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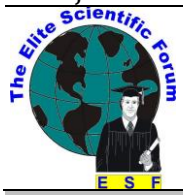

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RESEARCH ARTICLE

Antibacterial and Antioxidant Potential of Propolis against Skin Pathogens

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ABSTRACT

Skin, the body's largest organ serves as first line of defense against numerous insults caused by physical, chemicals and biological factors. Skin microbiota provides protection to the body if kept in balance. Propolis is a resinous substance that is collected by honey bees from different botanical sources. It serves as strong antimicrobial, antioxidant, antiviral and anti-inflammatory substance because of its polyphenolic and flavonoid constituents. Objective of the present study was to evaluate in vitro antibacterial and antioxidant potential of propolis from honey bee garden of University of the Punjab, Lahore, against skin pathogens. Antibacterial property of propolis was evaluated by preparing its extract in 70% ethanol. Susceptibility of isolates was analyzed by using disc diffusion method and MIC. DPPH ((2, 2-diphenyl-1-picrylhydrazyl hydrate radical) assay was performed to evaluate the antioxidant potential of ethanolic extract of proplis in terms of half maximal inhibitory concentration (IC 50). The ethanolic extract of propolis showed significant ($p<0.05$) inhibitory activity against all isolates with zones of inhibition ranging from 15.83±0.29 to 29.67±0.29mm whereas MIC values ranged from 4.50±0.50mg/ml to 6.67±0.58mg/ml. MBC values ranged from 5.83±0.29mg/ml to 7.67±0.58mg/ml. Inhibitory potential of propolis against bacteria expressed a dose dependent pattern. Ethanolic extract of propolis showed significant antioxidant activity with IC50 value of 12.28±0.49 µg/ml. Results of this study indicated that extract of the propolis can serve as important antibacterial and antioxidant substance for the maintenance of general skin health as well as for the treatment of skin diseases.

INTRODUCTION

Skin is a complex, highly specialized and largest organ of human body which acts as body's primary shield against a number of environmental insults like infections, mechanical trauma and chemical irritations (Hsu et al., 2014; Chen et al., 2018). Epidermis, the outer most layer of skin, serves as first line of defense by possessing huge amount of keratin in specialized cells named as keratinocytes (Zhang, 2018). Despites of the dead layer of keratin protein, skin also represents a living habitat for variety of microorganisms and interact with both inner and outer environments. These microorganisms are particularly known as "skin microbiota" (Grice and Sege, 2011; Mikamo et al., 2011). Sensitive balance between the host and its microbes maintains healthy skin physiology and also provides second defense line to the body. Disturbance in the balance on one or the other side of equation can bring about various diseases such as seborrhea dermatitis, atopic dermatitis, acne vulgaris, cutaneous candidiasis, green nail syndrome and tor web (Gupta et al., 2004; Cogen et al., 2008; Dessinioti and Katsambas, 2010; Findley and Grice, 2014).

Among all the skin disorders acne vulgaris is the most prevailing as it targets 85% of adult population. It leads to detrimental effects on psychology and self-esteem of young population (Jappe, 2003; Williams et al., 2012). *Cutibacterium acne*, the aerotolerant, anaerobic gram positive bacterium is the main role player in causing acne vulgaris. Other skin microbiota that represent as opportunistic pathogens, cause numerous skin infections include *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis* and *Peudomonas aeruginosa* etc (Jappe, 2003, Ekpo and Etim, 2009, Vu et al., 2015). Persistent use of antibiotics for the treatment of various skin ailments not only causes development of resistance in microorganisms but also exerts numerous side effects on human health i.e. skin allergies and eye infection (Das and Reynolds, 2014).

Due to emerging antibiotic resistance, world is shifting its concern towards the use of natural products for the treatment of different diseases. Natural products possess variety of molecules that ameliorate different diseases through novel mechanism of action and enhance the effect of drugs (Svendsen et al., 2017).

Honey and other honey bee products are renowned natural substances being used as remedies since ancient times (Abd Jalil et al., 2017). Propolis is the one of honey bee product that is collected by worker bees from the buds and exudates of different plants. It is a sticky substance; also known as bee glue (Salatino et al., 2011). Physical appearance, composition, consistency and colour of propolis depend on various factors such as time and place of collection, vegetation, geographic origin and season of the year. Propolis represents huge diversity in its composition by possessing more than 300 chemical compounds including phenolic acid, flavonoids, trepenoids, quinones, steroids, amines and minerals (Bankova et al., 2000; Viuda-Martos et al., 2008). Toreti et al., 2013 have described main component of propolis as resin (50%), wax (30%), essential oil (5%), pollen and other chemical compounds (10%).

Propolis has been used in traditional medicines due to its broad spectrum of biological activities such as antimicrobial, antioxidant, anti-carcinogenic, antiviral, anti-ulcerogenic and anti-inflammatory properties (Wang et al., 2011; Sun et al., 2015; Kismet et al., 2017; Migliori et al., 2017). These biological properties can mainly be attributed to phenolic compounds specifically flavonoids and phenolic acid. Da Silva et al (2006) documented linear relationship between polyphenols and flavonoids constituents and antioxidant activity of propolis through DPPH assay. It is one of the simplest assays used to determine antioxidant potential of natural substances. For medical therapy propolis is used in the form of extracts. Ethanolic extract of propolis is highly effective against a number of gram-positive and gram negative bacteria (Jorge et al., 2008; Umthong et al., 2011; Kubiliene et al., 2015).

Natural remedies have long been practiced without scientific validation in this country. The present work was aimed to evaluate the antibacterial and antioxidant properties of propolis collected from research honey bee gardens of University of the Punjab, Lahore, against opportunistic pathogens of skin infections including acne vulgaris.

MATERIALS AND METHODS

Chemicals

DPPH, methanol (HPLC grade), ethanol (analytical grade), ethyl acetate, sodium hydroxide and hydrochloric acid purchased from Sigma-Aldrich. Distilled water was used in the preparation of extract of propolis.

Collection of propolis sample and preparation of extract

Raw propolis was collected from Research Honey Bee Garden of University of the Punjab, Lahore during the month of December, 2019. Raw propolis sampled was frozen at -4˚C for 12 hours and then was ground to fine powder by using pestle and mortar. Extracts of propolis were prepared by using the method of Kacániová et al (2012) with mild modifications. For the preparation of ethanolic extract 5g of powdered propolis was dissolved in 70% ethanol. Ethanolic extract was acidified with hydrochloric acid to pH 2. Propolis samples was then extracted at 80 ˚C through reflux for one hour. After cooling, the mixture was centrifuged at 5000 rpm for 10 minutes and the supernatant was evaporated at 40˚C in heat dry oven (Dhg-9030a). The residue was dissolved into 80ml solvent mixture of ethyl acetate and distil water (1:1 v/v) and gently shaken for 5 minutes. Organic phase containing ethyl acetate was separated and solvent was evaporated. Residues were weighed, dissolved into absolute methanol to obtain 70% solution and kept in refrigerator until further use.

Susceptibility test

Disc diffusion method was used to access the antibacterial potential of the extracted propolis. The tested microorganisms included *Bacillus cereus* ATCC 14579, *Bacillus subtilis* KX881940, *Propionicbacterirm acne* ATCC-6919 (gram-positive) and *Escherichia coli* NBRC102203 (gram-negative). *B.cereus*, *B.subtilis* and *E.coli* were retrieved from the conservatory of Industrial Microbiology Lab of Institute of Zoology, University of the Punjab, Lahore, Pakistan. All these bacterial strains were isolated from the patients having different skin infections. *P.acne* was purchased from Musaj Adam & Sons. The tested organisms were cultivated in nutrient broth/agar except *P.acne* for which brain heart infusion was used. One hundred micro liter of 24 hours old broth culture of a bacterium was spread on a respective nutrient agar plate. Three Whatman filter paper No.1 discs each of 9mm in diameter were used. One disc was loaded with 50 µl of propolis extract along with a disc of ciprofloxacin used as a positive control and a negative control disc loaded with 50 µl of 70% ethanol. All these discs were placed at equal distance from each other on a pre-inoculated petri plates. All the plates were incubated at 37˚C for overnight period. Inhibition zones around the discs were measured in mm. All the experiments were repeated thrice and the results were demonstrated in the form of mean ± standard error.

To verify the dose dependent antimicrobial potential, different amounts of the propolis extract ranging from 10µl to 50µl were employed to record the growth inhibition zones as described before.

Determination of Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of propolis extract

The MIC and MBC were evaluated following the method of macro-dilution described by Kashi et al (2011) with little modifications. All the bacterial strains were revived from the conservatory of industrial biotechnology lab from the Institute of Zoology, University of the Punjab, Lahore, Pakistan. Accordingly,1 ml of 24 hours old bacterail culture containing CFU/ml in the range of 3.1×10^8 to 3.9×10^8 for aerobic bacteria and 2.4×10^6 for anaerobic *P.acne* was taken into sterilized glass tubes. The 1ml of 70% ethanolic propolis extract containing 1mg to 15mg were added and the tubes were incubated at 37˚C for 24 hours. Tubes containing liquid culture medium (without propolis) served as negative control whereas tube containing methanol and bacterial culture represented the positive control. For the determination of both MIC and MBC, 50 µl of experimental culture was spread onto nutrient agar plate and incubated for 24 hours at 37˚C. MIC was defined as the lowest concentration that gives minimum colonies on petri plate and MBC was defined as the concentration that did not allow the growth to appear on the plates.

Antioxidant activity of propolis extract

Antioxidant activity of ethanolic extract of propolis was determined by using DPPH (2, 2-diphenyl-1-picrylhydrazyl hydrate radical). For this purpose, 3.5 ml of ethanolic extract of propolis containing 5µg/mL to 60µg/mL of propolis was mixed with 1.5 ml of DPPH (0.1mM). After thorough shaking, mixture was incubated for 60 minutes in dark at room temperature (22˚C). After 60 minutes' absorbance was noted at 515nm by using UV-VIS spectrophotometer. Blank contained 1.5 ml of 5µg/mL to 60µg/mL of propolis dissolved in 3.5 ml of methanol.1.5ml methanol along with 3.5ml of DPPH solution served as negative control. Absorbance of all the concentrations were converted into % radical scavenging activity by using the following formula

DPPH radical scavenging activity (%) = $100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$

Half maximal inhibitory concentration (IC50) was calculated by using plots of linear regression where the % radical scavenging activity was represented along Y-axis and concentration of propolis along x-axis (Pontis et al., 2014).

All the experiments were performed in triplicates and results were presented as mean ± SEM. One way analysis of variance (ANOVA) and Dunnett T3 post hoc test was used to compare the sensitivity of bacteria against extract of propolis by using SPSS software (v 20; IBM Corporation). The significance level decided was $P<0.05$.

RESULTS

Antibacterial activity of ethanolic extract of propolis

Ethanolic extract of propolis showed antibacterial activity against both gram positive and gram negative, aerobic and anaerobic bacteria (Figure 1). Among all the strains *B.cereus ATCC14579*, *B.subtilis* KX881940 and NCBI3610 and *E.coli NBRC102203* showed significant sensitivity against propolis with the growth inhibition zones of 23.83±0.29mm, 20.67±0.58mm 23.50±0.87 mm and 25.83±0.29mm, respectively. Largest inhibition zone was observed against *E. coli* and smallest against *P.acne*. Propolis demonstrated greater antibacterial activity against all the bacteria except *B.subtilis* KX881940 and *P.acne* ATCC-6919 in comparison with antibiotic used as positive control. No growth inhibition zone was observed against negative control.

Susceptibility of the bacteria against different concentrations of propolis was also checked. The growth inhibition zones increased in a dose dependent manner when 20µl of 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml and 50µg/ml of propolis extract were loaded on the filter paper discs in correspondingly the growth inhibition zones against the bacterium *B.cereus* were 12mm, 15mm, 18mm, 19mm and 20mm, respectively.

MIC and MBC of propolis

MIC and MBC of propolis against the bacterial species have shown in Figure 2. Ethanolic extract of propolis showed MIC in the range of 4.50±0.50mg/ml to 6.67±0.58mg/ml and MBC from 5.83±0.29mg/ml to 7.67±0.58mg/ml for the *B.subtilis* NCBI3610 and *P.acne* ATCC-6919 respectively. *P.acne* proved as more resistant against propolis with MIC value of 6.67±0.58mg/ml and MBC value of 7.67±0.58mg/ml. Significant difference was observed in the sensitivity of *B.subtilis* NCBI3610 (MIC=4.50±0.50mg/ml) and *P.acne* (MIC=6.67±0.58) for extract of propolis. *B.cereus* ATCC14579 (MIC= 5.83±0.16mg/ml), *E.coli NBRC102203* (MIC=4.83±0.76mg/ml) and *B.subtilis* KX881940 (MIC=4.5±0.29mg/ml) demonstrated comparable susceptibility to propolis (Figure 2).

Regarding to antioxidant activity, IC50 (µg/ml) value of ethanolic extract of propolis was 12.28±0.49 µg/ml.

DISCUSSION

Emerging era of multi-drug resistance in both gram-positive as well as gram-negative bacteria turned the attention towards the use of natural substance in the treatment of skin ailments (Baysallar et al., 2004). Propolis, a sticky honey bee product depicts a number of pharmacological, biological and antimicrobial activities and has been investigated by numerous researchers worldwide against a number of pathogens (Cunha et al., 2013). The present study focused on antioxidant and antibacterial property of ethanolic extract of propolis.

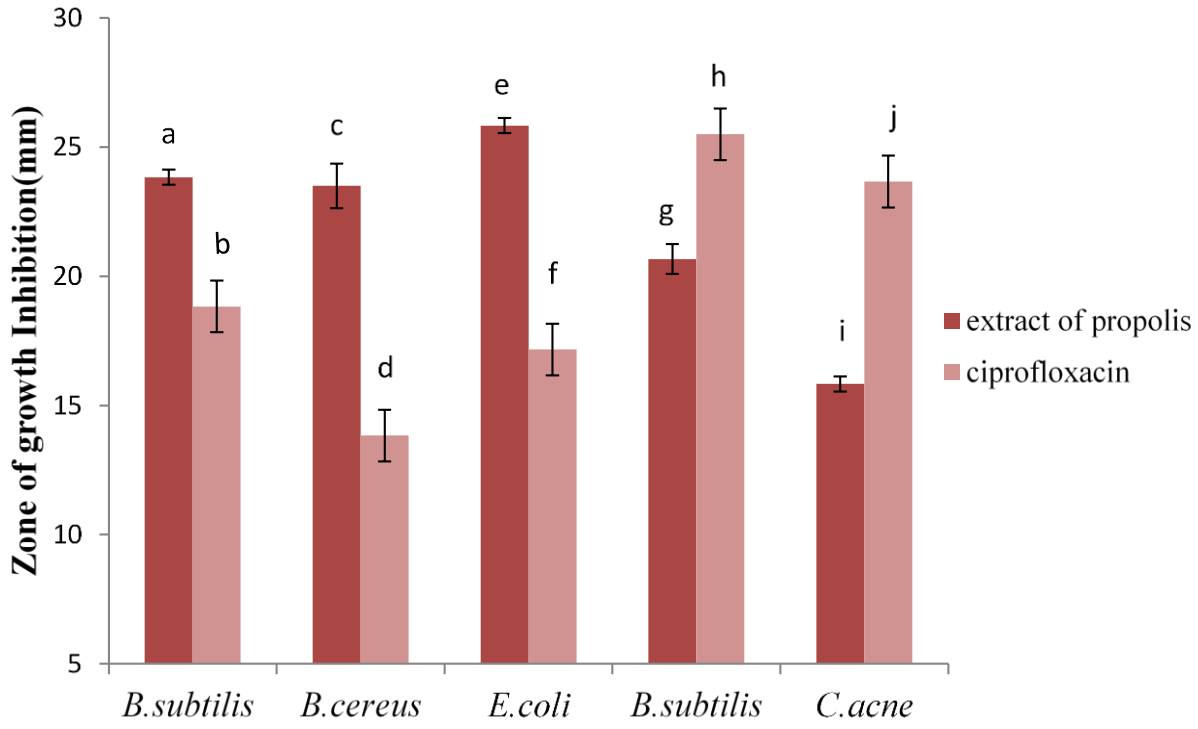

Figure 1: Growth inhibition zone of 70% ethanolic extract of propolis and the antibiotic, ciprofloxacin. For different bacterial species bars representing different letters differ significantly ($p<0.05$), negative control depicted no inhibition zone around the disc.

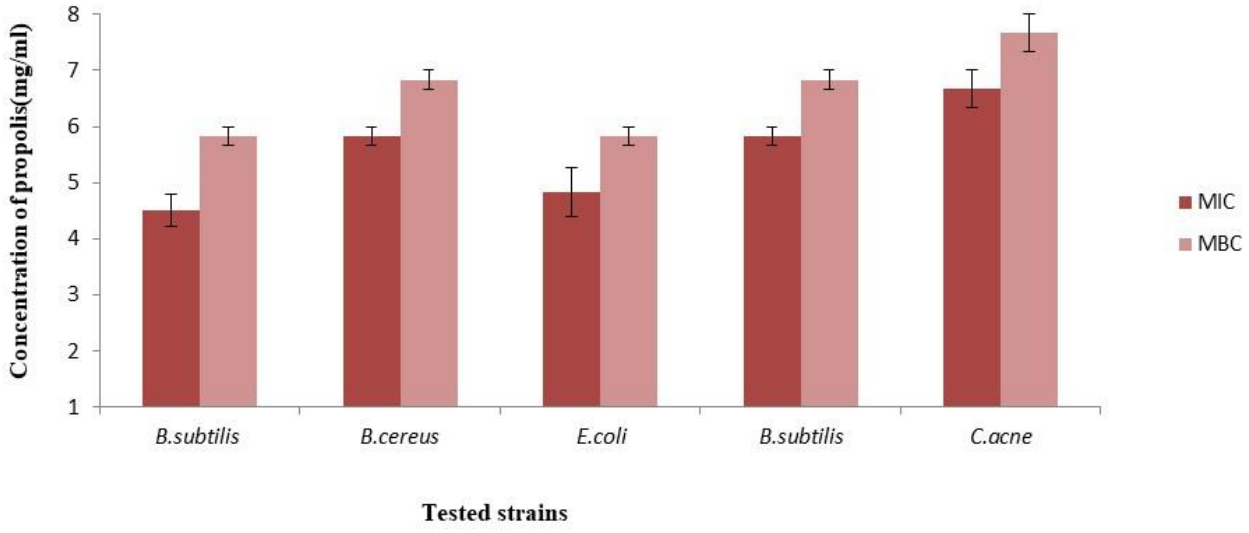

Figure 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanolic extract of propolis against different bacterial species

Ethanolic extract of propolis showed antibacterial activity against all the tested strains by yielding significant growth inhibition zones. Our results are comparable with earlier reports (Seidel et al., 2008, Kashi et al., 2011; Al-Ani et al., 2018). While reporting effects of bee propolis from the District Faislabad, Pakistan, Shahbaz et al (2015) documented that 65% ethanolic extract of propolis showed significant antibacterial activity against *S.aureus*, *B.subtilis* and *E.coli* with growth inhibition zones of 29.18±1.19, 26.37±1.13and 22.19±0.61mm,respectively. In the present study two strains of *B.subtilis NCBI3610*

andKX881940 exhibited different levels of sensitivity against 70% ethanolic extract of propolis. Manuharan et al (2021) and Shahbaz et al (2015) have already shown that 70% ethanolic extract of propolis showed growth inhibition zones ranging from 15 mm to 26 mm against *B.subtilis*. In the present work *Bacillus cereus* showed high sensitivity against ethanolic extract of proplois with growth inhibition zone of 25.5±0.87mm. Kubiliene et al (2015) showed that gram-positive bacteria showed greater sensitivity against propolis with inhibition zone of 15.8 – 17.2 mm than gram-negative bacteria (14.8 – 15.4 mm). However, in the present study both gram positive as well as gram negative bacteria did not show any difference in response to the application of propolis extract. Variations in antibacterial activity of propolis might be due to differences in botanical source (Koo et al., 2002) as well as differences in the susceptibility of isolates, owning to inherited resistant factor and exposure to different drugs in the environment (Ekpo and Etim, 2009). Antibacterial activity of propolis against *P.acne* though of moderate level in the present study is promising for further investigations. Ali et al (2015) had also reported use of propolis for the treatment of acne due to its effective antibacterial activity.

MIC and MBC values of propolis against the different bacterial isolates ranged from 4 to 7mg/ml. Highest MIC and MBS were for the acne bacterium. Ozen et al (2010) reported antibacterial activity of propolis against a number of oral anaerobic bacteria with MIC ranging from 0.4mg/ml to 6.1mg/ml against gram-positive bacteria and 5.8 to 108.1mg/ml against gram-negative bacteria. Al-Ani et al (2018) documented MIC values from 0.08 mg/ml to 2.5mg/ml against gram-positive bacteria and 0.6mg/ml to 5mg/ml against gram-negative bacteria. Various types of phenolic and flavonoid compounds determine the antibacterial activity of propolis. Inhibitory activity of propolis greatly depends on solvents that are used for extraction. Numerous studies evaluated the antimicrobial property of propolis prepared in different solvents against pathogenic bacteria and found that extracts of propolis prepared in ethanol exert greater inhibitory potential in comparison with other solvents such as methanol and water, due to high concentrations of flavonoid and phenolic compounds in the ethanolic extracts (Kalia et al., 2013; Shahbaz et al., 2015). Varying degrees of antibacterial activity observed for propolis extract of different origins and their different concentrations might be attributed to varying quantities of flavonoid and phenolic compounds in the extracts.

In the present study ethanolic extract of propolis gave IC 50 value of 12.28±0.49µg/ml that is supported by Valente et al (2011) who reported IC 50 value from 6.3 to 10.4µg/ml. Different IC 50 values for ethanolic extract of propolis have been reported by different researchers; 50µg/ml (Choi et al., 2006), 25.53 µg/ml to 69.96 µg/ml (Pratami et al., 2018) and 32.47µg/ml (Ramón-Sierra et al., 2019). Antioxidant potential of propolis depends on various factors i.e solvent used for extraction, chemical composition of propolis and its geographical region (Mihai et al., 2011). However, because of its effective antibacterial and antioxidant properties, propolis can served as remedy for the treatment of various skin disorders including acne and to escalate the process of wound healing (Dzialo et al., 2016).

Conclusion

Ethanolic extract of propolis exhibited greater antibacterial activity than ciprofloxacin for some bacterial isolates. This activity may be attributed to phenolic and flavonoid constituents of propolis. Antioxidant potential in propolis render the natural products as constituents in remedies used for the treatment of skin disorders in the era of emerging multi-drug resistance. Further studies are needed to compare the antibacterial as well as antioxidant potential of products of different botanical origins and that of which had specifically bee derived from monofloral origin. Such surveillance programs are likely to identify specific natural remedies for specific skin problems

Author's contribution

JI conceived the idea and designed the research work. MN performed the whole experiment and wrote the manuscript. Both authors read and approved the manuscript.

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