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RESEARCH ARTICLE

In-vitro Antibacterial Activity of Hydroalcoholic Extract of Propolis against Pathogenic Bacteria

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ABSTRACT

Propolis is a resinous substance produced by honey bee (*Apis mellifera* L.) from the exudations of plants. Propolis varies in composition due to change in climate and phytogeographical location. This study was designed to assess the *in vitro* antibacterial activity of locally available propolis against Gram positive and Gram negative bacteria. Bee propolis samples were collected from local apiaries. Hydroalcoholic extracts of propolis was prepared using different concentrations of ethanol (65%, 80% and 95%), methanol (65%, 80% and 95%) and water. All the extracts were assessed for antibacterial effects against *Staphylococcus* (*S.*) *aureus, Bacillus (B.) subtilis* and *Escherichia (E.) coli* using disc diffusion assay. The minimum inhibitory concentrations (MIC) were determined by measuring the optical density on spectrophotometer. The results showed highly significant variations ($P<0.05$) in antibacterial activities of propolis extracts used in this study. Among all the extracts, ethanolic extract of propolis showed highly significant ($P<0.05$) effect at 65% concentration with 29.18±1.19mm zone of inhibition for *S. aureus* and 26.37±1.13mm for *B. subtilis*; whereas, 22.19±0.61mm zone of inhibition was measured against *E. coli*. Significant difference ($P<0.05$) was shown for MIC values among the treatments with minimum value for 65% ethanolic extract of propolis followed by 65% methanolic extract while higher values were noticed for aqueous extract. In conclusion, 65% ethanolic extract showed more antimicrobial activity against *S. aureus* among all the extracts; however, aqueous extract represented least inhibitory potential. Due to rich phytochemistry, propolis could be used as an alternative source of natural antimicrobial agents.

INTRODUCTION

Propolis, a highly adhesive substance produced by honey bee (*Apis mellifera* L.), contains tree seep and plant exudates. Bees use propolis to cover the small holes and fissures in hives to protect colonies from foreign invaders. Chemically, propolis contains resinous substances (40-45%), fatty acids (25-30%), aromatic oils (10%), pollens (5%) and compounds of inorganic and organic nature (5%) (Silva et al., 2012). More than 300 compounds have been identified in resinous part of propolis including polyphenols, the bioactive moieties of propolis (Afrouzan et al., 2012).

Honey bee propolis is a rich source of polyphenols and flavonoids and has great potential as a natural antimicrobial agent against pathogenic micro-organisms without causing any adverse action. It inhibits the growth of bacteria by inhibiting the enzyme activity of bacteria to diminish their effects on biological systems Both Gram positive and Gram negative bacteria are susceptible to propolis (Zeighampour et al., 2013). Propolis can retard the development of biofilm formation among different pathogenic domains of microorganisms including *Listeria* spp., *Streptococcus* spp., *Staphylococcus (S.)* spp., *Bacillus (B.)* spp., *Escherichia (E.) coli and Pseudomonas* species (Stan et al., 2013). Different researchers have explored the antibacterial and antifungal properties of propolis against various food spoiling organisms and reported that phenolic

substances of propolis were responsible for antimicrobial activities (Wojtyczka et al., 2013).

Propolis is considered as a natural food preservative for various fruit juices. It is recommended to use against yeast and fungi to extend the shelf life of fruit juices (Koc et al., 2007). Application of propolis extract in fruits reduces the fungal attack, retards water loss, fixes the color, maintains quality attributes, inhibits the postharvest changes and microbial load during storage and transit (Ozdemir et al., 2010). Ethanolic extract of propolis has strong inhibitory effect on the growth of coliform bacteria in meat and meat based product. Substances found in propolis are generally recognized as safe (GRAS) and regarded as constituents of food and food products. They could be used as alternate of chemical preservative perishable food commodities (Tosi et al., 2007). The bioactive constituents of propolis that exhibit antimicrobial behavior differ by change in climate and geographical location. Flavonoids and phenolic acid of propolis are considered effective agents for antimicrobial perspective. Furthermore, the mechanism involved in antimicrobial activity is much complex and attributed due to synergistic association between sesquiterpenes and flavonoids hydroxyl acids (Lu et al., 2005).

In spite of numerous evidences regarding antimicrobial activity of propolis, no previous report was found on antimicrobial activity of propolis obtained from bee hives from geoclimatic conditions of Pakistan. This is the first study on the locally available propolis to check its *in vitro* antimicrobial activity against *S. aureus, B. subtilis* and *E. coli.*

MATERIALS AND METHODS

Collection of Propolis

Honey bee propolis was collected from the apiaries located in the surroundings of district Faisalabad, Pakistan and stored at room temperature until further analysis.

Preparation of alcoholic extract

Hydroalcoholic extract of propolis was prepared using 65, 80, and 95% of ethanol and methanol, each. The weighed amount of sample continuously agitated at room temperature for 24 hours under dark conditions. The extract was filtered twice with Whatman filter paper # 2 and centrifuged at 3000×g for 15 min. The polyphenols rich supernatant was collected and concentrated through rotary evaporator (SB-651, Eyela, Japan) under reduced pressure at 40°C. (Yaghoubi et al., 2007; Christov et al., 2006).

Preparation of inoculum

Cultures of *S. aureus*, *B. subtilis*, and *E. coli* were obtained from Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan (UAF) and prepared in nutrient broth by incubating at 37°C for 24 hours to get desired growth of microflora (1×10^6 cfu/mL) (Dhale et al., 2011).

Disc diffusion assay

For primary screening, disc diffusion assay was performed using nutrient agar (oxoid®, Japan). Culture media (15-20 mL) was transferred in each petri plate and 100 µL of inoculum added for the desired growth of respective strain on the petri plates. Discs of wicks sheets (10 mm diameter) soaked in 100 µL of 12mg/mL of each extract were placed on the petri plates with rifampicin (Sigma-Aldrich) in a dose rate of 10mg/10mL as positive control. Petri plates were incubated at 37°C for 24 hours and zone of inhibition was measured in millimeters using digital Vernier Caliper (NCCLS, 1997).

Determination of minimum inhibitory concentrations (MIC) of extracts

Broth micro-dilution method was used to determine MIC values according to the method described by Mehmood et al. (2012). In order to determine the MIC value for each extract, serial dilutions of all the extracts were made in a 96 well microtitre plate using Muller Hinton (MH) broth medium. Each extract was used at a concentration of 12mg/ml to make the serial dilutions. Thereafter, 10µl inoculum was added to each well containing nutrient broth and a standard drug Rifampicin (10mg/10mL) was used as positive control. The microtiter plates were incubated at 37°C for 24 hours followed by the addition of 10µL of resazurin solution (indicator) in each well and absorbance was recorded at 620nm using ELISA reader.

Statistical analysis

Data thus obtained were subjected to statistical analysis. Completely Randomized Design (CRD) and Analysis of variance technique (ANOVA) techniques were applied using Cohort version 6.1 (Steel et al., 1997).

RESULTS

Qualitative screening of propolis extract

According to results of antibacterial activity of propolis by disc diffusion method, zone of inhibition for *E. coli* exhibited a significant variation among the different extracts from minimum value of 8.72±0.51 mm for aqueous extract to maximum for EEP_{65} (22.19±0.61 mm) followed by MEP_{65} (19.89±0.43mm). *S. aureus* was found more susceptible to propolis extract with minimum zone of inhibition of 16.36±0.13 mm for EEP_{95} and maximum zone of inhibition of 29.18±1.19 mm with EE_{65} whilst least activity was noticed for aqueous extract (12.43±0.86mm). Regarding the antimicrobial activity against *E. coli*, maximum inhibition zone of 26.37±1.13mm was found in EEP_{65} followed by MEP_{65} as 20.37±0.63 mm (Figure 1 & 2).

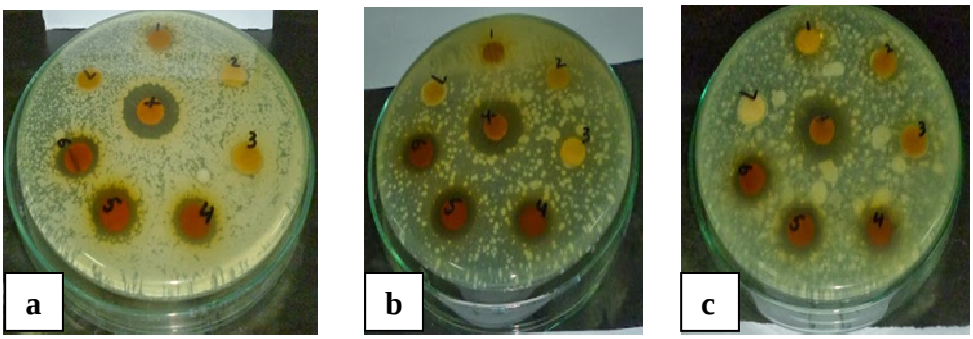

Fig. 1: Qualitative screening of antibacterial activity of propolis against a) *Bacillus subtilus* b) *Escherichia coli* c) *Staphylococcus aureus*

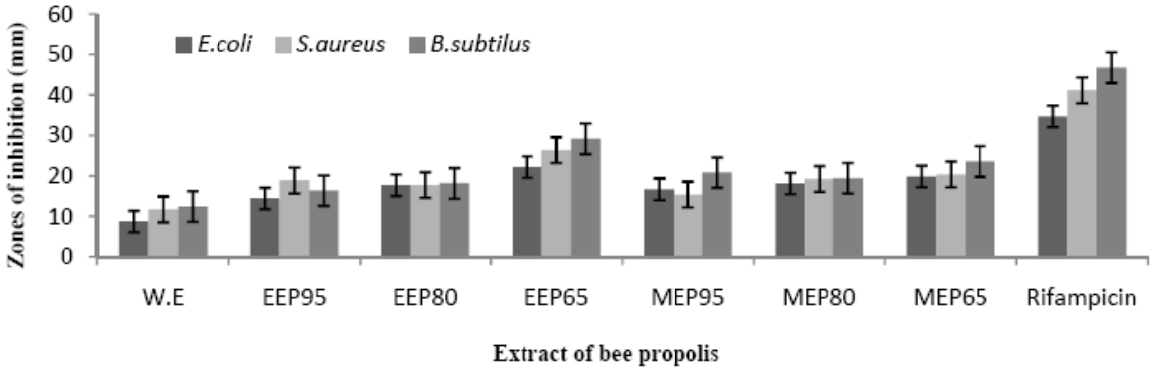

Fig. 2: Zones of inhibitions (Mean±standard deviation) against different bacterial isolates (P<0.05)
W.E: water extract, EEP_{95}: 95% ethanolic extract, EEP_{80}: 80% ethanolic extract, EEP_{65}: 65% ethanolic extract, MEP_{95}: 95% methanolic extract, MEP_{80}: 80% methanolic extract, MEP_{65}: 65% methanol

Table 1: Minimum inhibitory concentration (MIC) values of hydroalcoholic extracts of propolis

	Minimum Inhibitory concentration (g/g)			Mean
Propolis extracts	*E. coli*	*S. aureus*	*B. subtilis*	
W.E	1273.4±11.61^{a}	1123.4±9.61^{c}	1244.4±14.61^{b}	1213.7^{a}
EEP_{95}	855.5±12.46^{d}	438.5±16.52^{k}	648.3±11.42^{h}	647.4^{b}
EEP_{80}	784.3±9.27^{e}	372.7±9.23^{l}	469.3±13.68^{j}	542.1^{c}
EEP_{65}	626.4±13.91^{i}	225.7±10.37^{q}	286.5±9.56^{p}	379.5^{g}
MEP_{95}	731.5±10.43^{f}	338.6±14.64^{m}	432.3±14.73^{k}	500.8^{d}
MEP_{80}	693.6±8.61^{g}	309.4±13.69^{no}	373.7±12.38^{l}	458.9^{e}
MEP_{65}	645.6±11.45^{hi}	289.5±12.71^{op}	318.6±9.69^{mn}	417.9^{f}
Rifampicin	164.6±8.13^{r}	106.7±7.16^{t}	142.5±10.87^{s}	137.9^{h}

Table: 1. Mean values showing different letters differs significantly (p<0.05). W.E: water extract, EEP_{95}: 95% ethanolic extract, EEP_{80}: 80% ethanolic extract, EEP_{65}: 65% ethanolic extract, MEP_{95}:95% methanolic extract, MEP_{80}: $_{80}$% methanolic extract, MEP_{65}: 65% methanol

Determination of minimum inhibitory concentrations
The results regarding minimum inhibitory concentration (MIC) against *E. coli* were recorded as 855.5±12.46, 784.3±9.27, 626.4±13.5, 731.6±10.43, 693.6±8.61 and 645.6±11.45µg/mL for EEP_{95}, EEP_{80}, EEP_{65}, MEP_{95}, MEP_{80} and MEP_{65}, respectively, whereas water extract showed 1273.4±11.61µg/mL in comparison to positive control (Rifampicin) which was 164.67±8.13µg/mL (Table:1). The MIC values for the extracts of propolis extracts like EEP_{95}, EEP_{80}, EEP_{65}, MEP_{95}, MEP_{80} and MEP_{65} against *B. subtilis* measured as for 648.3±11.42, 469.3±13.68, 286.67±9.5, 432.32±14.3, 373.7±12.38 and 318.43±9.6µg/mL, respectively; whereas, the value for positive control was noticed as 142.33±10.87µg/mL (Table 1). Likewise, MIC against *S. aureus* were examined as 438.5±16.52, 372.7±9.23, 225.7±10.37, 338.6±14.64, 309.4±13.69, 289.5±12.71µg/mL in response to EEP_{95}, EEP_{80}, EEP_{65}, MEP_{95}, MEP_{80} and MEP_{65}respectively; whereas aqueous extract showed values as 1123.4±9.61 in comparison to positive control exhibiting 106.88±7.16 µg/mL (Table 1).

DISCUSSION

Bee propolis, a resinous product exhibits a number of biological, pharmacological and antimicrobial properties and has been investigated worldwide against various pathogens (Cunha et al., 2013). Different studies from Argentine explored that ethanolic extract of propolis successfully retarded the growth of *E. coli* thus could be used as a natural food preservative to inhibit the bacterial spoilage of perishable food commodities (Tosi et al., 2007).

The results of the present study are consistent with previous findings which showed that propolis inhibited the biofilm formation in both G-positive and G-negative bacteria including *S. aureus* (Stan et al., 2013). Propolis exhibited antimicrobial potential against different domains of bacteria; whereas, among the G-positive domain *S. aureus* showed more sensitivity against ethanolic extract of propolis (Kujumgiev et al., 1999) as observed in the present study. Similarly, values of zone of inhibition measured in present study are in line with the previous investigations of Zeighampour et al. (2013) who examined the inhibitory role of ethanolic extract of propolis using well diffusion method and observed that 70% of ethanolic extract showed better response against *S. aureus* as compared to *P. aeruginosa*. Chemical composition and antimicrobial status of propolis varies with climatic change, season of collection and geographical location. Propolis extract exhibited better antibacterial activity against G-positive bacteria and it is in accordance with the findings of Lu et al. (2005). Bee propolis being as nontoxic substance has potential usage against food spoilage microorganisms. It could be considered an efficient antimicrobial and antioxidant agent which could be incorporated in food systems to control microflora (Kalogeropoulos et al., 2009). Previously, the antimicrobial activity of propolis from Mangolia, Albania, Egypt and Brazil was identified against *S. aureus* which showed zone of inhibition of 24, 21.8, 24.3 and 21.8mm, respectively (Kujumgiev *et al.,* 1999) and those are comparable with the findings of present study. A number of studies relate antimicrobial activity of propolis with its bioactive compounds like caffeic acid, cinnamic acid, benzoic acid, quercetin, galangin and pinocambrin. As a mechanistic approach components of propolis mainly destruct the cell wall of bacteria to retard further growth/multiplication of microorganisms (Gatto *et al.,* 2002). Previously, ethanolic extract of propolis showed a significant inhibitory effect on the growth of bacteria and fungi due to its polyphenols and flavonoids content; whereas, extracts revealed more activity against G-positive bacteria than the G-negative bacteria (Yaghoubi et al., 2007). Earlier, comparative antimicrobial activity of propolis and honey had also been assessed which showed that propolis possessed more activity against *S. aureus* as compared to honey (Rahman et al., 2010). As a whole, in our study locally found propolis showed highest activity against *S. aureus* followed by *B. subtilis* and *E. coli,* whilst among all the treatments ethanolic extract prepared (65%) showed maximum zone of inhibition 22.19±0.61, 26.37±1.13 and 29.18±1.19mm for *E. coli, B. subtilis* and *S. aureus*, respectively.

Conclusion

In conclusion, Pakistani propolis contains novel compounds for inhibition of bacterial growth and could be used to control the problems associated with G-positive and G-negative bacteria. Further studies required to evaluate the chemical constituents of Pakistani propolis for its utilization as a natural antimicrobial agent in various ways.

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Authors' contribution

MS perceived idea, conducted research work during PhD study and wrote the manuscript. TZ and HN guided to design project and conduction of study regarding experimental protocols and statistical analysis. MAR participated in extract preparation for the analysis and helped in writing the manuscript.

REFERENCES

Afrouzan H, A Cyrus, AM Sayed, E Alireza, V Narges and T Golamhosein, 2012. Evaluation of antimicrobial activity of propolis and nanopropolis against *Staphylococcus aureus* and *Candida albicans*. African Journal of Microbiology Research, 6: 421-425.

Christov R, B Trusheva, M Popova, V Bankova, MB Middleton and EC Kandaswami, 2006. Chemical composition of propolis from Canada, its antiradical activity and plant origin. Natural Product Research, 20: 531-536.

Cunha MG, M Franchin, LCC Galvao, ALTG Ruiz, JE Carvalho, M Ikegaki, SM Alencar, H Koo and PL Rosalen, 2013. Antimicrobial and antipro-liferativeactivities of stingless bee *Melipona scutellaris* geopropolis. BMC Complementary and Alternative Medicine, 13: 23-31.

Dhale DA and SK Markandeya, 2011. Antimicrobial and phytochemical screening of *Plumbago zeylanica* Linn. (Plumbaginaceae) Leaf. Journal of Experimental Sciences, 2: 4-6.

Gatto MT, S Falcocchio, E Grippa, G Mazzanti, L Battinelli, G Nicolosi, D Lambusta and L Saso, 2002. Antimicrobial and anti-lipase activity of quercetin and its C2-C163-O-acyl-

esters. Bioorganic and Medicinal Chemistry, 10: 269-272.

Kalogeropoulos N, SJ Konteles, E Troullidou, I Mourtzinos, VT Karathanos, 2009. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chemistry, 116: 452-461.

Koc AN, S Silici, FM Sariguzel, O Sagdic, 2007. Antifungal activity of propolis in four different fruit juices. Food Technology and Biotechnology, 45: 57-61.

Kujumgiev A, I Tsvetkova, Y Serkedjieva, V Bankova, R Christov and S Popov, 1999. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. Journal of Ethnopharmacology, 64: 235-240.

Lu L, Y Chen and C Chou, 2005. Antibacterial activity of propolis against *Staphylococcus aureus*. International Journal of Food Microbiology, 102: 213-220.

Mehmood N, M Zubaır, K Rızwan, N Rasool, M Shahid and VU Ahmad. 2012. Antioxidant, Antimicrobial and Phytochemical Analysis of *Cichorium intybus* Seeds Extract and Various Organic Fractions. Iranian Journal of Pharmaceutical Research, 11: 1145-1151.

NCCLS (National Committee for Clinical Laboratory Standards), 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7-A. NCCLS, Villanova, PA.

Ozdemir AE, EE Çandir, M Kaplankiran, EM Soylu, N Şahinler and A Gul, 2010. The effects of ethanol-dissolved propolis on the storage of grapefruit. Turkish Journal of Agriculture, 34: 155-162.

Rahman MM, A Richardson and M Sofian-Azirun, 2010. Antibacterial activity of propolis and honey against *Staphylococcus aureus* and *Escherichia coli*. African Journal of Microbiology and Research. 4: 1872-1878.

Silva JC, S Rodrigues and LM Feas X, Estevinho, 2012. Antimicrobial activity, phenolic profile and role in the inflammation of propolis. Food Chemistry and Toxicology, 50: 1790-1795.

Stan T, L Marutescu, CM Chifiriuc, C Mateescu and V Lazar, 2013. Study of the antimicrobial and antibiofilm activity of romanian propolis. Biointerface Research and Applied Chemistry, 3: 541-550.

Steel RGD, JH Torrie and D Dickey, 1997. Principles and procedures of statistics: a biometrical approach, 3rd ed. McGraw Hill Book Co., Inc., New York.

Tosi EA, E Re, ME Ortega and AF Cazzoli, 2007. Food preservative based on propolis: Bacteriostatic activity of propolis polyphenols and flavonoids upon *Escherichia coli*. Food Chemistry, 104: 1025-1029.

Wojtyczka RD, M Kwpa, D Idzik, R Kubina, A KabaBa-Dzik, A Dziedzic, and TJ Wdsik, 2013. *In vitro* antimicrobial activity of ethanolic extract of polish propolis against biofilm forming *Staphylococcus epidermidis* strains. Evidence Based Complementary and Alternative Medicine, 2013: 1-11.

Yaghoubi SMJ, GR Ghorbani, ZS Soleimanian and R Satari, 2007. Antimicrobial activity of Iranian propolis and its chemical composition. DARU, 15: 45-48.

Zeighampour F, M Mohammadi-Sichani, E Shams and NS Naghavi, 2013. Antibacterial activity of propolis ethanolic extract against antibiotic resistance bacteria isolated from burn wound infections. Zehedan Journal of Research in Medical Sciences, 16: 25-30.