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IRANIAN PROPOLIS ETHANOL EXTRACT AGAINST *STAPHYLOCOCCUS AUREUS* INFECTION IN WOUND MICE MODEL

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ABSTRACT

Propolis as a honeybee's product is famous for its biological and pharmacological effects. The subject of this study was to evaluate the antimicrobial effects of the Iranian propolis ethanol extract against different kinds of microorganisms by disc diffusion method and microbroth dilution assay. Due to the high antimicrobial effect of propolis extract, the efficacy of topical propolis cream was compared with mupirocin against *Staphylococcus aureus* wound infection model in mice.
The *in-vitro* antimicrobial evaluations showed the more sensitivity of Gram positive bacteria to propolis ethanol extract, followed by yeast. In wound infection model, propolis cream significantly reduced the log CFU of *S.aureus*,compared to placebo cream ($p<0.05$), but its efficacy was lower than that of mupiricin ointment ($p<0.05$).
Although, propolis showed the acceptable antibacterial effects against *S. aureus in vitro* condition, but its efficacy against *S. aureus* infection was lower than that of mupirocin ointment in wound infection model. Therefore, propolis can be used as an alternative antiseptic for preventive purposes.

Keywords: Propolis, antimicrobial activity, wound infection model, mice

INTRODUCTION

Propolis or "bee glue" is a resinous compound that is collected by honey bees from plant's flowers (**Marcucci, 1995**). Propolis is called Russian penicillin and according to the folklore believes are said it has healing power. Propolis traditionally is used for treatment of dental caries and oro-pharyngeal infections **(Kuropatnicki *et al.* 2013)**. Investigation on chemical profiles of propolis revealed the presence of complex mixture of compounds, more than 300 types of various constituents **(Cardoso *et al.* 2010)**, that they are belonged to different types of chemical substances such as resins, balsams, waxes, essential oils, flavonoids, phenolic and cinnamic acid derivatives **(Duarte *et al.*, 2006; Huang *et al.* 2014; Marcucci, 1995)**. Many biological activities of propolis such as antimicrobial **(Marcucci, 1995; Scazzocchio *et al.*, 2006)**, anti-viral **(Marcucci, 1995)**, anti-cancer **(Khacha-Ananda *et al.* 2013)**, anti-inflammatory **(Paulino et al., 2003)**, antioxidant **(Khacha-ananda *et al.*. 2013; Marcucci, 1995)** and anesthetic **(Paintz & Metzner, 1979)** properties are related to its chemical compositions **(Cardoso et al., 2010; Krol et al., 1996)**. The chemical compositions of propolis can affect by many different factors such as bees species, regional flora, time of collection **(Libério *et al.*, 2009; Paulino *et al.*, 2003)**, and environmental conditions, but in spite of differences, flavonoids, phenolic and cinnamic acid derivatives are the major groups of compounds in propolis samples **(Popravko *et al.* 1969)**.
Although, the antimicrobial activities of propolis samples especially Iranian ones were the subjects of many investigations **(Ghasem *et al.* 2007; Jafarzadeh Kashi *et al.*, 2011; Massaro *et al.* 2015; Nina *et al.*, 2015; Yaghoubi *et al.*, 2007),** but evaluating the antimicrobial activity of Iranian propolis (Isfahan province, central parts of Iran) against a large groups of microorganisms (Gram positive, Gram negative, yeast and fungi) was performed for the first time in this study. Among screened microorganisms, *S. aureus* as Gram positive bacteria was sensitive to Iranian propolis, thus, the efficacy of propolis cream was compared with mupirocin ointment and placebo against *S. aureus* wound infection model.

MATERIAL AND METHODS

Propolis extract

Propolis was purchased from NajafAbad, Isfahan, Iran and was identified by Agriculture Department, Medicinal Plant, Research Center of Barij, Kashan, Iran. The ethanol extract was extracted by ethanol 96% by percolator. Then, ethanol was evaporated until the sticky-waxy compound was produced. This compound was kept in sterile dark vials and stored in cool places at 4 ℃ until the experiments. The extract had the total phenolic content of 37.5 mg galic acid and flavonoid content of 27.5 mg quercetin equivalent per 1 g extract.

Microbial strains and antimicrobial activity evaluations

The microbial strains were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus saprophyticus* ATCC 15305, *Bacillus cereus* ATCC 1247, *Bacillus subtilis* ATCC 6051, *Streptococcus pyogenes* ATCC 8668, *Shigella dysenteriae* RI 366, *Streptococcus sanguis* ATCC 10556, *Pseudomonas aeruginosa* ATCC 9027, *Streptococcus salivarius* ATCC 9222, *Klebsiella pneumoniae* ATCC 10031, *Salmonella enterica* ser. Typhimurium ATCC 4028, *Enterobacter aerogenes* NCTC 10009, *Enterococcus faecium* ATCC 25778, *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Shigella flexneri* RI 366, clinical isolate of *Streptococcus agalactiae*, *Aspergillus niger* ATCC 16404, *Aspergillus parasiticus* ATCC 15517, clinical isolate of *Aspergillus flavus*, *Candida albicans* ATCC 10231. The bacterial strains were cultured and incubated in suitable conditions. The Antimicrobial evaluations were performed by disc diffusion and micro broth dilution assays.
In disc diffusion method, the adjusted microbial suspensions to 0.5 McFarland were spread on the surface of agar medium (Muller Hinton Agar for bacteria, Sabauraud dextrose agar for fungi) depend on the type of microorganism by sterile cotton swabs. Different concentrations of diluted propolis extract in DMSO (4, 8, 12, 16 mg/disc) were impregnated on the blank disc papers (Padtan teb, Tehran, Iran) and were put on the surface of cultured media. Appropriate antibiotic and DMSO discs were used as controls. The plates were incubated in 30-35 ºC for 24 h for bacteria and 20-25 ºC for 5-7 days for fungi and the inhibition zone diameters were measured in millimeter with dial calipers. The experiments were performed in triplicates **(Mahboubi *et al.* 2014)**.
Minimal Inhibitory Concentration (MIC) and Minimal Lethal Concentration (MLC) of propolis ethanol extract against microorganisms were determined by micro-broth dilution assay **(Mahboubi *et al.*, 2014)**. Propolis ethanol extract was dissolved in DMSO and serially diluted in broth medium. The final concentrations were in the ranges of 64-0.15 mg/ml. Then, 100 µl of each concentration of propolis extract and 100 µl of diluted microbial suspensions (10^6

and 10^4 CFU/ml for bacteria and fungi, respectively) were added to each well. The plates were incubated as above and the MICs were reported as the lowest concentration of extract that show no visible microbial turbidity in the wells. The first well that had no growth on agar media was defined as MLC value (mg/ml).

Efficacy of propolis cream and mupirocin ointment in wound infection model

Male mice Balb/C with average weighing of 23 grams were obtained from Razi institute of Iran. The animals have free access to food and water ad libitum. They were kept in a constant temperature of 21±2 ºC, humidity of 55±5% and under 12-h light/dark cycle in polycarbonate cages. All experiments were in according to the UK Animals Scientific Procedures Act 1986 (86/609/EEC). The mice were divided into three groups (n=10), including propolis cream, mupirocin and placebo groups.

Skin suture wound model was applied at the Microbiology Department, Animal House of Research Center of Barij, Kashan, Iran, as described by Gisby and Bryant **(Gisby & Bryant, 2000)**. Clinical methicilin resistant *S. aureus* (MRSA) was inoculated in nutrient broth containing 0.2% yeast extract and incubated for overnight. Sterile silk sutures number 3.0 (Supasil, Karaj, Iran) were cut into 20 cm length and soaked in undiluted overnight broth culture for 30 min. Infected sutures were dried on sterile filter and cut into 1 cm, then CFU/cm of sutures were determined. Anesthesia was induced by intraperitoneally injection of 1.43 mg/kg diazepam (Khemidaru, Iran), and 13 mg/kg ketamin 10% (alfasan, Woerden-Holland). The stripped back of mouse was swabbed with ethanol 70%. One cm of infected suture was inserted under the skin and knotted. One incision was made along with the suture. The wound was covered with a plaster. Treatment was initiated after 4 h. A 0.1 ml mupirocin ointment, propolis cream (5%) and placebo were spread over the area. Treatments were carried out three times daily for further 7 days. A 16-20 hours after the last topical application, animals were killed by CO_2. A 1-2 cm of involved skin area was excised and homogenized. The bacterial counts of homogenized skin were estimated and expressed as means log CFU ± Standard deviations (SD).

Statistical analysis

All experiments were analyzed by SPSS software (version 17, Chicago, IL, USA). ONE-Way ANOVA test was used to compare the differences between compounds and then the P-values were calculated. All tests were performed on an overall 5% significance level, meaning that *P*- values less than 0.05 were considered as statistically significant differences (P<0.05).

RESULTS AND DISCUSSIONS

Antimicrobial activity of propolis ethanol extract

For screening the antimicrobial activity, the propolis sample was tested against the standard strains, which associated with important infections.

In disc diffusion assay, the inhibition zone diameters were increased dose dependently. The higher inhibition zone diameters were for Gram positive bacteria, followed by *C. albicans* and filamentous fungi. There were no inhibition zone diameters for Gram negative bacteria. Among Gram positive bacteria, the larger inhibition zone diameters were for *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *B. cereus*, *B. subtilis*, and *S. pyogenes*, while there were no inhibition zone diameters for propolis extract against oral *Streptococcus* (*St. sanguis*, *St. agalactiae*, *St. salivarius*), *E. faecalis* and *E.faecium*. The inhibition zone diameters for antibiotics were higher than that of propolis ethanol extract even in higher concentrations (Table 1).

Table 1 Antimicrobial effect of propolis ethanol extract on different microorganisms

Microorganisms	Inhibition zone diameter (mm)					Inhibitory concentrations (mg/ml)	
	Propolis extract (mg/disc)				Antibiotic	Propolis	
	4	8	12	16		MIC	MLC
S. aureus	7.95±0.21	10.25±0.21	11.1±0.07	12.1±0.56	17.3±0.21^{V}	0.34	0.78
S. epidermidis	8.0±0.14	9.3±0.14	10.6±.56	12.1±0.14	18.1±0.14^{V}	0.17	0.17
S. saprophyticus	7.05±0.35	9.1±0.14	10.6±.56	11.9±0.14	20.7±0.42^{V}	0.17	0.34
B. cereus	7.05±0.35	9.1±0.14	10.6±.56	11.9±0.14	21±0.22^{V}	0.17	0.17
B. subtilis	7.0±0.0	7.8±0.13	8.8±.28	11.3±0.42	22±0.28^{V}	0.17	0.17
St. salivarius	NI	NI	NI	NI	21.9±0.14^{V}	0.34	0.78
St. agalactiae	NI	NI	NI	NI	23.6±0.84^{V}	1.56	3.12
St. sanguis	NI	NI	NI	NI	22.9±0.14^{V}	0.78	1.56
St. pyogenes	8.5±0.71	10.3±0.71	11.0±.0.	12.9±0.14	23.7±0.71^{V}	0.78	1.56
E. faecium	NI	NI	NI	NI	15.4±0.56^{V}	3.12	6.25
E. faecalis	NI	NI	NI	NI	15.6±0.56^{V}	3.12	6.25
S. flexneri	NI	NI	NI	NI	12.0±0.14^{V}	1.56	3.12
E. coli	NI	NI	NI	NI	18.7±0.42^{G}	1.56	3.12
K. pneumoniae	NI	NI	NI	NI	20.7±0.42^{G}	0.39	0.78
E. aerogenes	NI	NI	NI	NI	19.7±0.42^{G}	3.12	6.25
S. enterica ser. Typhimurium	NI	NI	NI	NI	19.9±0.14^{G}	1.56	3.12
P. aeruginosa	NI	NI	NI	NI	21.0±0.0^{G}	1.56	3.12
C. albicans	NI	NI	7.1±0.2	8.2±0.98	12.95±71^{A}	0.17	0.34
A. niger	NI	6.5±0.42	7.2±0.28	7.9±0.21	16.4±0.56^{A}	0.78	1.56
A. flavus	NI	NI	7.1±0.07	8.3±0.21	11.02±0.16^{A}	0.78	1.56
A. parasiticus	NI	NI	8.0±0.14	9.3±0.21	12.15±0.35^{A}	0.78	1.56

Legend: NI= no inhibition, Antibiotics=V [Vancomycin (30 µg/disc)], G [Gentamycin (10 µg/disc)], A [amphotericin-B (100 U/disc)] for Gram positive, Gram negative and mold, respectively

It's believed that disc diffusion method is not a suitable assay for evaluating the antimicrobial effects, because the results strongly influence by the solubility and diffusion of tested compounds in agar mediums **(Rios & Recio, 2005; Srisukha *et al.*, 2012)**. So, the antimicrobial activity of propolis was also evaluated by micro-broth dilution method.

In microbroth dilution assay, MIC and MBC values (mg/ml) of propolis ethanol extract against different microorganisms were in the ranges of 0.17-6.25 mg/ml. The lower MIC and MBC values of propolis ethanol extract were for *S. epidermidis*, *B. cereus* and *B. subtilis* (MIC=MBC= 0.17 mg/ml), followed by *C. albicans* and *S. saprophyticus* (MIC, MBC=0.17 and 0.34 mg/ml) and *S. aureus* , *St. salvarius* (MIC, MBC=0.34, 0.78 mg/ml). *E. aerogenes*, *E. faecalis* and *E. faecium* (MIC=3.12 and MLC=6.25) showed less sensitivity to this extract (Table 1).

Although antimicrobial activity of propolis have been confirmed in some studies **(Ghasem *et al.*, 2007; Jafarzadeh Kashi *et al.*, 2011; Yaghoubi *et al.*, 2007)**, but the the reported values for MIC/MBCs are different. Evaluating the antibacterial activity of aqueous and ethanol extracts of propolis against oral pathogenic bacteria (*St. mutans*, *St. salivarius*, *S. aureus*, *E. faecalis* and *Lactobacillus casei* showed the MIC values of 0.25-0.5 mg/ml **(Jafarzadeh Kashi *et al.*, 2011)**. The antimicrobial activities of Iranian propolis ethanol extract from northwest of Iran was also confirmed against *S. aureus* and *C. albicans* **(Ghasem *et al.*, 2007)**. Complexity in results of the antimicrobial activities is related to differences in chemical composition of propolis, extraction method, experimental conditions and tested bacterial strains **(Lazar *et al.* 2013)**.

According to the results, Gram positive bacteria and *C. albicans* showed more sensitivity to propolis ethanol extract than the others. Our results are in compliance with **Yaghoubi et al (2007)** that confirmed propolis ethanol extract

had higher inhibition zone diameters against Gram-positives than the Gram-negative ones **(Yaghoubi *et al*., 2007)**. In total, Gram positive bacteria due to the structure of cell walls and presence of peptidoglycan in cell wall structures are more sensitive than Gram negative to essential oil and plant extracts **(Kareem, 2015; Lotfy, 2006; Ristivojević *et al.*, 2016; Tukmechi et al. 2010).**

The antimicrobial activity of propolis is related to its complex composition (**Gebara *et al*., 2002)**. The propolis with high concentrations of flavonoids have showed the antibacterial activity **(Bosio *et al*. 2000; Cheng & Wong, 1996)**. It has been shown, the pure flavonoid components of propolis (quercetin and naringenin) influence on the permeability of inner bacterial membrane and damage to the cell membranes **(Mirzoeva *et al.* 1997)**.

Phenolic compounds in propolis may also be responsible for its antimicrobial activities in dose dependent manner; low doses of phenolic compounds influences on the enzymes involved in energy production and its higher doses denature the protein structures, and change the cell surface hydrophobicity, charge and cause the cytoplasmic content leakage **(Borges *et al.* 2013; Ristivojević *et al*., 2016; Tiwari *et al.*, 2009)**.

The efficacy of propolis cream (5%) in wound infection model

Although, the antimicrobial effects of propolis have been the subject of different studies, but a few studies are present in regard of its antimicrobial activity in animal. For this, we evaluated the propolis efficacy in treatment of *S. aureus* infection wound model in mice. Topical application of propolis cream on infected wounds significantly reduced the log CFU of *S.aureus* higher than placebo group and lower than mupirocin group ($p<0.05$)(Table 2). Therefore, although, the propolis 5% can reduce the bacterial load in treated mice, but its efficacy was not comparable with mupirocin group. Indeed, propolis ethanol extract at this concentration (5%) has been acted as an antiseptic agent. Expected therapeutic effects from propolis need higher doses of propolis or use the propolis as supporting compound in combination with other herbal antibacterial compounds.

Table 2 Statistical indicators in studied groups.

Log CFU	propolis	mupirocin	placebo
	5.15±0.3	1.0±0.29	6.3±0.29

*= significant in the level of 0.05

Other biological effects of propolis, such as its anti-inflammatory **(Tsarev *et al.* 1985)**, antioxidant **(Khayyal *et al.* 1992; Krol *et al*., 1996)** and immune-stimulatory effects **(Ansorge *et al.* 2003)** may involve in its wound healing activities. The wound healing effects of propolis in treatment of hamester oral wounds were confirmed (**de Oliveira *et al*., 2013)**. The efficacy of propolis in microbial colonization was comparable with silver sulfadiazene **(Gregory *et al.* 2002)**. Therefore, the antiseptic properties of propolis along with its anti-inflammatory activity and wound healing effect make it as a good supporting compound in designing of new antiseptic herbal formulations. In addition, further investigations are needed to demonstrate some standards related to quality and safety of propolis for determination of precise therapeutics doses.

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