International Journal of Herbal Medicine
Available online at www.florajournal.com

IJHM International Journal of Herbal Medicine

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2016; 4(5): 01-04
Received: 01-07-2016
Accepted: 02-08-2016

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Phytochemical antioxidant and antimicrobial study of Libyan propolis ethanolic extract

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Abstract
The propolis is a substance collected by bees from the leaf buds of several different kinds of trees. The chemical composition and biological activity of propolis has been reported to be variable between different geographical regions. The current study designed to investigate of chemical constituents and antioxidant and antimicrobial activity of propolis sample collected from area around Tripoli. The phytochemical analysis of the propolis extract display presence of compounds belonged to phenolic, flavonoids, terpenoids, phytosterol and coumarine. The results of TLC illustrate DPPH free radical scavenging potential of EEP as the color changed from violet to yellow. The 1mg/ml of EEP showed significant antibacterial effect against *S. aureus, Ps. aeruginosa* and *S. poona,* and lower effect against *MRSA, K. pneumonia* and *E. coli* However other concentration had lower activity against tested bacteria.

Keywords: Libyan propolis; antimicrobial activity, antioxidant

1. Introduction
Propolis is a substance collected by bees from the leaf buds of several different kinds of trees. The worker bees collect the plant secretions or exudates from plant wounds such as the lipophilic material on leaves; mucilage; gums; resins and latexes. During the collection of propolis bees mix the plant exudates with beeswax and β-glucosidase in order to use it later on for sealing holes and to protect the hive from environmental change all year around. Propolis has been reported to be used in the folk medicine since 300 BC [1, 2]. The variation in the biological activity of the propolis has attracted the attention of researchers into investigating the constituents and in finding the relationship between its constitution and biological effects. Over 300 chemical components belonging to the flavonoids, terpenes, and phenolics have been identified in propolis. For example; flavonoids (flavones, flavonols, flavanones, dihydroflavonols and chalcones); phenolic acids; phenolic aldehydes; polyphenolic derivatives (cinnamic and benzoic acid, caffeic acid esters and terpenes) [3, 4] and other compounds such as 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens and 5% other organic substances [5].
Numerous activities of propolis have been reported in the literature including antibacterial, antiviral, antifungal antiprotozal, antioxidant, anti-inflammatory, anticarcinogenic, immunomodulating activities [3, 4, 6, 7].
It has also been used for treatment of diabetes and some cardiovascular diseases [1]. In addition propolis has been used in cosmetics, food, and beverage to produce much health promoting formulation [8]. The antimicrobial properties of propolis have get attraction of many researchers around the world, due to increasing of antibiotic resistance. Many research has revealed that propolis has a high antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* [1, 6] *Salmonella enteritidis* and *Salmonella typhimurium* [9], *Bacillus subtilis*, Bacillus cereus, *Klebsiella pneumoniae*, *Proteus vulgaris, Candida albicans*, and *Escherichia coli* and other pathogenic microorganism [10]. The antimicrobial activity of propolis has been suggested to be due to abundance of flavonoids constituents [11, 12] including flavonones such as pinocembrin, and flavonols such as galangin [13], antifungal activity may be due presence of coumaric and caffeic acid compounds. Even the volatile oil fraction and diterpenic acids show antibacterial and cytotoxic activities in some propolis samples [1]. It may be that antibacterial activity results from the presence of mixture of compounds which have synergistic effect with each other producing an antibacterial effect [13]. The chemical composition and biological activity of propolis has been reported to be variable between different geographical regions [6, 11]. That the propolis samples from Europe, South America, and Asia has variable chemical

composition between each other. This suggests that researchers should investigate the composition of the propolis from different geographical regions using different analytical methods to identify the compounds in the different samples [8].

2. Materials and Methods

2.1 Collection and extraction of propolis

Propolis was obtained from honey bee hives from Tarhona 80 Kilometer south east Tripoli during spring 2014 and kept in the refrigerator until used.

Raw propolis (20 g) was manually grinded and placed in a flask with 100 ml of ethanol. The macerate was kept for 7 days with occasional shaking then it was filtered through a Whatman filter paper (after left over night in fridge to remove the waxy part). Then extract was evaporated to dryness by air.

2.2 Phytochemical Screening

Chemical tests were carried out of propolis extract using standard procedures to identify the constituents as described by literatures.

2.3 Thin layer chromatographic test

The ethanolic extract of propolis was performed on thin layer chromatographic (TLC) plates, composed of Silica gel 60 GF 254. The plate was developed in chamber was previously saturated by mobile phase. The mobile phase was hexane/ ethyl acetate/ acetic acid 60:40:1, (all solvent were analytical grade). After drying, plate was investigated; visually, under UV 254/366 waves and vanillin/sulphuric acid spray reagent.

2.4. Antioxidant activity

The propolis extract and Ascorbic acid (positive control) were applied on to a silica gel plate and allowed to dry. The TLC plate was sprayed with 0.2% methanolic solution of diphenyl picryl hydrazyl (DPPH) reagent. Change of color from violet to yellow considered positive result.

2.5 *In vitro* antibacterial activities of plant extract

Determine antibacterial activity of propolis extract against G+ ve bacteria and G- ve bacteria. Test was carried out using disk diffusion method and propolis extract activity was tested by using cup- cut diffusion method.

2.5.1 Microorganism

Standard bacterial strains used in this study were *Pseudomonas aeruginosa* NCTC 12903/ATCC, *Escherichia coli* NCTC 12241/ATCC 25922, *Staphylococus aureus (MRSA*) NCTC 13373/ATCC 43300, *Staphylococcus aureus* NCTC 12973/ATCC, *Salmonella poona*, NCTC 4840, *Klebsiella pneumoniae*, NCTC 9633/ATCC, the standard bacterial strains were activated and cloned three successive times in nutrient agar and stored on nutrient agar slants at 4 °C.

2.5.2 Screening of antimicrobial activity

Antimicrobial susceptibility was tested using paper disk diffusion method [14]. Cup-cut agar method was used throughout this study to find out if the extract has ability to inhibiting bacteria growth. This method was performed using freshly prepared Mueller Hinton agar with overnight culture of bacteria inoculums were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusted to a 0.5 McFarland standard. On each plate wells (5 mm) were made by sterile cork borer. Each well was filled with 100µL of the propolis extract and the plates were then re-incubated for 24 h at 37 °C. The diameter of zones of inhibition was measured [15]. Inhibition zones were then measured in millimeter. Inhibition zones were indicated by a lack of microbial growth due to inhibitory concentrations. The antibiotics Ciprofloxacin (5µg) and A 5% (v/v) phenol were used as standards to compare the activity of propolis in inhibiting the growth of bacteria. Each experiment was carried out three times.

2.6 Statistical Analysis of Data

The data was tested for normality using a QC Analyses/K-S Normality Test. Normally distributed data was analyzed by student's t-test using the Statview® version 5.0.1 software package (SAS Institute Inc, Abacus Concept, *Inc*., Berkeley, CA, USA). A p value of < 0.05 was considered significant

3. Results & Discussion

The chemical profile of propolis is varying as several authors have studied propolis collected from different area around the world. The composition of propolis is manipulated by plants cover diversity as well as biotic and biotic factors [2, 12, 16, 17].

The current study display presence of different compounds as TLC experiment separates numbers of spots, which was detected by using UV and chemical reagent (figure 1). The phytochemical screening tests were demonstrated that those separated phytoconstituents might be belonged to phenolic, flavonoids, and phytosterol, terpenoids and coumarine compounds (Table 1). A previous studies in literature have identified around 300 compound correlated to those group [16]. Predictably, no alkaloids have been detected in this sample, which similar to previous discover in literature [16].

In the present study, qualitative analysis of antioxidant activity was done by test of the 2, 2, diphenyl-1-picrylhydrazyl (DPPH) on spots of propolis extract and ascorbic acid (reference) on TLC plate. The radical scavenging ability results appear as yellow spot of propolis compound against purple background after TLC sprayed by DPPH (figure 2). The antioxidant ability of propolis has been reported in many studies, which associated to the phenolic and flavonoids content of propolis [18, 19]. Results of the evaluation of antimicrobial activities of propolis extract at concentration 1, 0.5 and 0.25 mg/ml were correlated to phenol and Ciprofloxacin (5µg) antibiotic as standard references. The results obtained by agar well diffusion method, showed significant antimicrobial activities against bacteria (Table 2). The Propolis was affective against bacteria. The zone of inhibition was higher as compare to standard antibiotic. Propolis extract at 1mg/ml showed remarkable antibacterial effect against *S. aureus, Ps. aeruginosa* and *S. poona,* which has a zone of inhibition that no significant different to positive control phenol and Ciprofloxacin. However, propolis extract at 1mg/ml concentration showed a lower antibacterial effect against *MRSA, K. pneumonia* and *E. coli* which have zone of inhibition significant lower to the positive control and antibiotics (p<0.05). Propolis extract at 0.5 and 0.25 mg/ml showed lower zone of inhibition against tested bacteria compared to positive control; phenol and Ciprofloxacin antibiotic. The results indicated that there is no any effect of solvent on bacteria as no zone of inhibition at all.

The chemical composition of propolis is very complex and depends on the flora in the areas where it was collected. Therefore variations shown in the antimicrobial activity according to the propolis origin [20] are not surprising. Propolis had demonstrable antibacterial effect in this study.

Previous study the antibacterial activity of an alcoholic extract of propolis from Apis mellifera was investigated using a method of growth inhibition in the culture medium of microorganisms, The largest inhibition zone developed around *S. aureus* (27 mm) [21], that is similar to our result where the zone of inhibition against *S. aureus* is 26±0.06. Whereas, other work, ethanolic extracts of propolis was effective only against Gram-positive bacteria, they concluded that have antibacterial activity mainly on Gram-positive bacteria, showing a positive correlation with flavonoids content [22]. They verified that propolis inhibited *S. aureus* growth (10 to 14mm inhibition diameters), obtained diameters varying from 0 to 11mm, suggesting variability in the biological activity of propolis. Differences in propolis biological activity may be related to its botanical origin, reflecting differences in its chemical composition [23]. Orsi research group that verified moderate antibacterial action of ethanolic extract of propolis against *E. coli* (inhibition diameters from 8.0 to 8.8 mm) [24] demonstrated an elevated minimal inhibitory concentration of propolis against *Salmonella* sp, concluding that propolis shows limited action on Gram-negative bacteria [23]. Researcher that manifested the greatest sensitivity to propolis, followed by *E. coli* (23 mm), *P. aeruginosa* (22 mm), and *Salmonella spp*. (19 mm), results who are in concordance with those obtained by other researchers. Important is that all bacterial species examined none showed resistance to propolis, this demonstrates again the reason for which propolis is called natural antibiotic [21]. In this study the propolis had the lower zone of inhibition *E. coli* (16 mm), *P. aeruginosa* (6mm), and *Salmonella spp*. (15 mm). That different in the efficacy of the ethanolic extract of propolis could relate to different in the geographic origins area. As the previous work concluded that different in the geographic origin of Lebanon had the different efficiency against selected tested bacteria [25].

Fig 1: Results of visual, UV 336, and vanillin/sulphuric acid reagent

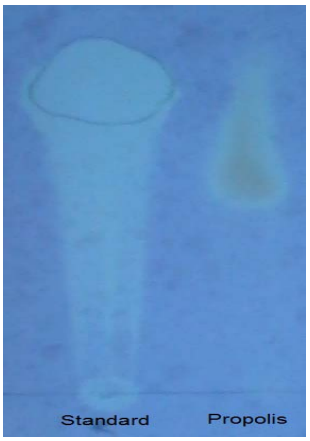

Fig 2: TLC photography of standard (ascorbic acid) and ethanolic extract of propolis colorized with 0.05% DPPH.

Table 1: Phytochemical screening of extract of propolis

Phyto constituents	Tests preformed	Results
Tannins and phenol	Ferric chloride test	++
Saponins	Foam test	-
Anthraquinone glycosids	Borntrager's test	+
Phytosterol	Libermann Burchard 's test	++
Flavonoids	Alkaline Reagent test Shindo's test	++ ++
Alkaloids	Mayer's test Dragendroff's test	- -
Carbohydrate	Fehling test	+
Coumarine	UV test	++

Table 2: Antimicrobial activity of Propolis extract.

Zone of inhibition of microbial growth (mm)							
Bacteria	Gram-positive bacteria		Gram-negative bacteria				Yeast
	S. aureus	*MRSA*	*E. coli*	*Ps. aeruginosa*	*S. poona*	*K. pneumonia*	*C. albican*
Propolis extract 1mg/ml	26±0.06	19±0.06	16 ± 0.06	6±0.05	15±0.05	16±0.06	4 ± 0.2
Propolis extract 0.5mg/ml	9±0.06	11 ±0.09	9 ± 0.06	1±0.03	10 ±0	5±0.03	1.2 ± 0.1
Propolis extract 0.25mg/ml	9±0.03	9±0.03	8 ± 0.03	0 ± 0	9 ±0	4±0.03	0 ± 0
Phenol	28±0.1	28±0.08	30±0.03	14±0.08	27±0.1	30±0.03	25±0.03
Ciprofloxacin	20±0.03	30±0.03	39±0.03	10±0.06	27±0.08	30±0.03	-
Fluconazole	-	-	-	-	-	-	30±0.02
DMSO (2%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

4. Conclusions

Libyan Propolis presented the interesting antioxidant and antimicrobial activity. The differences and variations in the susceptibility to propolis between the Gram negative and Gram positive bacteria still remains an important subject for further investigations.

5. Acknowledgments

We would like to thank: research assistants in faculty of pharmacy, University of Tripoli for helps and support.

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