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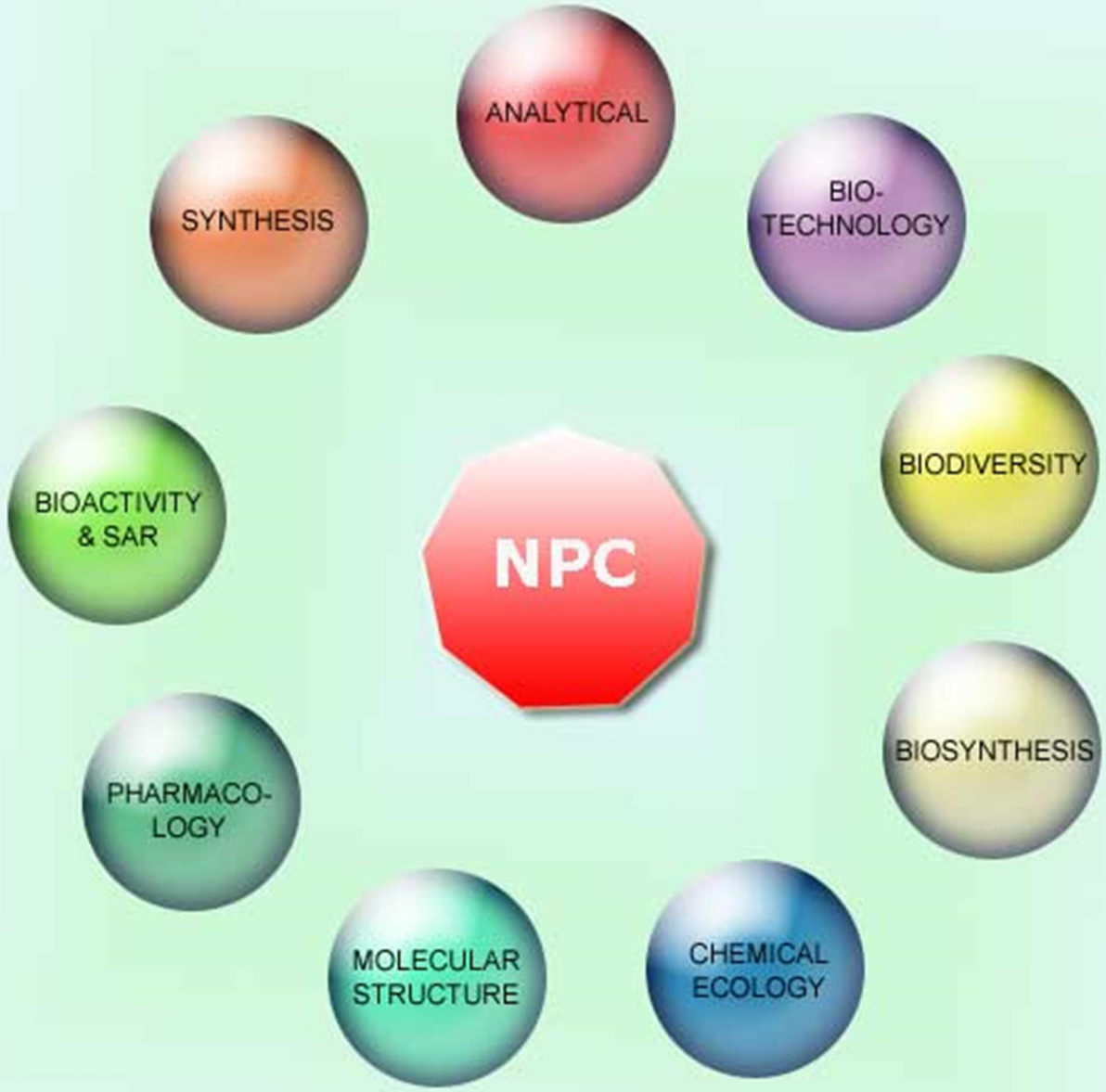

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Antioxidant and α-Glucosidase Inhibitory Properties and Chemical Profiles of Moroccan Propolis

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The chemical profiles of propolis hydroalcoholic extracts from different regions of Morocco were studied by gas chromatography coupled to mass spectrometry after silylation. Samples from Khamissat and Imouzzer belong to the poplar type, as typical poplar flavonoids predominated. Propolis from Rabat also contained high percentage of flavonoids, but had significantly lower amount of phenolic acid esters and contained no pinobanksin-3-*O*-acetate. Propolis from Bhalil demonstrated a profile rich in diterpenes (74.3%), and is a typical Mediterranean propolis sample. All samples had the capacity for inhibiting glucosidase and amylase enzymes, as well as the capacity for scavenging free radicals and preventing lipid peroxidation. Both activities were significantly higher in the flavonoids-rich samples.

Keywords: Flavonoids, Diterpenes, Propolis, Antioxidant activity, Glucosidase inhibition, Amylase inhibition.

Bees collect resinous materials actively secreted by plants or exuded from wounds in plants, which they mix with wax to produce propolis or bee glue, used as a building material in the hive [1,2]. The chemical composition of propolis depends on factors such as the botanical origin, seasonality, altitude and collector type [3-11].

The diverse and valuable biological properties of propolis are attributed mainly to the presence of polyphenols [2,12]. However, the diterpenes occurring in propolis from the Mediterranean basin also possess biological properties [11]. Flavonoids have been reported to be predominant compounds in propolis collected in Morocco after analysis by HPLC-ESIMS [13]. The antioxidant, anti-inflammatory, anti-acetylcholinesterase and anticancer activities of propolis from different regions of Morocco have been reported [13,14]. In the present work, the chemical composition of hydroalcoholic extracts of propolis from different regions of Morocco (Table S1) was studied in detail by GC-MS, together with their antioxidant and hypoglycaemic activities. Samples were collected taking in three different regions of Morocco: Region Fez (Bhalil and Immouzzer); region Rabat (Rabat and Khamissat); and Region Gharb (Moulay Bouslham).

The contents of waxes, resins and balsams of propolis samples from different regions of Morocco are depicted in Table 1. Great variability of wax percentage was observed. Propolis from Moulay Bousselham had the highest wax content (87.7%), in contrast to that from Rabat, which presented the lowest wax content and the highest percentage of resin. According to Burdock [15] the ratio between wax and plant resin may be a compromise between resins availability and use of propolis. If plant resins are scarce or difficult to collect, bees incorporate more wax in propolis. If bees need to repair honeycomb, they use larger quantities of wax to enhance the firmness of propolis.

Chemical profiling of the propolis samples was performed by GC-MS after silylation of dried extracts and the compounds identified in each sample are depicted in Table S2. The concentrations of groups of bioactive constituents are represented in Figure 1. The observed chemical profiles clearly separate the samples with respect to their source plant(s). Samples from Khamissat and Imouzzer were typical poplar propolis, rich in flavonoid aglycones (pinocembrin, galangin, chrysin, pinobanksin-3-*O*-acetate) and phenolic acid esters (pentenyl caffeates and phenylethyl caffeate CAPE).The Rabat sample was close to them in its high amount of "poplar type" flavonoids, but had significantly lower percentage of phenolic acid esters and contained no pinobanksin-3-*O*-acetate.

Table 1: Wax, balsam, and resin contents of propolis samples.

Sample	Waxes (%, w/w)	Resins (%, w/w)	Balsams (%, w/w)
Moulay Bouslham	87.7	3.3	0.9
Bhalil	16.7	30.8	0.2
Rabat	2.8	51.8	0.5
Khamissat	16.2	41.1	1.8
Imouzzer	16.3	39.0	1.9

In propolis from Bhalil, diterpenes prevailed (Figure 1). Major diterpenes in the extract were isocupressic acid (19.5%), agathadiol (11.3%), totarol (10.1%), 13-*epi*-cupressic acid (9.5%), communic acid (7.9%) and imbricataloic acid (4.8%) (Table S2). This is a typical Mediterranean propolis sample [10,11], which originates from *Cupressus sempervirens* resin [16], although in the same region (Fez), in the sample from Immouzzer flavonoid aglycones and phenolic acid esters predominated.

Relatively low and almost similar levels of flavonoids (8.5%), diterpenes (10.5%) and triterpenes (9%) characterized the chemical profile of the sample from Moulay Bousselham. Among the diterpenes, the major ones were isocupressic acid (3.2%), imbricataloic acid (2.7%) and totarol (1.5%), whereas in the flavonoid group, galangin (1.2%), chrysin (1.1%), pinocembrin and its chalcone (1% and 1.7%) and pinobanksin 3-*O*-acetate predominated. This sample is like a mixed poplar-Mediterranean type. Diterpenes were absent in propolis extracts from Rabat and Imouzzer (Table S2 and Figure 1).

Table 2: Antioxidant and hypoglycaemic activities of hydro-alcoholic extracts of Moroccan propolis.

Sample	Superoxide (IC_{50}=mg/mL)	NO (IC_{50}=mg/mL)	Molybdate (AAE=mg/mL)	Liposomes (IC_{50}=mg/mL)	Chelating (IC_{50}=mg/mL)	Glucosidase (IC_{50}=mg/mL)	Amylase (IC_{50}=mg/mL)
Moulay Bousselham	2.31±0.05^{a}	2.90±0.08^{a}	12.47±0.33^{d}	0.61±0.01^{a}	1.17±0.14^{d}	0.07±0.01^{a}	0.52±0.01^{a}
Bhalil	1.22±0.05^{b}	0.27±0.08^{c}	28.41±0.33^{a}	0.05±0.01^{c}	1.79±0.14^{c}	0.02±0.01^{b}	0.39±0.01^{b}
Rabat	0.15±0.05^{c}	0.73±0.08^{b}	22.77±0.33^{c}	0.03±0.01^{d}	0.13±0.14^{e}	0.01±0.01^{c}	0.09±0.01^{c}
Khamissat	0.23±0.05^{c}	0.13±0.08^{cd}	24.36±0.33^{b}	0.07±0.01^{b}	7.17±0.14^{b}	0.02±0.01^{b}	0.13±0.01^{d}
Imouzzer	0.25±0.05^{c}	0.08±0.08^{d}	24.81±0.33^{b}	0.04±0.01^{cd}	7.83±0.14^{a}	0.02±0.01^{b}	0.31±0.01^{c}

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

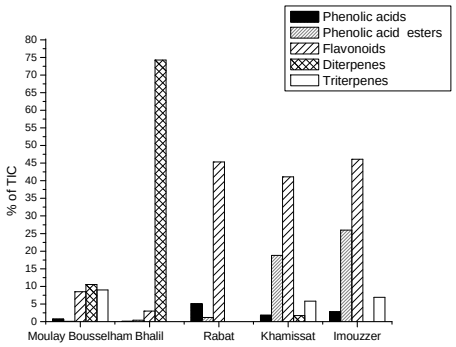

Figure 1: Groups of compounds present in propolis extracts from diverse locations of Morocco.

It is interesting to note that triterpenes were found in samples from Moulay Bousselham (9%), Imouzzer (6.1%) and Khamissat (4.5%,). However, the triterpenes in these samples were not identical. In Moulay Bousselham propolis, the major ones were lupeol, α-amyrin, lupeol acetate, and α-amyrin acetate. In the Imouzzer sample, lanosterol, cycloartenol and 24-methylene cycloartenol were identified as major triterpenes. In the sample from Khamissat, all the above mentioned triterpenes were present as major constituents of the triterpene mixture. Obviously, there are different sources of triterpenes used by bees in these regions, but their identification is difficult, because these triterpenes are widespread and are produced by many plants.

Extracts of propolis have already been reported to control blood glucose and modulate the metabolism of glucose and blood lipid, diminishing lipid peroxidation and level of free radicals in rats with diabetes mellitus [17-20]. Some of these studies demonstrated that phenolics were responsible for such activities [17].

The antioxidant and hypoglycemic activities of hydro-alcoholic propolis extracts from different locations in Morocco are presented in Table 2. All of them demonstrated both activities, but differences were found among them. These differences may be connected to their distinct chemical composition. The propolis sample from Rabat had the best capacity for inhibiting *in vitro* glucosidase and amylase. This sample also had the best capacity for preventing lipid peroxidation by inhibiting the oxidation of lecithin of liposomes. Propolis from Imouzzer was the most active in scavenging nitric oxide. The capacity for scavenging superoxide anion radicals was not significantly different among the samples from Rabat, Khamissat and Imouzzer (Table 2).

Small intestinal α-glucosidase and pancreatic α-amylase are two enzymes involved in the dietary carbohydrate digestion in humans [21]. The inhibition of these enzymes can significantly retard carbohydrate digestion and glucose absorption and, therefore, decrease the postprandial increase of blood glucose after a mixed carbohydrate diet [22,23].

Our work demonstrated that the capacity of inhibiting glucosidase and amylases, as well as the capacity for scavenging free radicals and preventing lipid peroxidation were better in those samples in which phenolics and particularly flavonoids predominated. There was a statistically significant correlation between chelating ability and the amount of phenolic acid esters ($R = 0.97221$, $p < 0.05$), and between superoxide radical inhibition and the amount of flavonoids ($R < -0.86575$, $p = 0.05$) in our samples. Flavonoid concentration was correlated with the glycosidase and amylase inhibition, but these correlations were not statistically significant. For the hydro-alcoholic extract of Bhalil propolis, in which diterpenes predominated, the antioxidant and hypoglycemic activities were lower compared with the samples in which flavonoids prevailed. The sample from Moulay Bousselham, with low amounts of phenolics and diterpenes, had the lowest activities. Our results demonstrate the importance of phenolics with respect to the antioxidant and hypoglycemic activities.

Hydro-alcoholic extracts of propolis from 5 different regions of Morocco were chemically analysed. The results demonstrated that the propolis composition in Morocco varies and poplar type propolis is present along with Mediterranean (cypress type) and mixed type propolis. The capacity for inhibiting glucosidase and amylase enzymes, as well as the capacity for scavenging free radicals and preventing lipid peroxidation were better in those samples in which phenolics and especially flavonoids predominated.

Experimental

Propolis: The coordinates (latitude, longitude and altitude) of locations where propolis samples were collected in 2012 are presented in Table S1.

Extraction: Hydroalcoholic extracts were obtained, as described in [14]. Wax, balsam and resin extractions were carried out as previously described in [24].

GC–MS analysis: This was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 60 to 300°C at a rate of 5°C/min, and a 10 min hold at 300°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:10, the injector temperature 280°C, the interface temperature 300°C, and the ionisation voltage 70 eV. The identification of the compounds was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds. Semiquantification was carried out by internal normalisation with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

Superoxide anion scavenging activity: Measurements of superoxide anion scavenging activity of samples were based on the method described by Soares [25]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide

adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). The superoxide anion was generated in 300 µL of phosphate buffer (19 mM, pH7.4) containing NBT (43 µM) solution, NADH (166 µM) solution and different concentrations of propolis. The reaction was started with the addition of PMS solution (2.7 µM) to the mixture. The reaction mixture was incubated at 20°C for 7 min and the absorbance reading was performed at 560 nm in a microplate reader. The percentage of inhibition was calculated using the following equation: Inhibition = [(*A*0-*A*1)/*A0*] × 100 (%), where *A*0 is the absorbance of the control (without sample) and *A1* is the absorbance in the presence of the sample. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against propolis concentrations.

Nitric oxide scavenging capacity: The nitric oxide (NO) scavenging activity of samples was measured according to a described method [26]. Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. In this method, 50 µL of a serially diluted sample of propolis was added to 50 µL of 10 mM sodium nitroprusside in phosphate buffer saline (PBS) in a 96-well plate and the plate was incubated at room temperature for 90 min. Finally, an equal volume of Griess reagent was added to each well and the absorbance was read at 546 nm. Several concentrations of samples were made and the percentage inhibition calculated from the formula: [1-(A_{sample} - $A_{sample\ blank}$)/($A_{control}$-$A_{control\ blank}$)]*100, where (A_{sample} −$A_{sample\ blank}$) is the difference in the absorbance of a sample, with or without 10 mM sodium nitroprusside, and ($A_{control}$ − $A_{control\ blank}$) is the difference in the absorbance of the PBS control, with or without 10 mM sodium nitroprusside. Percentage inhibition was plotted against sample concentration and IC_{50} was determined as the concentration of sample able to scavenge 50% of NO free radical.

Evaluation of total antioxidant capacity by phosphomolybdenum method: The antioxidant activities of samples were evaluated by the phosphomolybdenum method [27] and expressed relative to that of ascorbic acid. Briefly, an aliquot of 0.1 mL of propolis sample was mixed with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Ethanol 70 % was used as blank instead of propolis solution. The reaction mixture was vortex-mixed and let to stand in a water bath at 95ºC for 90 min. Absorbance was measured at 695 nm. The experiment was conducted in triplicate and values are expressed as equivalents of ascorbic acid in µg per mg of extract.

Liposomes: Liposomes were obtained from 0.4 g lecithin in 80 mL chloroform. This solution was dried and submitted to flux for 30 s. Liposomes were then subjected to vacuum for at least 2 h until complete dryness. Eighty mL of phosphate saline buffer 0.01 M, pH 7.0 were added and kept at 4ºC until the assay. A mixture that contained 0.2 mL of liposomes homogenate, 50 µL of 0.1 mM ascorbic acid, 50 µL of 4 mM $FeCl_2$, and 50 µL of test compounds was incubated for 1 h at 37°C. After incubation, 2 mL of 0.6% TBA was added. The mixture was heated for 10 min in a boiling water bath. After cooling, at room temperature, the contents of the tubes were stirred and centrifuged at 3,000 rpm for 10 min. The absorbance of the sample was read at 532 nm against a blank, which contained all reagents except liposomes homogenate.

Chelating metal ions: The degree of chelating of ferrous ions by propolis was evaluated [28]. Briefly, samples were incubated with 0.05 mL of $FeCl_2.4H_2O$ (2 mM). The addition of 0.2 mL of 5 mM ferrozine initiated the reaction, and after 10 min, the absorbance at 562 nm was measured. An untreated sample served as the control. The percentage of chelating ability was determined according to the following formula: [(A0–A1)/A0*100], in which A0 is the absorbance of the control and A1 the absorbance of propolis sample.

α-Glucosidase inhibitory activity: The α-glucosidase inhibitory activity was assessed by the method previously reported [29], with slight modifications. Briefly, 25 µL of propolis extract at different concentrations and 50 µL of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/mL) was incubated in 96 well plates at 37ºC for 20 min. After pre-incubation, 50 µL of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 ºC for another 20 min. Then the reaction was stopped by adding 160 µL of 0.2 M Na_2CO_3 into each well, and absorbance readings (A) were recorded at 405 nm by a micro-plate reader and compared with a control which had 25 µL of ethanol 70% in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows: Inhibition = [(A0-A1)/A0] × 100 (%), where A0 is the absorbance of the control (without sample) and A1 is the absorbance in the presence of the sample.

α-Amylase inhibitory activity: Starch-iodine color assay: Starch-iodine color assay screening of Moroccan propolis extracts for α-amylase inhibitors was carried out [30] with slight modification based on the starch-iodine test. Hydro-alcoholic extract of Moroccan propolis of varied concentrations (100 µL) was added to 100 µL of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) containing 0.04 units of α-amylase solution and incubated at 37°C for 10 min. Then 100 µL soluble starch (1%, w/v) was added to each reaction well and incubated at 37°C for 15 min. 1 M HCl (20 µL) was added to stop the enzymatic reaction, followed by the addition of 100 µL of iodine reagent (5 mM I_2 and 5 mM KI). The color change was noted and the absorbance was read at 620 nm on a microplate reader. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls without the enzyme were also included. Inhibition of enzyme activity was calculated as: Inhibition of enzyme activity (%) = (Abs2 -Abs1) / (Abs4 -Abs3) × 100, where Abs 1 is the absorbance of incubated solution containing sample, starch, and amylase, Abs 2 is the absorbance of incubated solution containing sample and starch, Abs 3 is the absorbance of incubated solution containing starch and amylase, and Abs 4 is the absorbance of incubated solution containing starch. A dark-blue color indicates the presence of starch; a yellow colour indicates the absence of starch while a brownish colour indicates partially degraded starch in the reaction mixture. In the presence of inhibitors from the extracts the starch added to the enzyme assay mixture is not degraded and gives a dark blue colour complex whereas no colour complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolysed by α-amylase.

Supplementary data: Tables S1 and S2, Chemical profile of Moroccan propolis samples obtained by GC–MS. Results are shown as % of total ion current.

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