

ASSESSMENT OF PHENOLIC AND FLAVONOID CONTENTS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF MOROCCAN PROPOLIS

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ABSTRACT

Background. Despite the extensive literature focused on propolis extract, few data exists on the bioactive compounds and biological activities in the Moroccan propolis and its economic value is low.

Objective. In this research, the aim was to evaluate the total content of phenols and flavonoids as well as the antioxidant, antibacterial and antifungal activities of Moroccan propolis.

Material and Methods. The polyphenol and flavonoid content of the Moroccan propolis from three geographic regions, was quantified in the ethanolic extract by colorimetric methods using folin-ciocalteu and aluminum chloride. The antioxidant activity was evaluated by the DPPH test and expressed as IC50. Disk diffusion and broth microdilution methods were used to examine in vitro antimicrobial activity against known human microorganism pathogens.

Results. The obtained data revealed that Moroccan propolis samples presented significant variations in total polyphenols and flavonoids. All samples showed significant antioxidant activity with IC50 values ranging from 4.23±0.5 to 154±0.21 µg/mL. A strong correlation between total phenolic activity, flavonoids and antioxidant activity was found. The in vitro study of antibacterial activity showed that the propolis samples exhibited a range of growth inhibitory actions against all bacterial strains tested with the highest activity against gram-positive bacteria. Only propolis from the Sidi Bennour region demonstrated an antifungal activity.

Conclusion. The study data show that Moroccan propolis extracts have a promising content of antioxidant and antimicrobial compounds that could be exploited to prevent certain diseases linked to oxidative stress and pathogenic infections.

Key words: propolis, polyphenols, antibacterial activity, antifungal activity, Morocco

INTRODUCTION

Propolis is a sticky, resinous, darker-green material produced by *Apis mellifera* honey bees using buds and exudates of various plants of the native vegetation near their hive [1]. Honeybees use propolis as an insulating material and as a sealant for cracks and openings in the hive, also as a barrier against external reats and climatic conditions [2]. Since the antiquity, propolis has been used by Romans, Egyptians, Greeks and Persians civilizations as a raw material for numerous pharmaceutical preparations [3]. The composition of this beekeeping product is quite complex and varied throughout the world. Generally, raw propolis consisting of resin and vegetal balsams (~50%), wax

(~30%), essential and aromatic oils (~10%), pollen (~5%) and other substances including, minerals, polysaccharides, proteins, vitamins, aliphatic and aromatic acids and esters, flavonoids and other plant phenolics and terpenoids [4]. The chemical composition is considerably affected by botanical source, collection region, harvest season, climate environmental conditions [5]. Different biological and pharmacological effects have been linked to propolis constituents. These include, but not limited to antioxidant, antimicrobial, antiviral, antitumor, antidiabetic, anti-inflammatory, antiallergic activities. It would also have immunomodulatory, cardioprotective, hepatoprotective, neuroprotective, and hypoglycemic properties [1, 2, 6]. Propolis

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has become the subject of extensive biological and chemical studies in recent decades, mainly for its applications in medicine, the food industry, cosmetics, veterinary medicine and animal husbandry. However, Moroccan propolis is little studied and few studies have recently addressed its chemical composition and biological properties [7-12]. With a view to contributing to the knowledge of Moroccan propolis, the aim of this present work was to determine the chemical composition, antimicrobial and antioxidant activities of three samples taken from regions of different geographical origins in Morocco.

MATERIAL AND METHODS

Propolis samples

Propolis samples were collected by professional beekeepers from *Apis mellifera* hives located in three regions of Morocco. Table 1 provides detailed information on the floral origin, area of origin, geographic location and date of collection of the propolis samples.

Equipment

A Spectrophotometer-UV/Visible (Jenway 6300, USA), biomerieux densitometer (ATB 1550, Italy), Büchi rotavapor (Type R110, Switzerland) were used.

Chemical reagents

Sodium carbonate, gallic acid, folin-ciocalteu reagent, sodium nitrite, aluminum chloride, sodium hydroxide, quercetin, 2,2-diphenyl-2 picrylhydrazyl and ethanol were obtained from Sigma – Aldrich (Germany).

Propolis extraction

After their collection, the propolis samples were cut into small portions and ground in a mortar to obtain homogeneous powders and then extracted by maceration method using ethanol 70% (70:30 ethanol: water) as a solvent, they underwent extraction by maceration method using 70% ethanol as a solvent (ethanol 70:30 water). The 70% ethanol is one of the most used solvents for propolis extraction, particularly for the extraction of phenolic and flavonoid compounds [13]. The extract obtained is then filtered through Whatman filter paper (No. 4) and the solvent is removed by evaporation at 40°C under reduced pressure using a rotavapor.

Determination of total phenolic content

The quantification of the total phenolic compounds (TPC) in the propolis extracts, was carried out by the colorimetric method of folin-ciocalteu according to Loizzo et al. [14]. Gallic acid was used to calculate the standard curve and the resulting values were expressed in milligrams per gram of gallic acid equivalent (mg GAE/g).

Determination of total flavonoids content

The content of the total flavonoid compounds (TFC) was quantified in the propolis extracts by the colorimetric method of aluminum chloride as previously used according to Aboukhalaf et al. [15]. The mean of three readings was used and total flavonoids was expressed as milligrams per gram of catechin equivalent (mg CE/g).

In vitro antioxidant evaluation

The free radical scavenging potentials of propolis ethanolic extracts were assessed using the 2,2-diphenyl-2 picrylhydrazyl (DPPH●) method described by Aboukhalaf et al [15]. The test was performed in triplicate and expressed as an IC50 value (concentration in µg/ml needed to scavenge the 50% of initial DPPH). Ascorbic acid was used as standard antioxidant.

Antimicrobial activity

Microorganism strains

Four strains of bacteria (two strains of gram-positive bacteria and two strains of gram-negative bacteria) and two strains of fungi were obtained from the American Type Culture Collection (ATCC) and the Institute Pasteur Paris Collection (CIP), and were used in this study: *Escherichia coli* (CIP54127), *Pseudomonas* sp, *Enterococcus faecalis* (ATCC19433), *Staphylococcus aureus* (ATCC25923), *Cryptococcus neoformans* (CIP 960) and *Candida albicans* (48.72). All the strains were maintained at freeze temperature until use.

Disk diffusion assay

Screenings of extract for antimicrobial activity was done using the disc diffusion method [16]. In order to do this, bacterial and fungal sterile physiological saline suspension was prepared to 0.5 of the McFarland standards (1.5×10^8 CFU/mL) from bacterial colonies grown on nutrient agar overnight

Table 1. Floral origin and geographical location of the propolis samples

Sample	Area	Geo-localisation	Vegetation origin	Collection date
1	Sidi Bennour	32°39' 7» N 8° 26' 35» W	<i>Eucalyptus- Rosmarinus</i> <i>Thymus- Citrus- Olea</i>	May 2021
2	Benslimane	33° 36' 44»N 7° 07' 16»W	<i>Eucalyptus</i> and <i>Quercus</i>	May 2021
3	Sidi Ifni	29° 23' 0" N, 10° 10' 0" W	<i>Argania</i> and <i>Euphorbia</i>	June 2021

at 37 °C and yeast grown on Sabouraud at 28 °C for 48 h. Then the bacterial suspensions were spread on Mueller Hinton Agar and the yeast suspensions were spread on Sabouraud Dextrose Agar. A paper discs (6 mm diameter) that were impregnated with 60 µl of extract at concentration of 100 mg/ml, were placed on the inoculated agar surface. Petri dishes were left for 2h at 4 °C to allow the diffusion of the extract before incubation at 37 ± 2 °C for 18–24 h for bacteria and at 28 ± 2 °C for 48 h for the yeast activity. After incubation, the diameters of the inhibition zones were measured in mm. Fluconazole and Ampicillin were used respectively as positive controls and methanol as negative control.

Determination of the minimum inhibitory concentration (MIC)

The MIC was defined as the least concentration of extracts that totally inhibited microbial growth. MIC was determined by the quantitative method of microdilution. Volume of 8.7 ml of Mueller-Hinton broth or Sabouraud broth were placed in test tubes and 0.2 ml of each extract, were adjusted to (0.5–100 mg/mL), and added to the test tubes. To each tube containing the mixture, 0.1 ml of the microbial suspension (1×10^7 CFU/ml) was added, prepared in Mueller-Hinton Broth for bacteria and prepared in Sabouraud broth for yeast. The tubes were incubated for 24–48 h at 37 ± 2 °C and for 48–96 h at 27 ± 2 °C respectively for bacteria and yeasts. Microbial growth in each tube is indicated by turbidity at the bottom of the tube.

Statistical analysis

SPSS software version 26 was used to statistical analysis. All studied data are expressed as mean ± standard error means (SEM). ANOVA one way with a confidence level of 95% was used to look for differences among the study results. A p value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Total phenolic content

The TPC determined in the Moroccan propolis samples analyzed in this study, are summarized in Figure 1. The data showed that TPC content in the sample extracts ranged from 12.26 ± 1.46 to 100.13 ± 1.23 mg GAE/g. The propolis of Sidi Bennour region demonstrated the highest value of phenols (100.13 ± 1.12 mg GAE/g). While, the propolis of Sidi Ifni region demonstrated the lowest value of phenols. These results are similar to those reported by El Menyiy el al. [9] and by Laaroussi et al. [8] in propolis samples collected from different regions of Morocco in which the TPC ranged from 6.74 ± 1.17 mg FAE/g to 149.13

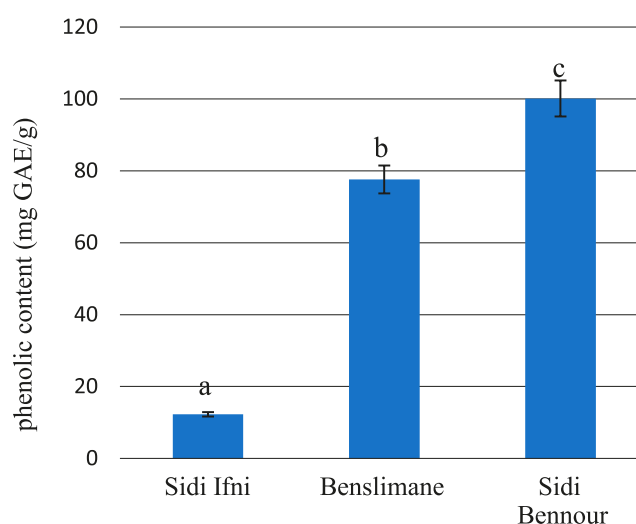


Figure 1. Total phenolic content of ethanolic extract of propolis samples collected from different Moroccan regions Means with different letters are significantly different at $P < 0.05$.

± 2.12 mg FAE/g and from 5.99 ± 0.86 mg GAE/g to 117.81 ± 5.43 mg GAE/g respectively. These values are however, higher than those reported for the neighbor countries as Tunisia (17.34 to 33.4 mg GAE/g) [17] and Algeria (0.81 ± 0.16 to 8.97 ± 0.25 mg GAE/g) [18].

Total flavonoids content

Flavonoids are one of the most important polyphenolic classes found in many plants and plant-derived products. Numerous studies have shown that flavonoids exhibit a broad range of biological properties, including, antifungal, antibacterial, antiviral, antioxidant anti-inflammatory, anti-allergic, hepatoprotective activity, anticancer and cardiovascular protection effects and so on [19, 20, 21]. In studied propolis samples the TFC was 73.46 ± 2.12 mgQE/g for the propolis of Sidi Bennour region, 34.7 ± 1.12 mgQE/g for the propolis of Benslimane region and 3.56 mgQE/g for the propolis of Sidi Ifni region. These values are similar to those obtained for different other Moroccan propolis samples reported to have TFC values ranging between 0.16–80 mgQE/g [22]. On the other hand, the present results are higher than those obtained for the propolis obtained from Indonesia 0.76–3.39 mgQE/g [23] and South Korea, Australia, Brazil, and China (33–53 mg QE/g) [24]. The obtained results were however, lower than those found in the propolis from Mexico (13–379 mg mgQE/g) [25]. The variations in TPC and TFC are dependent on type of vegetation predominant in the region of the sample collection, the environmental conditions, the seasonal variation as well as the extraction method [10], Table 2.

Antioxidant activity

The results found concerning the antioxidant activity of the selected propolis samples as determined

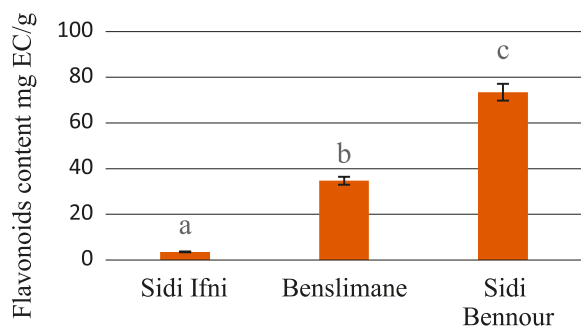


Figure 2. Total flavonoids content of ethanolic extract of propolis samples collected from different Moroccan regions Means with different letters are significantly different at $P < 0.05$.

using the free radical scavenging assay of the 2,2-diphenyl-2 picrylhydrazyl (DPPH●) method are expressed as IC₅₀ values and shown in Figure 3. All the propolis samples examined have a considerable antioxidant activity with IC₅₀ between 4.23 ± 0.5 and 154 ± 0.21 $\mu\text{g/ml}$. The sample from the Sidi Bennour region expressed the highest antioxidant potential (IC₅₀ = 4.23 ± 0.5 $\mu\text{g/ml}$) that was close to that of ascorbic acid used as a standard antioxidant. The sample from Sidi Ifni that was with the smallest contents of phenolic and flavonoids, demonstrated the lowest free radical scavenging capacity (IC₅₀ = 154 ± 0.21 $\mu\text{g/ml}$).

Using the Pearson's correlation analysis showed a significant negative correlation between IC₅₀ values and total phenols ($r = -0.946$), and total flavonoid content ($r = -0.817$), (Table 2). The high correlations prove the role of phenolic and flavonoids compounds as the major contributor to the antioxidant properties

Table 2. Spearman's correlation coefficients between total polyphenols, total flavonoids and antioxidant activity

	Flavonoids	Polyphenols	IC ₅₀
Flavonoids	1.000	0.879**	-0.817**
Polyphenols	0.879**	1.000	-0.946**
IC ₅₀	-0.817**	-0.946**	1.000

** Correlation is significant at the 0.01 level.

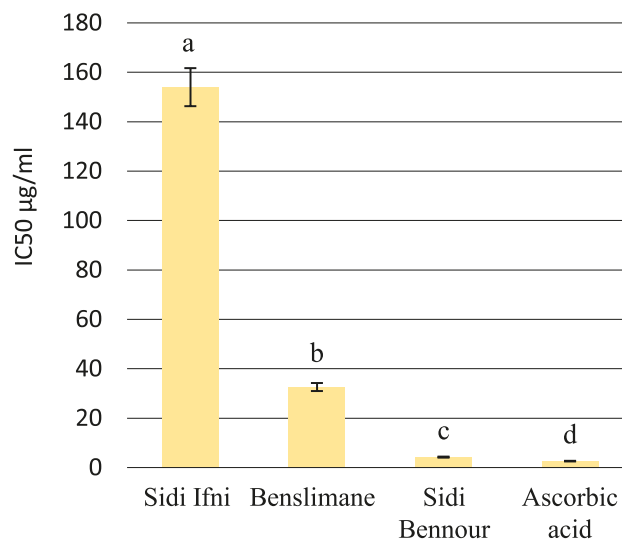


Figure 3. IC₅₀ values of ethanolic extract of propolis samples collected from different Moroccan regions Means with different letters are significantly different at $P < 0.05$.

of Moroccan propolis extracts. High correlation between the total phenolic and flavonoids content and the antioxidant capacity has also been revealed in other propolis samples from Morocco [9, 10, 22], Algeria [18], South Korea [24], Mexico [25] and Poland [26]. These data allow us to confirm the beneficial use of propolis as valuable tool in food and pharmaceutical industry.

Antimicrobial activity

The present study examined also the antimicrobial effects of the ethanolic extracts of propolis against known human microorganisms' pathogens, by the disk diffusion method. The results of the tested propolis samples using this method are shown in Table 3. All propolis samples exhibited varying degrees of antibacterial activity against all strains tested, markedly against gram-positive bacterial strains, with inhibition zones ranged between 12 ± 1.03 mm and 22 ± 0.5 for *S. aureus*, followed by *Pseudomonas* sp with a diameter ranged from 10 ± 0.08 mm to 20 ± 0.02 mm and *E. faecalis* with a diameter varied

Table 3. Disk inhibitory zone of propolis extracts against microorganisms

Microorganisms	Disk inhibitory zone (mm)					
	Gram negative bacteria		Gram positive bacteria		Yeasts	
Sample	<i>Pseudomonas sp.</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>C. albicans</i>	<i>C. neoformans</i>
Sidi Bennour	20 ± 0.02	17 ± 1.04	22 ± 0.5	19 ± 0.6	9 ± 0.6	10 ± 0.2
Benslimane	10 ± 0.08	13 ± 2.52	12 ± 1.03	13.5 ± 0.04	NI	NI
Sidi Ifni	11 ± 0.58	12 ± 0.35	NI	11 ± 0.4	NI	NI
Ampicilline	24 ± 0.77	22 ± 1.4	22 ± 0.83	23 ± 0.9	ND	ND
Fluconazole	ND	ND	ND	ND	20 ± 0.1	21 ± 0.1

ND: not determined; NI: no inhibition

from 11±0.4mm to 19±0.6mm. The *E. coli* has the lowest value of the inhibition diameter (12±0.35mm to 17±1.04mm). The propolis of Sidi Bennour region which has high phenolic and flavonoids contents and higher antioxidant potential was the most effective against all the tested bacteria.

The efficacy tests of the propolis extracts on the bacterial strains used, was assessed by determining the minimum inhibitory concentration (MIC) (Table 3). The MIC of tested bacteria was between 5 to 40 mg/ml. Among the bacterial strains tested, the gram-positive *E. faecalis* was the most susceptible as compared to other bacteria, while the gram-negative *E. coli* was the most resistant. These results have also been confirmed by numerous studies demonstrating that gram-positive bacteria are more sensitive to propolis extracts than gram-negative bacteria [9, 10]. The propolis sample from Sidi Bennour demonstrated the best antibacterial activity with the lowest value of MIC (MIC=1.25, MIC=1.25, MIC=2.25, MIC=5 mg/ml) for *S. aureus*, *E. faecalis*, *E. coli* and *Pseudomonas* sp respectively, while the propolis sample from Sidi Ifni has the low antibacterial effect, with MIC values of 40, 10, 5 and 5 mg/ml for *E. coli*, *Pseudomonas* sp, *S. aureus* and *E faecalis*, respectively. Only the propolis of Sidi Bennour region showed an antifungal activity against both *C. albicans* and *C. neoformans* with inhibition zones of 9±0.6 and 10±0.2 mm respectively, and MIC value of 100 mg/ml (Table 4). Several authors have reported antimicrobial activity of propolis from different regions of the world [27, 28, 29]. The antimicrobial effects of propolis extracts has been attributed to its phenolic and flavonoid content. As example, a study conducted by Hegazi et al. [30], has found that pinocembrin, benzyl caffeate and *p*-coumaric acid isolated from French propolis were partially responsible for its antimicrobial activity against *S. aureus*; *E. coli*, and *C. albicans*. Moreover, methyl ferulate, vanillin and 4-coumaric acid, are among the molecules reported to exhibit antibiofilm activity against several microorganisms, including *C. albicans* [31]. It is well known that phenolic compounds and flavonoids of propolis affects microbial viability through disruption membrane

permeability, inhibition of ATP and DNA and RNA synthesis, decreasing microbial mobility as well as other activities [32, 33, 34].

CONCLUSION

The present study report high contents of bioactive compounds in Moroccan propolis from three geographic regions. The data revealed also high antioxidant, antibacterial and antifungal activities in these samples. Propolis from Sidi Bennour region was found to have the highest amount of phenolic and flavonoids compounds, and a stronger antioxidant and antimicrobial activities, making of it promising natural antioxidant and antimicrobial agents useful to combat diseases related to oxidative stress and pathogenic infections. Moreover, it could be also used as a suitable natural product in food industry to ensure safety and improve the food products quality. More detailed studies are required to identify the qualitative chemical composition of this Moroccan propolis.

Conflict of interest

The authors declare no conflict of interest.

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Table 4. Minimum inhibitory concentration of propolis extracts

Microorganisms	Minimum inhibitory concentration (mg/ml)					
	Gram negative bacteria		Gram positive bacteria		Yeasts	
Extracts	<i>Pseudomonas</i> sp.	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>	<i>C. albicans</i>	<i>C. neoformans</i>
Sidi Bennour	5±0.0	2.25±0.0	1.25±0.0	1.25±0.0	100±0.0	100±0.0
Benslimane	7±0.0	5±0.0	6.5±0.0	4±0.0	ND	ND
Sidi Ifni	10±0.0	40±0.0	5±0.0	5±0.0	ND	ND

ND: not determined

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