_{50} values and concentration of phenols, flavones and flavanones was found. These activities corresponded to the generally higher amounts of phenols and flavonoids. In the same region, propolis samples have dissimilar phenol content and enzyme inhibitory activities.

Keywords α-amylase, α-glucosidase, hyaluronidase, hydroethanolic extracts, lipoxygenase, propolis.

Introduction

Propolis is a plant product–resinous mixture with a broad spectrum of biological properties, collected by the *Apis mellifera* bee from the bud and exudates of various plants and transformed in the presence of bee enzymes (Miguel *et al*., 2014a,b). The wide application of propolis in modern medicine and the increasing demand for it, due to its health benefits and use in cosmetic and food products, have drawn growing attention to its chemical composition, which is susceptible to the geographical location, collection site, botanical origin (Salatino *et al*., 2011), bee species (Silici & Kutluca, 2005) and the availability of water and climate (Boufadi *et al*., 2014). Flavonoids, phenylpropanoids, terpenoids, stilbenes, lignans, coumarins and their prenylated derivatives, alkaloids and iridoids are some groups of compounds identified in propolis worldwide. This chemical diversity is related to the bioactivity and potential uses of this product (Huang *et al*., 2014).

The biological properties attributed to the propolis of different parts of the world have been the target of studies, and very recently, Silva-Carvalho *et al*. (2015) compiled the recent published information about antibacterial and antifungal, antiviral, anti-inflammatory, antioxidant, immunomodulatory and antitumor activities of propolis.

Alzheimer's disease is a progressive, neurodegenerative disease that is clinically characterised by a progressive loss of cognitive abilities. The pathophysiology of that disease involves defective beta-amyloid protein metabolism, abnormalities of glutamatergic, adrenergic, serotonergic and dopaminergic neurotransmission and the potential involvement of inflammatory, oxidative and hormonal pathways (Kang *et al*., 2005; Chen *et al*., 2008). For the treatment of Alzheimer's disease, acetylcholinesterase (AChE) inhibitors are included, which increase the availability of acetylcholine at cholinergic synapses,

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although the modest benefits were obtained (Chen *et al*., 2008). Some natural products of plant origin have revealed to be effective acetylcholinesterase inhibitors – of them some were used as medicines, such as some alkaloids (galantamine). Other groups of compounds have been also checked and some flavonoids have also revealed the ability for inhibiting this enzyme (Scotti & Scotti, 2015). Only very few works regarding the capacity of propolis extracts to inhibit acetylcholinesterase have been published (Chen *et al*., 2008; Miguel *et al*., 2014b).

Postprandial hyperglycaemia plays an important role in the development of type 2 diabetes mellitus, and one strategy used to stabilise postprandial plasma glucose is the utilisation of specific enzyme inhibitors, such as α-glucosidase and pancreas α-amylase inhibitors (Murai *et al*., 2002). Natural bioactive compounds with the capacity to delay the glucose absorption by the inhibition of α-glucosidase or α-amylase activities and consequently to moderate the excess postprandial blood glucose level have been searched, including those present in the propolis from different geographical origins: China (Zhang *et al*., 2015), Morocco (Popova *et al*., 2015) and Brazil (Matsui *et al*., 2004).

The inhibition of lipoxygenase has been considered as an indicator of anti-inflammatory and antioxidant activities. 5-Lipoxygenase is the key enzyme of leukotriene biosynthesis, and it has been the target for many inhibitors for trying to find potential drugs for combating a variety of inflammation- and hypersensitivity-based human diseases including asthma, arthritis, bowel diseases such as ulcerative colitis and Crohn's disease and circulatory disorders such as shock and myocardial ischaemia (Sailer *et al*., 1996).

Propolis (Miguel *et al*., 2014b) and particularly some components (e. g. caffeic acid phenethyl ester) have been reported as lipoxygenase inhibitors with antioxidant properties (Sud'ina *et al*., 1993; Mirzoeva & Calder, 1996; Touaibia & Guay, 2011).

Hyaluronidase is an enzyme that depolymerises the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue, and it is involved in allergic reactions and inflammation (Moon *et al*., 2009). Hyaluronidase inhibitors may have therefore anti-allergic and anti-inflammatory properties. Inhibition of hyaluronidase by propolis from diverse geographical origins such as Portugal (Silva *et al*., 2012) and China and Brazil (Miyataka *et al*., 1997; Park & Ikegaki, 1998) has been reported, and therefore, propolis is considered as a potential anti-inflammatory natural product.

Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase. This enzyme is a key in melanin biosynthesis, which involves in the colour formation of mammalian hair and skin and prevents the skin from damage by ultraviolet (Nerya *et al*., 2003; Dong *et al*., 2014). Propolis from diverse origins (Australia, Brazil, China, Hungary, Japan, Ukraine and Uruguay) has been described to inhibit the tyrosinase activity (Tazawa *et al*., 2001).

The enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid. Overproduction of uric acid, termed hyperuricaemia, is the underlying cause of gout. During the reoxidation of xanthine oxidase, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide (Cos *et al*., 1998). During this process, beyond the production of uric acid, there is also the production of reactive oxygen species that contribute to the oxidative stress which is involved in several pathological processes such as inflammation, atherosclerosis, cancer, ageing, among other ones (Cos *et al*., 1998).

Propolis extracts and their components have the ability to inhibit xanthine oxidase, either *in vitro* or *in vivo* systems, which may be considered as potential agent against gout and oxidation (the production of superoxide radical and hydrogen peroxide is hampered) (Ilhan *et al*., 2004; Yoshizumi *et al*., 2005; Armutcu *et al*., 2015).

Only very few works have focused on Moroccan propolis. One of the few studies concerning propolis extracts from this country reported *in vitro* and *in vivo* anticancer properties (Ait Mouse *et al*., 2012), whereas antioxidant, anti-inflammatory (the inhibition of lipoxygenase activity) and anti-acetylcholinesterase activities of fourteen propolis samples from diverse regions of Morocco were evaluated by Miguel *et al*. (2014b) and α-glucosidase and α-amylase inhibitory activities were reported by Popova *et al*. (2015) from the propolis collected in five diverse locations of that country.

The main goal of the present work was to evaluate the inhibitory activities of 21 propolis samples of diverse geographical origins of Morocco on acetylcholinesterase, lipoxygenase, α-glucosidase and α-amylase activities, previously studied but with fewer number of propolis samples (Miguel *et al*., 2014b; Popova *et al*., 2015). At the same time, other enzyme inhibitory activities, such as xanthine oxidase and hyaluronidase, enzymes also involved in inflammatory processes, were evaluated, as well as the inhibitory activity of tyrosinase by the same samples. This work may contribute to ascertain the importance or not of the harvesting places on the enzyme inhibitory activities. In addition, three samples purchased from the market were also the target of study, due to the increasing demand and sell of propolis by Moroccan people.

Material and methods

Hydroalcoholic extract of propolis

Twenty-four propolis samples were provided by diverse Moroccan beekeepers or purchased from the market. The harvesting places and their coordinates, climate data as well as the surrounding plants of the beehives are given in Table S1.

Extracts were obtained as reported by Miguel *et al.* (2014b) with slight modifications. Briefly, one gram of propolis was chopped into small pieces and extracted by maceration using 30 mL of 70% ethanol and maintained for 1 week at 37 °C under agitation (200 rpm). The resulting solution was filtered under vacuum. For the successive analyses, a clear solution, without further purification, was used to prepare serial dilutions with the concentrations of 33.33, 16.66, 8.33, 4.17, 2.08, 1.04, 0.52 and 0.26 mg mL^{-1}.

Chemicals

Acetylthiocholine iodide (ATCI), AChE (type VI-S) from the electric eel Electrophorus electricus, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), hyaluronidase from bovine testes, sodium tetraborate, alpha-amylase, alpha-glucosidase, 5-lipoxygenase from soya bean, linoleic acid, mushroom tyrosinase, L-DOPA, xanthine oxidase, xanthine and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Nitrophenyl-β-D-glucopyranoside (PNPG), boric acid, ascorbic acid, Tris–HCl and hydrochloric acid (HCl) were purchased from Merck, Darmstadt, Germany. Starch, potassium iodide, iodine and Na_2CO_3 were purchased from Riedel de Haen (Seelze, Germany), Riedel-de-Haën Laboratory chemicals, Germany. Calcium chloride, Folin-Ciocalteu's phenol reagent and $AlCl_3$ were purchased from Panreac Química, Montcada i Reixac, Barcelona, Spain. Acetic acid, PDMAB (*p*-dimethylaminobenzaldehyde), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from VWR (Leuven, Belgium). Ferulic Acid, DNP (2,4-dinitrophenylhydrazine), quercetin, H_2SO_4 and KOH and eriodictyol were purchased from Fluka Biochemika (Sigma-Aldrich, Steinheim, Germany).

Total phenol content

The total polyphenol content in propolis samples was determined using the method of Gülcin *et al.* (2005). Hydroalcoholic extracts (25 μL) were mixed with 125 μL of Folin-Ciocalteu's phenol reagent (0.2 N) and 100 μL of 7.5% Na_2CO_3, and the absorbance was measured at 765 nm after 2 h of incubation at room temperature. The total polyphenol content was expressed as mg ferulic acid equivalents per mL (mg FAE mL^{-1}) using a calibration curve.

Flavones and flavonol content

The amounts of flavones and flavonols in the extracts were determined according to the method of Miguel *et al.* (2010) with a minor modification. An amount of 100 μL of $AlCl_3$ (20%) was added to 100 μL of extract, and after 1 h at room temperature, the absorbance was measured at 420 nm. Total flavone and the flavonol contents were calculated as mg quercetin equivalents per mL (mg QE mL^{-1}) using a calibration curve.

Flavanone and dihydroflavonol contents

The quantification of total flavanones and dihydroflavonols was performed as described by Popova *et al.* (2004) with slight modifications. Briefly, 75 μL of sample and 2 mL of DNP (2,4-dinitrophenylhydrazine) solution (1 g DNP in 2 mL 96% sulphuric acid, diluted to 100 mL with methanol) were heated at 50 °C for 50 min. After cooling at room temperature, the mixture was diluted to 10 mL with 10% KOH in methanol (w/v). A sample (1 mL) of the resulting solution was added to 10 mL methanol and diluted to 50 mL with methanol. The absorbance was measured at 486 nm.

Inhibition of acetylcholinesterase

Acetylcholinesterase degrades the substrate acetylcholine into 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. The acetylcholinesterase inhibition assay was adapted from the method of Mata *et al.* (2007). About 25 μL of sample, 425 μL of Tris–HCl buffer (0.1 M, pH 8) and 25 μL of enzyme (0.28 U mL^{-1}) were added, and then the mixture was agitated and incubated for 15 min at room temperature. After this period, 75 μL of substrate (0.005 g of iodine acetylcholine in 10 mL of buffer) and 475 μL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (0.059 g in 50 mL of buffer) were then added. The absorbance of sample (Abs_{sample}) was read after 30 min and compared with the absorbance of the control (Abs_{cont}) in which the sample was replaced by buffer. Inhibition percentage of enzyme was calculated as follows: $[(Abs_{cont} - Abs_{sample})/Abs_{cont}] \times 100$. The analyses were carried out in triplicate. The inhibition

percentage of enzyme was plotted against propolis extract concentration (w/v), and IC_{50} values were determined (the concentration of propolis extract able to inhibit 50% of acetylcholinesterase).

Inhibition of α-amylase and α-glucosidase

α-Amylase inhibition assay

α-Amylase inhibition assay was carried out by a modified starch iodine (Uddin *et al*., 2014). The total assay mixture consisting of 100 μL sodium phosphate buffer (0.02 M, pH 6.9 containing 6 mM sodium chloride), 50 μL of α-amylase (0.02 units) and propolis was incubated at 37 °C for 10 min. After the incubation, 200 μL of soluble starch (1%, w/v) was added to each test tube and the mixture was reincubated for 20 min at 37 °C. About 300 μL of 10% HCl was added to stop the enzymatic reaction, followed by the addition of 300 μL of iodine reagent (5 mM I_2 and 5 mM KI), and after that, 8 mL of distilled water was added. The absorbance was read at 620 nm. Sample, substrate and blank were undertaken under the same conditions. Each experiment was done in triplicate. The inhibition percentage of the enzyme was calculated using the following formula:

% of α-amylase inhibition:

$$\left[1 - \frac{[(A^{-}_{\text{control}}) - (A^{+}_{\text{control}})] - (A_{\text{sample}})}{(A^{-}_{\text{control}}) - (A^{+}_{\text{control}})}\right] \times 100$$

where A^{-}_{control} denotes the absorbance of 100% enzyme activity (ethanol 70% with enzyme), A^{+}_{control} denotes the absorbance of 0% enzyme activity (ethanol 70% without enzyme), and A_{sample} denotes the absorbance of sample. And then, the IC_{50} values were compared.

α-Glucosidase inhibition assay

The yeast α-glucosidase inhibitory activity of the 24 propolis samples was determined by a modified version of the method described by Popova *et al*. (2015). Briefly, a mixture of 25 μL of different concentrations of the samples, 30 μL of yeast α-glucosidase (2.4 U mL^{-1}) and 100 mM phosphate buffer (pH 6.8) was incubated in 96-well plates at room temperature for 10 min. After pre-incubation, 100 μL of 0.5 mM *p*-nitrophenyl-β-D-glucopyranoside (PNPG) solution in phosphate buffer was added to each well. The reaction mixtures were incubated at 37 °C for 30 min, and then 80 μL of sodium carbonate solution (0.4 mM) was added to stop the reaction. The absorbance was recorded with a microplate reader at 405 nm. The control had 25 μL of 70% ethanol instead of test samples. The inhibition percentage of the enzyme was calculated, and the IC_{50} values were compared. The analyses were carried out in triplicate.

Inhibition of lipoxygenase

The inhibition of lipoxygenase was carried out as previously reported by Frum & Viljoen (2006). The reaction was initiated by the addition of 5 μL 5-lipoxygenase solution (0.054 g in 1 mL borate buffer 0.005%, Tween 0.1 M, pH 9) to 937 μL borate buffer, 10 μL sample and 50 μL linoleic acid (0.001 M). The enzymatic reactions were performed in the absence or in the presence of propolis extracts and their kinetics were compared. The inhibition percentage of the enzyme was calculated, and the IC_{50} values were compared. The analyses were carried out in triplicate.

Inhibition of tyrosinase

The inhibitory action of propolis on tyrosinase was evaluated as previously described by El-Hady *et al*. (2014), but with the slight modifications. In short, 140 μL phosphate buffer (50 mM, pH 6.5), 25 μL of sample and 60 μL of mushroom tyrosinase solution (100 U mL^{-1}) were added to a 96-well microplate. The assay mixture was incubated at 25 °C for 40 min. Following incubation, 60 μL of 5 mM L-DOPA solution was added and the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm in a microplate reader. The percentage of inhibition of tyrosinase activity was calculated and the IC_{50} value was determined.

Inhibition of xanthine oxidase

Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine. Xanthine oxidase inhibiting activity was conveniently measured with a modified assay according to Umamaheswari *et al*. (2013). The assay mixture consisted of 100 μL of the sample, 350 μL of phosphate buffer (pH 7.5, 50 mM) and 45 μL of xanthine oxidase enzyme solution (0.4 units mL^{-1} in phosphate buffer, pH 7.5), which was prepared immediately before use. After pre-incubation at room temperature for 15 min, the reaction was initiated by the addition of 330 μL of 0.150 mM xanthine solution (substrate). The assay mixture was incubated for 30 min. The reaction was stopped by adding 100 μL of 1 M HCl, and the absorbance was measured at 290 nm using an UV/VIS spectrophotometer. The assay was done in triplicate, and the IC_{50} values were calculated from the percentage of inhibition.

Inhibition of hyaluronidase

Hyaluronidase inhibition was determined by measuring the amount of *N*-acetylglucosamine separated from

sodium hyaluronate as described by Sahasrabudhe & Deodhar (2010). Bovine hyaluronidase solution (150 μL) (7900 units mL^{-1}) dissolved in acetate buffer (pH 3.6) was mixed with 25 μL of propolis extracts and incubated for 30 min at 37 °C. After 30 min, 50 μL of calcium chloride (12.5 mM) was added to the reaction mixture and again incubated for 30 min at 37 °C. After this period, 250 μL sodium hyaluronate (1.2 mg mL^{-1}) was added and again incubated at 37 °C for 1 h 30 min. After incubation, 50 μL of 0.4 M sodium hydroxide and 100 μL of 0.6 M sodium borate were added to the reaction mixture and incubated in the boiling water bath for 3 min. After cooling in ice, 1.5 mL of PDMAB (*p*-dimethylaminobenzaldehyde) solution (4 g PDMAB dissolved in 50 mL of 10 M HCL and 350 mL of glacial acetic acid) was added to the reaction mixture. The absorbance was measured at 585 nm. The assay was done in triplicate.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 23.0 software (SPSS Inc., Chicago, IL, USA). Statistical comparisons were made with one-way analysis of variance followed by Tukey's multiple comparisons. The level of significance was set at $P < 0.05$. Correlations between phenol and flavonoid contents and enzyme inhibitory activities were achieved by Spearman's correlation coefficient (r) at a significance level of 95%. Hierarchical cluster analysis was used to investigate the similarities and dissimilarities between the 24 propolis samples with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols content and the capacity to inhibit xanthine oxidase and α-amylase activities. In 9 samples, due to its low inhibitory activity on α-glucosidase, lipoxygenase, acetylcholinesterase and tyrosinase, the IC_{50} values were impossible to determine in some assays. In this case, only 15 samples were submitted to hierarchical cluster analysis, to investigate the similarities and dissimilarities between the 15 propolis samples with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols contents and the capacity for inhibiting those enzymes. In any case, for the classification purposes, 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

Phenols and flavonoids

The concentrations of total phenols, flavones and flavonols, and flavanones and dihydroflavonols found for propolis samples collected in several places of Morocco are presented in Table 1. The results reveal a great variability in concentrations of all groups of phenols. Concerning total phenols, the concentrations ranged from 0.236 mg, in sample 24, to 8.846 mg FAE mL^{-1} in sample 17. Sample 23 (8.827 mg FAE mL^{-1}) had a concentration similar to that of sample 17. Total phenol concentrations of samples 1 and 10 were 6.861 and 4.802 mg FAE mL^{-1}, respectively. The amounts of flavones and flavonols were always superior to those of flavanones and dihydroflavonols (Table 1). As reported for total phenols, the concentrations of flavones and flavonols were higher in the sample 17 (4.320 mg QE mL^{-1}), immediately followed by the samples 23 (2.699 mg QE mL^{-1}) and 1 (1.784 mg QE mL^{-1}). Concerning dihydroflavonols, the sample 23 had the highest amount of this group of compounds (0.532 mg EE mL^{-1}) and not sample 17, as found for total phenols and flavones and flavonols, immediately followed by samples 1 (0.257 mg EE mL^{-1}) and 17 (0.222 mg EE mL^{-1}).

The highest concentrations of total phenols and flavonoids found in the hydroethanolic extracts of Moroccan propolis in the present work are within the

Table 1 Concentration of total phenols, flavonols, flavones, flavanones and dihydroflavonols in propolis extracts

Samples	Phenols (mg FAE mL^{-1} extract)	Flavonoids (mg QE mL^{-1} extract)	Dihydroflavones (mg (EE mL^{-1} extract)
1	6.861 ± 0.016^{b}	1.784 ± 0.012^{c}	0.257 ± 0.001^{b}
2	0.564 ± 0.016^{l}	0.097 ± 0.012^{jkl}	0.047 ± 0.001^{m}
3	1.101 ± 0.016^{i}	0.353 ± 0.012^{g}	0.092 ± 0.001^{h}
4	0.436 ± 0.016^{m}	0.088 ± 0.012^{kl}	0.050 ± 0.001^{m}
5	0.732 ± 0.016^{k}	0.143 ± 0.012^{ij}	0.070 ± 0.001^{k}
6	0.465 ± 0.016^{m}	0.107 ± 0.012^{jk}	0.060 ± 0.001^{l}
7	0.260 ± 0.016^{no}	0.028 ± 0.012^{mno}	0.031 ± 0.001^{o}
8	1.921 ± 0.016^{f}	0.402 ± 0.012^{f}	0.084 ± 0.001^{i}
9	0.443 ± 0.016^{m}	0.057 ± 0.012^{lmn}	0.057 ± 0.001^{l}
10	4.802 ± 0.016^{c}	0.781 ± 0.012^{d}	0.168 ± 0.001^{e}
11	0.699 ± 0.016^{k}	0.067 ± 0.012^{klm}	0.060 ± 0.001^{l}
12	0.318 ± 0.016^{n}	0.006 ± 0.012^{o}	0.038 ± 0.001^{n}
13	1.307 ± 0.016^{h}	0.186 ± 0.012^{i}	0.071 ± 0.001^{jk}
14	2.113 ± 0.016^{e}	0.409 ± 0.012^{f}	0.139 ± 0.001^{f}
15	1.929 ± 0.016^{f}	0.331 ± 0.012^{h}	0.176 ± 0.001^{d}
16	1.787 ± 0.016^{g}	0.297 ± 0.012^{h}	0.0142 ± 0.001^{f}
17	8.864 ± 0.016^{a}	4.320 ± 0.012^{a}	0.222 ± 0.001^{c}
18	0.802 ± 0.016^{j}	0.0136 ± 0.012^{j}	0.058 ± 0.001^{l}
19	0.477 ± 0.016^{m}	0.069 ± 0.012^{klm}	0.074 ± 0.001^{k}
20	2.429 ± 0.016^{d}	0.604 ± 0.012^{e}	0.109 ± 0.001^{g}
21	0.261 ± 0.016^{no}	0.020 ± 0.012^{mn}	0.077 ± 0.001^{j}
22	1.892 ± 0.016^{f}	0.0427 ± 0.012^{f}	0.138 ± 0.001^{f}
23	8.827 ± 0.016^{a}	2.699 ± 0.012^{b}	0.532 ± 0.001^{a}
24	0.039 ± 0.016^{o}	0.039 ± 0.012^{mno}	0.058 ± 0.001^{l}

Values with the same letter are not significantly different ($P < 0.05$) by Tukey's multiple range test.

range of those generally detected in methanolic extracts of Portuguese propolis (Miguel *et al.*, 2014a).

Inhibition of acetylcholinesterase

A possible strategy for the treatment of Alzheimer's disease, which is characterised by a decline in cognitive function and mental atrophy, may include the inhibition of acetylcholinesterase, the fundamental enzyme in the breakdown of acetylcholine by the termination of the nerve impulse transmission at cholinergic synapses (Miguel *et al.*, 2014b). Several propolis samples had similar and relatively high ability for inhibiting acetylcholinesterase: samples 1, 10, 14, 15, 16, 17, 21, 22 and 23 (Table 2). Although an inverse correlation between IC_{50} values and total phenols and flavonoids was observed ($P < 0.01$) (Table 3), which is representative of the importance of these groups of compounds for the inhibition of acetylcholinesterase activity, sample 8 had higher concentration of phenols than sample 21, but had lower activity. According to Miguel *et al.* (2014b), propolis samples collected in Moulay Bousselham, Sidi Sliman, Taza, Sefrou and Kenitra were not able to inhibit acetylcholinesterase, which was in contrast to the results obtained in the present study (Table 2). The importance of phenols and flavonoids present in Moroccan propolis samples was already reported (Miguel *et al.*, 2014b). In addition, the acetylcholinesterase inhibitory activity was also reported by Chen *et al.* (2008) for Chinese propolis, because water-soluble extracts, consisted of mainly flavonoids, significantly inhibited the acetylcholinesterase activity in the hippocampus of adult male Kunming mice compared to control mice given with normal saline solution. In spite of these findings by both Chen *et al.* (2008) and Miguel *et al.* (2014b), other group of compounds present in propolis samples has a role in the inhibitory activity of acetylcholinesterase, owing to the relatively good activity observed for sample 21 rather than the relatively low concentration of phenols and flavonoids.

Inhibition of α-amylase and α-glucosidase

Pancreatic α-amylase is an enzyme, such as α-glucosidase, of dietary carbohydrate digestion in humans. Inhibitors of α-amylase may be effective in retarding carbohydrate digestion and glucose absorption and, therefore, in suppressing postprandial hyperglycaemia (Tadera *et al.*, 2006).

α-Glucosidase is located in the brush border surface of the intestinal cell and catalyses the final step in the

Table 2 Enzyme inhibitory activities (IC_{50} = mg mL^{-1}) of hydroalcoholic extracts of Moroccan propolis harvested at different places

Samples	Acetylcholinesterase	α-Amylase	α-Glucosidase	Lipoxygenase	Tyrosinase	Xanthine oxidase
1	0.009 ± 0.051^{i}	0.065 ± 0.097^{l}	0.006 ± 0.016^{ef}	0.020 ± 0.019^{n}	0.050 ± 0.192^{j}	0.014 ± 0.052^{i}
2	–	2.020 ± 0.097^{f}	–	0.463 ± 0.019^{cde}	0.374 ± 0.192^{ij}	0.250 ± 0.052^{defg}
3	0.535 ± 0.051^{fg}	2.066 ± 0.097^{ef}	0.056 ± 0.016^{cdef}	0.406 ± 0.019^{efg}	–	0.245 ± 0.052^{defg}
4	0.446 ± 0.051^{g}	2.730 ± 0.097^{c}	0.068 ± 0.016^{cd}	0.653 ± 0.019^{a}	0.971 ± 0.192^{hi}	0.237 ± 0.052^{defg}
5	0.199 ± 0.051^{h}	3.304 ± 0.097^{b}	0.103 ± 0.016^{c}	0.568 ± 0.019^{b}	3.824 ± 0.192^{ab}	0.279 ± 0.052^{def}
6	1.453 ± 0.051^{c}	2.417 ± 0.097^{d}	0.062 ± 0.016^{cde}	0.646 ± 0.019^{a}	2.444 ± 0.192^{d}	0.585 ± 0.052^{c}
7	2.034 ± 0.051^{b}	1.263 ± 0.097^{hi}	–	–	–	0.753 ± 0.052^{b}
8	0.188 ± 0.051^{hi}	1.355 ± 0.097^{hi}	0.032 ± 0.016^{def}	0.478 ± 0.019^{cd}	1.595 ± 0.192^{fg}	0.362 ± 0.052^{de}
9	0.087 ± 0.051^{hi}	2.144 ± 0.097^{def}	0.027 ± 0.016^{def}	0.441 ± 0.019^{def}	1.844 ± 0.192^{ef}	0.245 ± 0.052^{defg}
10	0.007 ± 0.051^{i}	0.170 ± 0.097^{l}	0.007 ± 0.016^{ef}	0.086 ± 0.019^{m}	0.451 ± 0.192^{hij}	0.031 ± 0.052^{i}
11	1.341 ± 0.051^{cd}	1.249 ± 0.097^{hi}	0.018 ± 0.016^{def}	0.642 ± 0.019^{a}	–	0.179 ± 0.052^{efgh}
12	1.212 ± 0.051^{d}	2.853 ± 0.097^{c}	–	0.505 ± 0.019^{c}	4.286 ± 0.192^{a}	0.381 ± 0.052^{d}
13	0.648 ± 0.051^{f}	3.632 ± 0.097^{a}	0.746 ± 0.016^{a}	0.372 ± 0.019^{gh}	–	0.162 ± 0.052^{fgh}
14	0.002 ± 0.051^{i}	0.855 ± 0.097^{j}	0.019 ± 0.016^{def}	0.239 ± 0.019^{k}	0.295 ± 0.192^{j}	0.219 ± 0.052^{defg}
15	0.024 ± 0.051^{i}	1.484 ± 0.097^{gh}	0.027 ± 0.016^{def}	0.152 ± 0.019^{l}	0.612 ± 0.192^{hij}	0.076 ± 0.052^{hi}
16	0.007 ± 0.051^{i}	1.450 ± 0.097^{gh}	0.025 ± 0.016^{def}	0.256 ± 0.019^{jk}	3.600 ± 0.192^{bc}	0.125 ± 0.052^{fgh}
17	0.003 ± 0.051^{i}	0.146 ± 0.097^{l}	0.014 ± 0.016^{def}	0.065 ± 0.019^{mn}	0.110 ± 0.192^{j}	0.017 ± 0.052^{i}
18	0.123 ± 0.051^{hi}	2.338 ± 0.097^{de}	0.155 ± 0.016^{b}	0.574 ± 0.019^{b}	–	0.619 ± 0.052^{bc}
19	0.871 ± 0.051^{e}	0.524 ± 0.097^{k}	0.176 ± 0.016^{b}	0.324 ± 0.019^{hi}	2.288 ± 0.192^{de}	0.279 ± 0.052^{def}
20	0.160 ± 0.051^{hi}	0.493 ± 0.097^{k}	0.015 ± 0.016^{def}	$0.312 \pm 0.019_{ij}$	1.047 ± 0.192^{gh}	0.279 ± 0.052^{def}
21	0.009 ± 0.051^{i}	3.471 ± 0.097^{ab}	–	0.425 ± 0.019^{defg}	–	3.116 ± 0.052^{a}
22	0.013 ± 0.051^{i}	1.080 ± 0.097^{ij}	0.038 ± 0.016^{def}	0.253 ± 0.019^{jk}	3.173 ± 0.192^{c}	0.252 ± 0.052^{defg}
23	0.013 ± 0.051^{i}	0.018 ± 0.097^{l}	0.002 ± 0.016^{f}	0.046 ± 0.019^{mn}	0.037 ± 0.192^{j}	0.008 ± 0.052^{i}
24	3.555 ± 0.051^{a}	1.692 ± 0.097^{g}	–	0.393 ± 0.019^{fg}	–	0.391 ± 0.052^{d}

–: without activity.

Values in the same column followed by the same letter are not significantly different ($P < 0.05$) by Tukey's multiple range test.

Table 3 Spearman's correlation coefficients between total phenols and flavonoids and enzyme inhibitory activities

	Total phenol	Flavone + Flavonol	Flavanone + Dihydroflavonol
Total phenol	1	0.965**	0.858**
Flavone + Flavonol	0.965**	1	0.850**
Flavanone + Dihydroflavonol	0.858**	0.850**	1
Acetylcholinesterase	−0.638**	−0.648**	−0.750**
α-Amylase	−0.663**	−0.653**	−0.631**
α-Glucosidase	−0.729**	−0.663**	−0.687**
Lipoxygenase	−0.714**	−0.697**	−0.863**
Tyrosinase	−0.698**	−0.634**	−0.568*
Xanthine oxidase	−0.718**	−0.666**	−0.674**

*Correlation is significant at the $P < 0.05$ level. **Correlation is significant at the $P < 0.01$ level.

digestive process of carbohydrates, releasing absorbable monosaccharides. This process is responsible for the increased blood glucose levels (Zhang *et al*., 2015). When that enzyme is inhibited, the liberation of D-glucose from dietary complex carbohydrates can be retarded and may delay the digestion and absorption of carbohydrates and restrain postprandial hyperglycaemic episode (Zhang *et al*., 2015).

Concerning α-amylase, samples 1 and 23 had the lowest IC_{50} values (0.065 and 0.018 mg mL^{-1}, respectively), although not significantly different to those found for samples 10 and 17 (0.17 and 0.146 mg mL^{-1}, respectively) (Table 2). Such as observed for α-glucosidase, the α-amylase inhibitor activity of propolis extracts, expressed as IC_{50} values, is inversely correlated with total phenols and flavonoid concentrations ($P < 0.01$) (Table 3). Caffeoylquinic acid derivatives as well as flavonoids from Brazilian propolis have been reported as being able to inhibit both α-glucosidase and α-amylase activities and, therefore, with hypoglycaemic property (Matsui *et al*., 2004; Tadera *et al*., 2006).

Sample 23 (geographical origin unknown) was the best inhibitor of α-glucosidase (IC_{50} = 0.002 mg mL^{-1}), immediately followed by the samples 1 (Sefrou) and 10 (geographical origin unknown) (IC_{50} = 0.006 and 0.007 mg mL^{-1}, respectively) (Table 2). Such as reported for the inhibitory activities reported above, also in this case, the activity was inversely correlated with the amounts of total phenols and flavonoids (Table 3). The inhibitory activity of propolis extracts against α-glucosidase was already reported in other propolis extracts of different geographical origins (Zhang *et al*., 2015; Popova *et al*., 2015). Although two samples of propolis studied by Popova *et al*. (2015) were from the same city of those reported in the present work (2 and 9: Moulay Bousselhame; 8: Rabat), their activities were different. For the sample from Moulay Bousselham, the inhibitory activities, expressed as IC_{50} values, of α-glucosidase and α-amylase were 0.07 and 0.52 mg mL^{-1}, respectively, while in the present work such IC_{50} values for α-glucosidase inhibitory activities ranged from without activity (sample 2) up to 0.027 mg mL^{-1} (sample 9). Concerning α-amylase inhibitory activities, IC_{50} values were 2.0–2.1 mg mL^{-1} for samples 2 and 9, respectively. For the sample from Rabat, Popova *et al*. (2015) found 0.010 and 0.090 mg mL^{-1} as IC_{50} values for α-glucosidase and α-amylase inhibitory activities, respectively, that is lower than those observed for sample 8 (0.032 and 1.36 mg mL^{-1}) (Table 2).

The lowest IC_{50} values found in the present work are similar to those reported by Zhang *et al*. (2015), for aqueous ethanol extracts of Brazilian propolis. The hypoglycaemic activity of propolis when mixed with mulberry leaf extract was already reported by Murata *et al*. (2004) after the administration to patients with type 2 diabetes.

Inhibition of lipoxygenase

After the oxidation of arachidonic acid, 5-lipoxygenase is responsible for the formation of the proinflammatory leukotrienes. Linoleic acid is also a substrate for this enzyme, which is enzymatically converted by 5-lipoxygenase to a conjugated diene, which results in a continuous increase in absorbance at 234 nm (Miguel *et al*., 2011).

Sample 1 had the lowest IC_{50} value (0.020 mg mL^{-1}) – so that it had the ability to inhibit 5-lipoxygenase – immediately followed by the sample 17 and sample 23 (IC_{50} = 0.065 and 0.046 mg mL^{-1}, respectively) (Table 2). These values are significantly lower than those previously reported by Miguel *et al*. (2014b) for Moroccan propolis extracts from different origins. Although the samples possess the same harvesting place (Moulay Bousselham, Rabat, Sidi Sliman, Taza, Sefrou and Kenitra) as the samples investigated by Miguel *et al*. (2014b), only that from Rabat had higher IC_{50} value. A negative correlation was observed between the IC_{50} values and the concentration of total phenols and flavonoids (Table 3),

particularly flavanones and dihydroflavonols. This type of correlation was already reported for Moroccan propolis (Miguel *et al*., 2014b).

Mirzoeva & Calder (1996) found that propolis and its components caffeic acid phenethyl ester (CAPE), caffeic acid (CA), quercetin and naringenin significantly suppressed the lipoxygenase pathway of arachidonic acid metabolism during inflammation *in vivo*. However, CAPE was the most potent modulator of the arachidonic acid metabolism among the propolis components studied by the authors. However, according to Araújo *et al*. (2012), the anti-inflammatory activity of propolis seems to be particularly associated with the presence of flavonoids, especially galangin and quercetin. These flavonoids have been shown to inhibit the activity of lipoxygenase, among other enzymes and factors responsible for inflammation processes: inhibition of cyclooxygenase, reduction in the levels of PGE_2 as well as in the release and expression of the induced isoform cyclooxygenase-2 (COX-2). In the present work, the compounds were not identified; nevertheless, the correlation between the inhibitory activity and flavanones and dihydroflavonol contents was strong compared to the remaining correlations between phenol content and inhibitory activity, which may suppose to have higher importance of those kinds of compounds in the lipoxygenase inhibitory activity.

Inhibition of tyrosinase

The tyrosinase inhibition by propolis samples is depicted in Table 2. Samples 1 and 23 were the most active against this enzyme, with IC_{50} values of 0.050 and 0.037 mg mL^{-1}, respectively. These values were not significantly different (Table 2). An inverse correlation was observed between the inhibition activity presented as IC_{50} values and the concentrations of total phenols and flavonoids, demonstrating that these compounds have an important role in the inhibition of tyrosinase (Table 3). Some flavonoids have demonstrated to be able to inhibit mushroom tyrosinase by the chelation of active sites. Examples include the chelation reactions between the vicinal 3′,4′-dihydroxyl group of catechin and the Cu^{2+} on tyrosinase, or still the structure of 3-hydroxy-4-keto moiety in some flavonoids, such as quercetin (Fu *et al*., 2005). Other studies revealed that caffeic acid phenethyl ester suppresses melanogenic enzyme expression, but not via a direct inhibition of tyrosinase activity itself (Lee *et al*., 2013).

Inhibition of xanthine oxidase

The inhibitory activity expressed as IC_{50} values is presented in Table 2. Without significant differences ($P < 0.05$), the propolis samples 1, 10, 17 and 23 possessed the lowest IC_{50} values (0.014, 0.031, 0.017 and 0.008 mg mL^{-1}, respectively) and therefore are considered as the best ones for inhibiting xanthine oxidase activity (Table 2). As reported for the other inhibitory enzyme activities, there was an inverse correlation between IC_{50} values and the amounts of phenols and flavonoids (Table 3). According to Cos *et al*. (1998), flavonoids possess the capacity for inhibiting xanthine oxidase – mainly those showing the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 – and found that the presence of a hydroxyl group at C-3 would slightly decrease the inhibitory activity. Yoshizumi *et al*. (2005) reported that propolis from China with relatively high amounts of chrysin, galangin and caffeic acid phenethyl ester possessed higher inhibitory activity than the Brazilian propolis with higher amounts of *p*-coumaric acid and artepillin C and also concluded that a continuous intake of propolis could be effective for the prevention and the treatment of gout and hyperuricaemia.

Inhibition of hyaluronidase

Figure 1 presents the percentage of inhibition of hyaluronidase obtained from Moroccan propolis extracts when at a final concentration of 33.33 mg mL^{-1}. Samples 1, 8, 10, 14, 17, 20 and 22 had percentages of inhibition higher than 80%, whereas the samples 7, 12 and 21 even at those relatively high concentrations (33.33 mg mL^{-1}) did not present the ability for inhibiting the hyaluronidase enzyme.

The capacity of Portuguese propolis extracts for inhibiting hyaluronidase was already reported (Silva *et al*., 2012). The values found by these authors were also dependent on the place of harvesting, such as observed by us in the present work. Silva *et al*. (2012) did not find a correlation between phenol contents and activity, suggesting that other components present in the propolis extracts, such as proteins and vitamins, might also be involved in the activity.

Statistical analysis

A combination of cluster analysis using Ward's technique (Fig. 2a) revealed the presence of three principal clusters with respect to the phenol, flavone, flavonol, flavanone, dihydroflavonol content, α-amylase and xanthine oxidase inhibitory activities of 24 samples. Samples belonging to cluster 1 included propolis sample 1 (region Fez-Boulemane), 10 (purchased from the market), 17 (region Fez-Boulemane) and 23 (purchased from the market). These samples had the highest concentration of all types of phenols as well as the highest inhibitory activities of xanthine oxidase and α-amylase. Cluster 2 included samples 2–7, 9, 11-13, 18, 19, 21 and 24, which possessed the lowest activities as well as the lowest amounts of all types of

phenols. The harvesting places for these propolis samples are widespread all over the country. Samples 8 (region Rabat), 14 (region Gharb), 15 (region Rabat), 16 (region Gharb), 20 and 22 (both from Fez-Boulemane) possessed intermediate concentrations of phenols and inhibitory activities. Some of these samples (14 and 16) were collected in regions of Gharb in which other samples were also harvested and had lower amounts of phenols and lower activities (samples 9 and 13 from cluster 2) and therefore belonged to the cluster 2. Samples in cluster 3 (samples 20 and 22, which were harvested in Fez-Boulemane region) and the samples 1 and 17 in cluster 1 had the best inhibitory activities as well as the highest phenol content (Tables 1 and 2).

Three principal clusters with respect to the phenol, flavone, flavonol, flavanone, dihydroflavonol contents and α-glucosidase, lipoxygenase, acetylcholinesterase and tyrosinase inhibitory activities (Fig. 2b) were found for 15 samples. Samples 1, 10, 17 and 23 belonged to cluster 1; samples 4–6, 9, 15, 19 and 22 belonged to cluster 2; and samples 8, 14, 16 and 20 belonged to cluster 3. The distribution of samples by clusters was the same as that of reported above. A drawback of the present work is the limited number of samples, which makes it difficult to draw conclusions; nevertheless, it is possible to find propolis samples in the same region having dissimilar phenol contents and enzyme inhibitory activities. In addition, great part of samples had relatively small amounts of phenols and consequently weak enzyme inhibitory activities. In this way, the activities correlated well with the phenol content, but in the same region, it was possible to find the samples with dissimilar properties.

Conclusions

Propolis samples from different places of Morocco had diverse amounts of total phenols, flavones, flavonols, flavanones and dihydroflavonols. Those samples with higher concentrations of these groups of compounds generally had the best capacity to inhibit the activities of acetylcholinesterase, α-amylase and α-glucosidase, lipoxygenase, tyrosinase, xanthine oxidase and hyaluronidase. A correlation between harvesting place of propolis samples and phenol content or inhibitory enzyme activities was not found in the present work, because in the same place, samples with dissimilar phenol contents and enzyme activities were found. In addition, dissimilar activities and phenol contents were also found in the three propolis samples purchased from the market, which in turn reveals the importance of normalisation of these natural products because propolis sold in the market shows distinct efficacious activities, not consistent with the requirements of quality, efficiency and safety that these products should have.

Even in the absence of a deep chemical characterisation of samples, it was possible to conclude the importance of polyphenols in inhibiting enzyme activities given the correlations found between polyphenol contents and activities. For the inhibitory activity of the enzymes studied in the present work, the results may suggest that a phenol normalisation of the extracts may be enough, without the need for chemically characterising them. Such results must be the target of further study for deeply evaluating the possibility to use these products in the combat of some diseases involving inflammation, hyperuricaemia or gout,

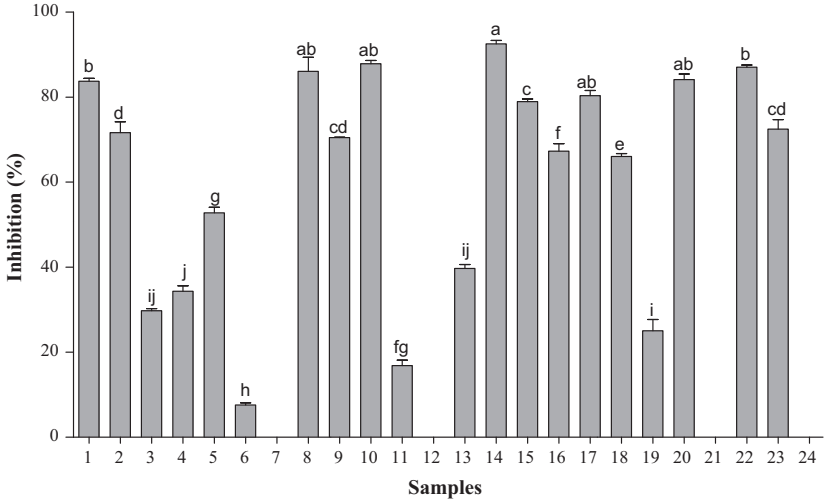

Figure 1 Percentage of hyaluronidase inhibition obtained from propolis extracts (33.33 mg mL^{-1}) collected at different places of morocco. Bars represent standard deviations ($n = 3$). In many cases, such bars may not be visible due to the very low standard deviations. Values with the same letter are not significantly different ($P < 0.05$) by Tukey's multiple range test.

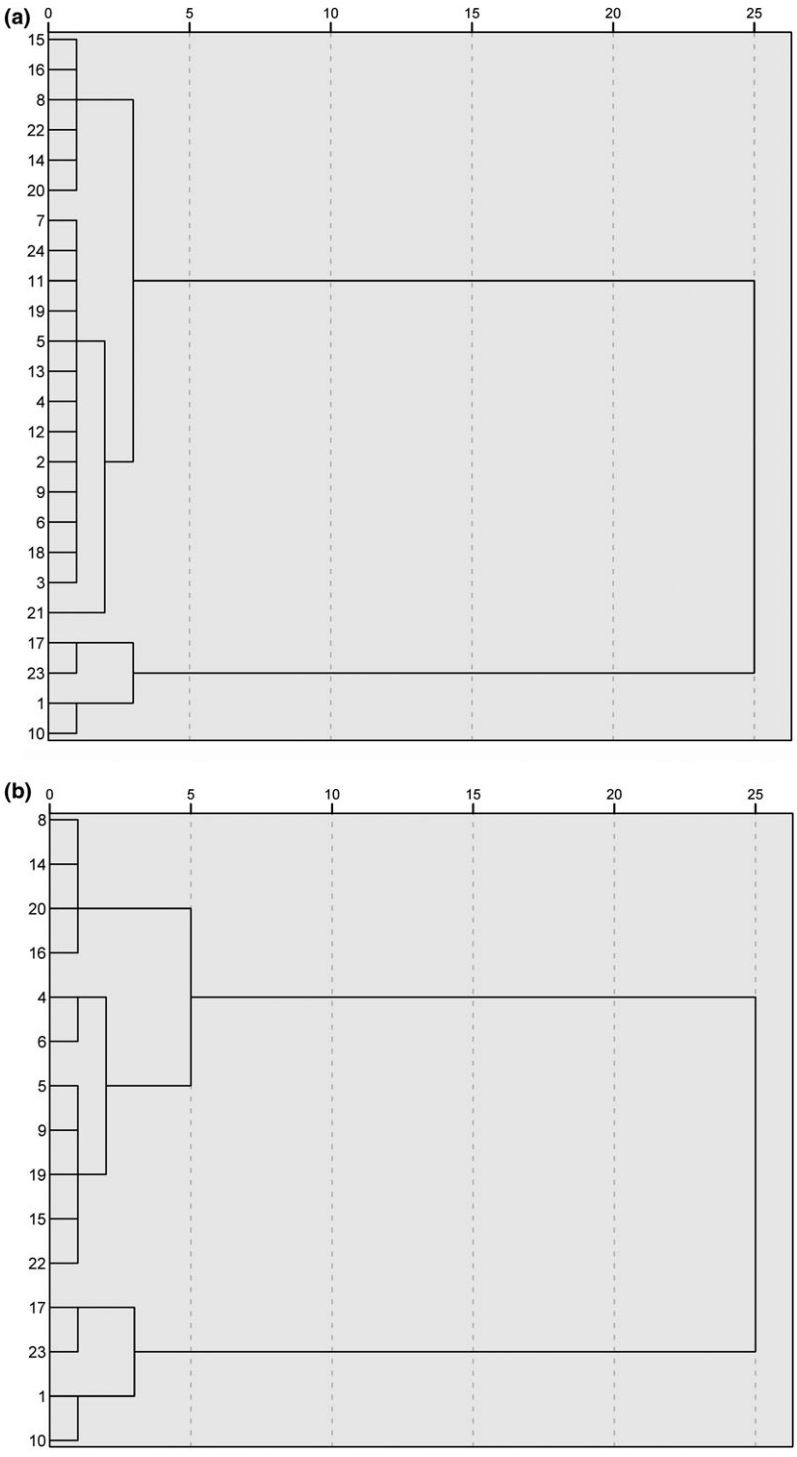

Figure 2 (a) Dendrogram obtained from the cluster analysis of 24 samples of propolis from Morocco with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols contents and the capacity to inhibit xanthine oxidase and α-amylase. Samples were clustered using Ward's technique with the squared Euclidean distance measure. (b) Dendrogram obtained from the cluster analysis of 15 samples of propolis from Morocco with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols contents and the capacity to inhibit acetylcholinesterase, α-glucosidase, lipoxygenase and tyrosinase. Samples were clustered using Ward's technique with the squared Euclidean distance measure.

hyperglycaemia, in those diseases characterised by a decline in cognitive function and mental atrophy, or as agents still for the treatment of hyperpigmentation of the human skin.

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Conflict of interest

The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Harvesting places of Moroccan propolis and their coordinates as well the surrounding plants.