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SHORT COMMUNICATION

In vitro Analysis of the Antimicrobial Properties of Propolis Collected from Jimma, Ethiopia

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Abstract

The effectiveness of antimicrobial drugs becomes uncertain due to the emergence of multidrug resistant microorganisms, which highlights the need for alternative antibacterial agents. Natural products are of great importance in the search for biologically active compounds. The present study aimed at investigating the antibacterial properties of propolis, one of the natural bee products, against *S. aureus*, *P. aeruginosa* and *E. coli*. Propolis was extracted using 30 %, 50 %, 70 %, and 99.9 % ethanol. The *in vitro* antibacterial activities of propolis extracts were evaluated by the disc diffusion method with concentrations between 500 and 4000 µg/ml. Among the extracts, the 50 % and 70 % propolis extracts showed strong antibacterial activity against all tested strains with inhibition zones ranging from 6.64 ± 0.15 to 11.99 ± 0.04 mm. *P. aeruginosa* was sensitive strain to the ethanolic extracts of propolis with the highest inhibition zone diameter of 11.99 ± 0.04 mm. Statistically significant differences in growth inhibition were observed among the types of extracts (30 %, 50 %, 70 % and 99.9 %) against *P. aeruginosa* ($p < 0.05$) and *E. coli* ($p < 0.05$), but the effect was not significant on *S. aureus* ($p > 0.05$). All propolis extracts showed no effect on *S. aureus* at concentrations below 2000 µg/ml. Propolis extracts showed a lower zone of inhibition compared to the effect demonstrated by the positive control. Overall, the results indicate that ethanolic extracts of Ethiopian propolis has a promising antibacterial activity which could be of an antibiotic development benefit.

Keywords: Antibacterial Activity, Disc Diffusion Method, Ethiopia, *In vitro*, Propolis

1. Introduction

Propolis, commonly known as the "bee glue", is a resinous natural sticky substance produced by bees, primarily to cover hive walls and seal openings and cracks[1]. It is also used as an "embalming" agent to cover hive invaders and dead bodies inside the beehive to ensure a clean environment. Bees collect plant resins from buds, exudates and other parts of plants, and combine them with their own salivary enzymatic secretions and beeswax to produce propolis[2].

Propolis contains a mixture of different secondary metabolites including flavonoids, aromatic acids, terpenes and tannins[3,4] that are responsible for various bioactivities such as antibacterial, antifungal, antiparasitic, antioxidant, anti-ulcer, anti-inflammatory, anti-viral activities and anti-angiogenic. The qualitative and quantitative composition of constituents of propolis is dependent on geographical regions from which propolis is collected and seasonal conditions. The diverse plant species found in different geographical locations could afford propolis of variable chemical composition and intensity of bioactivities[5,6]. More than 300 chemical compounds have been identified in propolis extracts including phenolic compounds,

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aromatic acids, essential oils, waxes, sugars and amino acids[7].

Despite great progresses made in the past, treatment of infectious diseases still presents a significant problem. Antibiotic related side-effects and emergence of drug-resistant pathogens necessitate the need for novel and effective antimicrobial compounds. Propolis has drawn attention as a potential source of bioactive chemicals since it has been used for thousands of years as a healing agent in traditional medicine[8,9]. Several studies showed that propolis extracts possess wide-spectrum medicinal values including antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, and anticancer[8,10,11]. The intensity of bioactivities of propolis extracts, however, varies depending on the geographic source of propolis. Although previous studies in other countries demonstrated the pharmacological properties of propolis, there has been only limited research on antimicrobial profile of Ethiopian propolis. Therefore, the aim of this study was to evaluate the antibacterial effect of propolis collected from the Southwestern Ethiopia.

2. Materials and Methods

2.1 Description of Study Area

Propolis samples were collected from apiaries found in Jimma areas, Southwestern Ethiopia, which is located 350 km away from Addis Ababa, Ethiopia. It lies between 7°33' N and 36°57' E. The area is midland (locally called Woyna-Dega) and has an average altitude of 1710 m above sea level. The average annual temperature and relative humidity range between 11.4°C and 26.8°C and 39.92 % and 91.4 %, respectively. The average annual rainfall is about 1500 mm[12]. The study was conducted in the School of Pharmacy and the Department of Veterinary Microbiology, Jimma University, Ethiopia.

2.2 Test Strains

Staphylococcus aureus (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were obtained from the Department of Bacteriology, Parasitology and Zoonosis of the Ethiopian Public Health Institute.

2.3 Collection and Preparation of Propolis

Propolis samples were collected using trap plates fixed on the top of beehives. The propolis was then scraped off from the plates and dried in the freezer at -20 °C. The dried material was crushed and homogenized[13].

2.4 Preparation of Crude Propolis Extracts

Propolis samples (30 g) were extracted using 100 ml of four different concentrations of ethanol: 99.9 % (absolute ethanol), 70 %, 50 % and 30 % (v/v) by mixing vigorously for 30 minutes followed by intermittent shaking for 7 days. After a week, the supernatant was filtered with Whatman # 1 filter paper and the alcohol was evaporated on a water bath at 50 °C[5]. The dry propolis extracts were weighed and the percentage yield was determined based on the weight of raw propolis.

2.5 Antibacterial Activity

Antibacterial activity of propolis extracts was tested using disc diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI, 2005) guideline[14]. Briefly, dried extracts of propolis were dissolved in 70 % ethanol to prepare 10 % stock solution of the extracts, from which eight different test concentrations (4000, 3500, 3000, 2500, 2000, 1500, 1000, and 500 µg/ml) were prepared using the same solvent. Sterile blank discs of 6 mm diameter were then loaded with 20 µl of each propolis test solution. The extract impregnated discs were then dried in an oven at 40°C for 6 hours to get 80, 70, 60, 50, 40, 30, 20, and 10 µg per disc, respectively. Standard Gentamicin disc (10 µg, OXOID, CT0024B) were served as positive controls, while discs loaded with 70 % ethanol and dried in the same manner as the test discs were used as negative controls.

Bacterial inocula were prepared in sterile normal saline (0.9 % NaCl) solution with the bacterial density corresponding to 0.5 McFarland standards. The discs were then placed on the bacterial lawn using sterile forceps and gently pressed down to ensure complete contact with the agar surface. After incubation of the plates for 24 hours at 37 °C, the zone of inhibition (in mm) was measured using digital caliper.

2.6 Data Analysis

The experiment was performed in triplicate and results are expressed as mean ± Standard Deviation (SD). Between group and within group analysis was performed using one-way ANOVA to test the statistical difference in antibacterial activity between the different ethanolic extracts, concentrations, and the bacterial strains. Post hoc multiple comparison was performed using Tukey's test. Differences between means were considered significant at $p < 0.05$. All tests were done using SPSS version 20.0 for windows and graphs were prepared using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1 Yield of Propolis Extracts

The percentage yield of extracts among the four ethanol concentrations was statistically different ($p < 0.05$) with maximum yield of dry extract was obtained with 99.9 % ethanol (31.9 % ± 2.46 w/w). The least extract yield (4.22 % ± 0.17w/w) was obtained with the 30 % ethanol (Figure 1).

3.2 Physical Properties of Propolis Extracts

The physical characteristics of propolis extracts are shown in (Table 1). The colours of extracts were observed as light yellow (99.9 %), yellow brown (70 %), reddish brown (50 %) and dark brown (30 %). The stickiness of extracts was examined through palpation, and it was found that extracts obtained with 30 and 50 % ethanol were stickier than those obtained from 70 % and 99.9 % ethanol.

3.3 *In vitro* Antibacterial Activities of Propolis

Results of the antibacterial activities of propolis extracts were evaluated by disc diffusion method and presented in Table 2.

The propolis extracts generally exhibited a dose/concentration dependent increase in antibacterial response (Figure 2). The 50 % and 70 % ethanolic extracts of propolis had a strong antimicrobial activity against all the tested bacterial strains (Table 2), while the 30 % and 99.9 %extracts were found to be inactive against *E. coli* and *P. aeruginosa*. These later two bacterial strains were more sensitive to the 50 % propolis extract at all concentration range explored (Figure 2).

The highest inhibition zones were recorded with the 50 % ethanol extracts at 4000 µg/ml (80 µg extract disc) against *P. aeruginosa* (11.99 ± 0.04 mm), *E. coli* (10.47 ± 0.12 mm) and *S. aureus* (10.46 ± 0.12 mm). In contrast, the lowest inhibition zone (6.64 ± 0.15 mm) was observed with 70 % propolis extract at

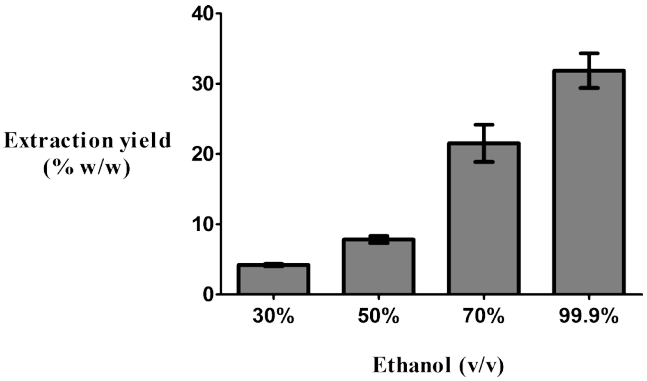
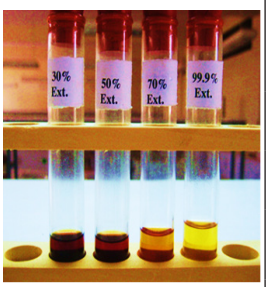


Figure 1. Percentage yield of propolis extracts produced with different strengths of ethanol used for extraction. The percentage yield was calculated based on the following formula: Percentage yield (% w/w) = Wd/Wr × 100 (where Wd; weight of dry extract and Wr; weight of raw propolis).

Table 1. Physical properties of ethanolic extracts of propolis*

Extracting solvent (Ethanol, v/v)	Colour	Stickiness	
30 %	Dark brown	Very Sticky	
50 %	Reddish brown	Sticky	
70 %	Yellow brown	Slightly sticky	
99.9 %	Light yellow	Slightly sticky	

*The image represents the colour of each extract.

Table 2. Antibacterial activity of ethanolic extracts of propolis at different concentrations against *S. aureus, P. aeruginosa,* and *E. coli*

Ethanolic extracts (v/v)	Propolis concentration (µg/ml)	Zone of inhibition (mm)[a]		
		S. aureus	*P. aeruginosa*	*E. coli*
	500	-	-	-
	1000	-	-	-
	1500	-	-	-
30 %	2000	-	-	-
	2500	-	-	-
	3000	-	-	-
	3500	6.97 ± 0.09	-	-
	4000	7.65 ± 0.10	-	-
	500	-	8.01 ± 0.07	7.09 ± 0.06
	1000	-	9.22 ± 0.04	7.91 ± 0.14
	1500	-	9.95 ± 0.09	7.98 ± 0.07
50 %	2000	-	10.04 ± 0.06	9.27 ± 0.30
	2500	9.63 ± 0.24	10.11 ± 0.10	9.09 ± 0.10
	3000	9.73 ± 0.17	10.61 ± 0.11	9.98 ± 0.04
	3500	10.05 ± 0.05	11.56 ± 0.10	10.25 ± 0.14
	4000	10.46 ± 0.12	11.99 ± 0.04	10.47 ± 0.12
	500	-	6.64 ± 0.15	-
	1000	-	7.19 ± 0.06	-
	1500	-	8.13 ± 0.15	-
70 %	2000	-	8.82 ± 0.13	-
	2500	8.59 ± 0.11	7.87 ± 0.08	6.96 ± 0.10
	3000	8.91 ± 0.10	8.21 ± 0.09	7.11 ± 0.11
	3500	9.65 ± 0.23	8.77 ± 0.12	8.19 ± 0.12
	4000	10.17 ± 0.07	9.14 ± 0.08	9.00 ± 0.08
	500	-	-	-
	1000	-	-	-
	1500	-	-	-
99.90 %	2000	-	-	-
	2500	-	-	-
	3000	7.96 ± 0.08	-	-
	3500	8.83 ± 0.11	-	-
	4000	9.57 ± 0.10	-	-
	70 % Ethanol	-	-	-
Control	Gentamicin (10µg)	19.06 ± 0.08	17.51 ± 0.14	24.05 ± 0.05

[a] value is expressed as mean ± SD of three replicates (-) = no inhibition

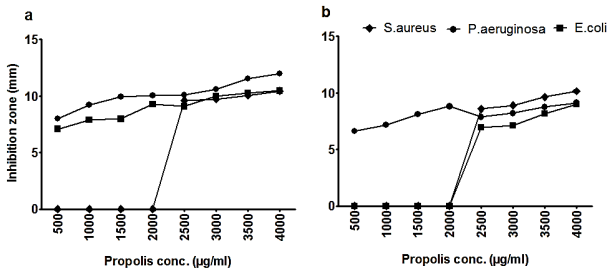


Figure 2. Concentration dependent effects of 50 % propolis extract **(a)** and 70 % propolis extract **(b)** on the growth of *S. aureus* (diamond), *P. aeruginosa* (circle), and *E. coli* (square).

500 µg/ml concentration (10 µg extract disc) against *P. aeruginosa*. All propolis extracts showed no effect on *S. aureus* at concentrations below 2000 µg/ml. Statistically significant difference in growth inhibition was observed among the types of extracts (30 %, 50 %, 70 % and 99.9 %) against *P. aeruginosa* ($p < 0.05$) and *E. coli* ($p < 0.05$), but the effect did not reach to significance against *S. aureus* ($p > 0.05$). All propolis extracts resulted in lower zone of inhibition compared to the effect demonstrated by the positive control. Whereas, the negative control disc did not show antibacterial activity on the studied strains.

4. Discussion

One of the major public health problems globally and especially in developing countries is infectious diseases. Many of the pathogenic bacteria have become resistant to commonly used antibiotics, and thus there is an increased need to search for alternative antimicrobial agents. Natural products, such as propolis, have been proven as a potential source of bioactive compounds. In this work, we have investigated the antibacterial activity of Ethiopian propolis against standard bacterial strains.

The percentage yield of dry propolis extract obtained in the current study is in line with Popova et al.,[5] who reported 12 to 41 % w/w yield with the 70 % ethanol. Koru and co-worker[15] also found the percentage yield ranging from 4.6 % to 17.5 % w/w, which is comparable to our study. The percentage yield of propolis extract in other studies have been noted as high as 61.3 % w/w[5,16,17]. These differences in yield may reflect the compositional variability in propolis from region to region due to variations in the types of trees and shrubs from which the bees harvest the resins. Moreover, propolis samples collected from different areas showed different solubility in ethanol even if the same amount of propolis were dissolved in the same volume of ethanol[15]. In the present study, the yield of ethanolic extract of propolis increased with the proportion of ethanol in the solvent mixture. However, the antibacterial activity did not increase equally as an increased dry extract yield may not necessarily indicate a proportional increase in relevant bioactive chemicals. A high yield with a 99.9 % alcohol may be an indication of the extracting power of the solvent with respect to non-polar components. This high yield, if the fraction was found to be active and promising, could also add advantage to the antimicrobial property of the 99.9 % extract. However, the finding of our study revealed that the 50 % and 70 % extracts were better in antibacterial activity. This could be an indication that the active principles in propolis have semi-polar nature. Furthermore, in addition to the chemical and structural constitution of ingredients that determines the intrinsic bioactivity, an important factor that contributes to antibacterial activity of extracts is concentration. In our study, we found a consistent increase in antibacterial activity with increase in concentration of extracts, in 50 % and 70 % extracts, but not with 99.9 % extracts. This could further indicate that, either constituents in the 99.9 % extracts are NOT active to the tested strains OR the extracts contain only trace quantities of the active principles to migrate into the extracting solvent (*i.e.*, 99.9 % alcohol).

In our study, the preliminary physical characteristics of propolis extract have also been established. The color and stickiness of propolis extracts became intense as the proportion of water in the extracting solvent increased. The difference in colour and glueyness of the extract may also be due to the nature of resins and other constituents found in propolis. It has previously been reported that more than 300 chemical compounds have been identified in propolis extracts including phenolic compounds, aromatic acids, essential oils, which could impart different color intensities in the various proportions of extracting solvent[7].

The strength of aqueous-ethanol mixture used for extraction of propolis exhibited significant difference ($p < 0.05$) with respect to the antibacterial properties of propolis extracts. The 50 % and 70 % aqueous-ethanol mixture produced the most efficient extracts for inhibiting the growth of tested bacterial strains. On the other hand, the 30 % and 99.9 % ethanol extracts were relatively ineffective in inhibiting bacterial growth. Results shown here are supported by several other studies in which they mostly used 60 % and 70 % ethanol as effective extracting solvents for propolis[17–19]. Furthermore, Mavri and colleague[20] reported that extraction of Slovenian propolis with 70 % ethanol was more efficient than that of 96 % ethanol, as the 70 % ethanolic extracts was found to have more phenolic compounds. Therefore, this may justify that the 50–70 % alcohol may be optimum composition to better extract biologically active constituents out of propolis.

The antibacterial activity of propolis demonstrated in this study against *S. aureus, P. aeruginosa* and *E. coli* is consistent with previous research findings[18,21]. It is believed that the antimicrobial properties of propolis are mainly attributed to its bioactive substances, such as phenols, flavonoids, alkaloids, etc. Ethanolic extracts of the Brazilian propolis inhibited the growth of *S. aureus* and *E. coli* with inhibition zones diameters ranged between 7 mm and 13 mm[22], which is consistent with our results (between 6.97 mm and 10.47 mm). The present findings are also in accordance with Hendi *et al.,*[23] who verified the antibacterial activity of Iraqi propolis against *S. aureus, P. aeruginosa,* and *E. coli* with inhibition zone diameters of 25 mm, 10 mm, and 15 mm respectively. However, in contrary to our results, Marghitas and associates[18] reported about the complete resistance of *P. aeruginosa* to the Romanian propolis extracts. Such discrepancies in the biological activities of propolis might be due to the difference in chemical compositions originated from diverse botanical sources. The antibacterial effect of gentamicin (a positive control) on *S. aureus* (19.06 ± 0.08), *P. aeruginosa* (17.51 ± 0.14) and *E. coli* (24.05 ± 0.05) significantly varied ($p < 0.05$) compared to propolis extracts; and this might be due to less amount of biologically active principles of propolis presented in the discs.

Previous studies showed that the antibacterial effect of propolis is more pronounced on Gram-positive bacteria than on Gram-negative ones (such as *E. coli* and *P. aeruginosa*). This has been explained by the fact that Gram-negative bacteria have got a fatty phospholipid outer layer which could act as a diffusion barrier for the crude extracts[24]. Regardless of this permeability issue, some propolis extracts still have a strong inhibitory effect on Gram-negative strains[25–28], as it was found in the current study. This could be attributed to the richness of plant biodiversity in the country, which may afford unique bioactive chemicals in the Ethiopian propolis.

This study has some limitations. First, our findings would be more appealing if we could identify and characterize biologically active constituents to verify potential antimicrobial substances. Further chemical characterization studies should be carried out as the Ethiopian propolis is assumed to have unique bioactive components. Second, we only evaluated the *in vitro* antibacterial activity of propolis in three bacteria; and other microorganisms, such as fungi, have not been tested. Third, the propolis samples were collected from a single region, which makes it hard to draw a strong conclusion about the antimicrobial properties of Ethiopian propolis. Despite all these limitations, our results still reveal that ethanolic extracts of propolis collected from the Southwest Ethiopia possess a promising antibacterial activity.

5. Conclusion

The results presented suggest that antibacterial activities of propolis depends on the concentration of ethanol used for extraction as well as the extract concentration loaded in the discs. Propolis extracts showed interesting antibacterial effects on *P. aeruginosa* and *E. coli*, the well-known multi-drug resistant bacteria. To the best of our knowledge, this is the first report published on the antibacterial profile of propolis collected from Southwest Ethiopia and thus it can be used as baseline data for subsequent studies on chemical characterization and antimicrobial properties of Ethiopian propolis.

6. Conflicts of Interest

The authors declare no conflicts of interest.

7. Acknowledgement

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