Antimicrobial properties of Libyan propolis against Staphylococcus aureus

Najat Hossin Sarkez
Department of Microbiology, National Medical Research Center, Zawia, Libya
Correspondence to Sarkez@nmrclibya.com.ly

Abstract: Libyan propolis Libyan propolis, bee glue" produced by honey bees feeding on plant buds, leaves and exudates was obtained from hives of apiary in Zawia area of western Libya. Its antimicrobial properties against pathogenic Staphylococcus aureus, coagulase positive obtained from Central hospital, Tripoli. The effects of ethanloic extract of propolis against S. aureus under different incubation temperatures (6, 20 and 37 °C), salt concentrations (5 and 10%), and pH values (3.5, 6.3) was investigated. Several fractions were obtained during the partial purification of propolis: crude ethanolic extract, resinous material ethanolic solution, and alkaline hydrolysis of water soluble compounds solution. Tests conducted included measurement of inhibition zone by the disk diffusion method, minimal inhibitory concentration by tube dilution method, and minimal bactericidal concentration by agar plating. Only crude ethanolic extract of propolis exhibited effective inhibition zone at different seed concentrations: a diameter of 19.62 mm at 30 mg/150 µl (w/v) and 19.73 mm at 40 mg/ 200 µl (w/v). There was no antibacterial effect of the extract at 10 mg/50 µl concentration. The minimal inhibitory concentration and minimal bactericidal concentration were found at 26.04 mg/ml, and 34.72 mg/ml respectively. Higher temperature (37 ºC) under natural acidic pH of 3.5 enhanced the antimicrobial activity of ethanolic extract of propolis, while high salt concentration, 10%, showed no added antibacterial effect. Possible use of propolis extract as food or pharmaceutical preservative, or topical treatment for skin diseases caused by S. aureus is encouraging and feasible.

Introduction

Propolis" bee glue" is a mixture of beeswax and resins collected by honeybees from plant buds, leaves and exudates such as (Willow, Poplar, Birch, Fir, Pine, Chestnut, etc) (1). Bee mixes the original propolis with bee wax and β-glycosidase they secrete it during propolis collection (2). Bees use propolis not only as a building material for their hives but also as a means to guard against growth of bacteria and fungi in the hives properties (3). Propolis and its constituents are reported to have biological effects, such as being antibacterial, antifungal, cytotoxic, antiprotozoan, antioxidant, anti-inflammatory, antiseptic, spasmolytic and having anaesthetic properties (3). From these activities, the antibacterial action is the most extensively investigated and the differences between the ethanolic extracts being due to some factors such as bee species, propolis origin, extract preparation and bacteria tested (2). Propolis is very dependent on the botanical origin of the exudates and very complex in that it contains at least 140 different known compounds (4). The major bioactive components of propolis are aromatic acids, esters and the flavanoids: galangin, quercetin, kaempferol, acacetin, pinocembrin and pinostobin (4).

The antibacterial activity of the isolated compound of propolis was determined against Gram-negative strains (Serratia sp., Pseudomonas sp. and Escherichia coli) and Gram-positive bacteria (B. cereus, S. aureus) (5). Cells in late exponential phase were most susceptible to Ethanolic Extract (EEP).Higher temperature (37 ^{o}C) and acidic pH enhanced the antibacterial activity of EEP (6).
The in vitro activity of Brazilian ethanolic extract of propolis (EEP) against 118 strains of

Staphylococcus aureus isolated from human infections was studied by the agar dilution method. The average minimum inhibitory concentration (MIC) was 22.5 mg per ml) (7). S. aureus causes a wide variety of suppurative diseases and toxinoses by virtue of many virulence factors it possesses, (8). There has recently been a dramatic increase in the incidence of nosocomial infections caused by strains of S. aureus that are resistant to many antimicrobial compounds, including antibiotics, antiseptics and disinfectants (4).

One hundred and seventy S. aureus isolates previously identified as methacilin-resistant S. aureus MRSA were obtained from three hospitals in Tripoli, Libya. (9). This research aimed to investigate antimicrobial properties of propolis collected from bee hives in Zawia area, northwestern part of Libya, against pathogenic isolate of S. aureus, employing different propolis extracts under different pH, temperatures, and Salt concentrations. Successful results may encourage employment of propolis in skin wound infections, food or pharmaceutical goods.

Materials and methods

Propolis: 250 gm of propolis was collected. The propolis was made by Italian honey bees (Apis mellifera ligustica) from plants around bee hives such as (pine trees, acacia, eucalyptus) in Zawia area, northwest of Libya, in the autumn of 2009. Microorganisms: Pathogenic strain of S. aureus (coagulase positive), isolated from otitis media patients at Tripoli Central Hospital, Tripoli, Libya.

Extraction and partial purification: The extraction process was performed according to Funayama et al. (2). Propolis was then cut it to small pieces and ground to powder by an electrical blender. 600 ml of 75% ethanol were added to propolis in blender, which was turned on five times during a period of 3 min. each to enhance full dissolution. Such extraction process was repeated after three hours. The suspension was left to stand for about 18 hr in maceration and then filtered, through double Whatman filter paper (No. 2). A filtrate of about 450 ml of crude ethanolic extract of propolis (CEEP) was obtained as such in glass beaker. Beaker was covered with muslin cloth for 2 days at cold and dry place to enhance evaporation and prevent contamination. After settlement, two fractions were obtained: an upper aqueous solution, and a yellowish insoluble resinous material. About 110 gm of insoluble resinous material was harvested and wash-filtered with sterile distilled water until the rinse water was not cloudy. Washed resinous material was later dissolved in 150 ml of 99.8% ethanol and tagged as resinous material ethanolic solution (RMES), covered with muslin cloth, and stored in a dry place at 20 °C. After 17 days the volume of this hydrolyzed mixture was reduced to half. Liquid and resinous fractions were recollected separately as described in step 3 above. Fractions of rinsed water were added to the former one (rinsed water from CEEP). After storage at 2 °C for 48 hr, the aqueous solution became cloudy. The preparation was centrifuged at 10,000 x g for 10 minutes. The supernatant was collected, tagged as alkaline hydrolysis water-soluble compounds (AHW-SCS), and the pellet was discarded.

Preparation of filter paper discs with different concentrations of propolis: Filter paper discs (10 x 1 mm) were dried by incubation at 50 °C for 48 hr and their weights were determined. Propolis discs were impregnated by 10 µl of each crude extract for 5 times on each paper disc, and incubated at 30 °C for 20 minutes between subsequent applications, to ensure full diffusion and saturation of propolis in disc tissue. Discs were kept at 30 °C for 24 hr for drying after which time their weights were again determined. Difference in weight is considered weight of extract. The steps were repeated for different extract amounts, 20, 30 and 40 µl of propolis.

A control paper discs were prepared by impregnation with 75% ethanol. Disc Diffusion Assay of propolis extracts. Kirby-Bauer Disc Diffusion Method was employed

as detailed by Clinical and Laboratory Standards Institute (10). An overnight culture of S. aureus (0.1 ml), grown in nutrient broth, was spread over the surface of the Mueller-Hinton agar (MHA) plate using a sterile glass spreader in circular motion, until the agar plates had absorbed the broth culture. Filter paper discs containing propolis extracts and disc of ethanol were placed on the inoculated MHA plates, and incubated at 37 °C for 24 hr before reading diameter of zone of inhibition for each disc. A control plate of MHA was free of discs and contained only the same amount of culture inoculums.

Minimal inhibitory concentration (MIC) of CEEP: Using Broth dilution assay following the method described by the Clinical and Laboratory Standards Institute for Bacteria (11), CEEP was tested in doubling dilutions ranging from 13.02 mg/ml to 208.35 mg/ml as a final concentration in broth dilution assay. Inoculums of S. aureus from stock culture was merged into 100 ml nutrient broth, and left at 37C overnight. One ml portion was inoculated in a tube containing 9-ml nutrient broth, properly mixed, and 2-ml sample was measured spectrophotometrically at 480 nm, and properly diluted, if necessary, to get an OD equivalent to 4 x 105 cfu of S. aureus/ ml broth, as elucidated from determination of OD steps above. One ml nutrient with 4 x 105 cfu of S. aureus/ml was added to 3 ml of sterile nutrient broth in 9 ml test tube. To this suspension, one ml containing appropriate CEEP amount in mg to make a final CEEP concentration in the test tube to multiples of 13.02 mg/ml (26.04, 52.08, 104.17 or 208.35 mg/ml) as carried by serial dilution before introduction of the culture. Control broth tubes, without CEEP, included one ml of 75% ethanol. MICs were determined as the lowest concentration of CEEP resulting in optically clear broth tube. Tests were repeated at least three times and model MIC values were determined. Minimal Bactericidal concentration (MBC) of CEEP was tested by plating one ml of each inoculated tube with no visible growth, on blood agar plates, in duplicate and incubated at 37 °C per 24 hr.

Effect of CEEP under different conditions:

Effect of pH values. pH of crude CEEP was 3.5. It was adjusted to 6.3 by addition some ml of 2.5 N NaOH, then tested its effect on S. aureus by disc diffusion assay with the same preparation of filter paper discs containing 150 µl of CEEP, as found to be the best concentration resulting in measureable inhibition from disc diffusion assay above. MHA plates were incubated at 37 °C per 24 hr.

Effect of temperatures: Discs of CEEP (150 µl) were tested on S. aureus at different temperatures (6 °C per three days, 20 °C per two days and 37 °C per one day).

Effect salt concentrations: Concentrations of salt were added to MHA using MHA with 5% and 10% (w/w) NaCl, and then discs of 150 µl of CEEP were placed on MHA plates inoculated with suspension of S. aureus, as above and incubated at 37 °C per 24 hr.

Results

Table 1 summarizes the extraction, partial purification and activities determination of propolis extracts fractions on Staphylococcus aureus culture. Table 2 summarizes minimal inhibitory concentrations determinations of CEEP. The MIC determined for S. aureus was 26.04 mg/ml. The Minimal Bactericidal Concentration MBC determined for S. aureus was 34.7 mg/ml. Table 3 summarizes the effect of CEEP under different conditions on S. aureus.

Table 1: Zone of inhibition (mm) of propolis extracts and ethanol (control) at 37˚C/24 hr against S. aureus

Extract	Dry Wt (mg)	Volume (µl)	Inhibition (mm)
CEEP	10	50	0
	20	100	13.53
	30	150	19.62
	40	200	19.73
RMES	20	50	0
	50	100	0
	90	150	0
	100	200	0
AHWSCS	5	50	0
	10	100	0
	20	150	0
	30	200	0
ETHANOL (75%)	0	50	0
	0	100	0
	0	150	0
	0	200	0

Table 2: Effect of different CEEP conc. on S. aureus after incubation in nutrient broth for 24 hr at 37 °C.

CEEP (mg/ml)	Visible growth in nutrient tube	Growth on blood agar
208.35	-	-
104.17	-	-
52.08	-	-
41.67	-	-
34.72	-	- [B]
29.76	-	+
26.04	- [A]	++
13.02	++	+++
0	+++	+++

A: MIC value. B: MBC value.

Table 3: Inhibition zone (mm) caused by 30 mg/150 µl CEEP

Temperature			NaCl		pH	
37˚C/24 hr	20˚C/48 hr	6˚C/72 hr	5%/24 hr	10%/24 hr	3.5/24 hr	6.3/24 hr
19.62	12.62	0	11.66	11.66	19.62	11.20

Discussion

Libyan propolis has effective inhibitory effect against S. aureus. Other researchers concluded similar observations on other propolis in Brazil (12) and Iran (13). Crude ethanolic extract of Libyan propolis (CEEP) was found to be effective using the disc diffusion methodology on Mueller-Hinton agar; however (2) reported that AHWSCS was more effective than CEEP and REMS. Such difference is anticipated and referred to the variations in chemical compositions of propolis based on botanical and seasonal origins (14). Only crude ethanolic extract of propolis exhibited effective inhibition zone at different seed concentrations: a diameter of 19.62 mm at 30 mg/150 µl (w/v) and 19.73 mm at 40 mg per 200 µl (w/v). Other propolis extracts e.g. REMS and AHWSCS have been subjected to vigorous washing which may have freed effective propolis component. Values of MIC and MBC were found at 26.04 mg/ml, and 34.72 mg/ml respectively. This indicates clear antagonistic effect of propolis against S. aureus. Dilutions of raw honey exhibited effective bactericidal effect against the pathogen (15). The mode of action of either propolis or honey against S. aureus remains to be uncovered, however. Higher temperature (37 ºC) under natural acidic pH of 3.5 resulted in most effective antimicrobial activity of ethanolic extract of propolis. Lower temperature like 6 or 20 °C may retard

transport and entry of propolis into the cytoplasm. Lower temperatures are known to retard bacterial growth, in general. Similar effect of greater bacteriostatic effect of CEEP at 37 °C than lower temperatures, or at lower pH 5.0 than 6.0, was noticed by Chang et al. against S. aureus (16). High salt concentration, 10%, showed no added antibacterial effect of CEEP against S. aureus. The bacterium is notoriously salt-tolerant (17). Many factors may influence the antibacterial activity of propolis. Flavonoids (pinocembrin and galangin) and esters of phenolic acids have been associated with the antibacterial activity of European propolis (18). The chemical composition of propolis exhibits considerable geographic differences. Propolis from Bulgaria, Turkey, Greece and Algeria usually contains mainly flavonoids and esters of caffeic and ferulic acids (12). German propolis has been very active against S. aureus. The effect of Brazilian propolis on Helicobacter pylori has been associated with lambdane-type diterpenes and some prenylated phenolic compounds (18).

In conclusion, a possible use of propolis extract as food or pharmaceutical preservative, or topical treatment for skin diseases caused by S. aureus is encouraging and feasible, although the effect of propolis on S. aureus in vitro is promising, further microbiological, pharmacological and clinical trials are required.

References

1. Hegazi AG. Propolis an overview. International symposium on Apitherapy, Cairo 8-9 March, 1997.
2. Funayama S, Andrade UC and Werner PR. Action of inhibitory compounds on growth cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from propolis of Apis mellifera. X Seminário de Pesquisa e IV de Iniciação Cientifica. Anais Virtuais da UTP Publicação."on line" 2006.
3. Silva MS, De-Lima SG, Oliveira EH, Lopes JD, Chaves MH, Reis F M and Cito AL. Anacardic acid derivatives from Brazilian propolis and their antibacterial activity. Ecletica Química. 2008, 33; 3: 69-76.
4. Dolci P and Ozino OI. Study of the in vitro sensitivity to honey bee propolis of *Staphylococcus aureus* strains characterized by different sensitivity to antibiotics. Ann Microbiol. 2003, 53: 233-243.
5. El-Bassuony AA. New prenilated compounds from Egyptian propolis with antimicrobial activity. Revista Latino americana de Química. 2009, 37; 1: 85-90.
6. Yiyang H, Wei-L, Cheng-Ming C and Cheng-Chun C. Antibacterial activity of propolis ethanol extract against *S. mutans* as influenced by concentration, temperature, pH and cell age. J Food Drug Analysis. 2007, 15; 1: 75-81.
7. Fernandes A, Sugizaki F, Fogo ML, Funari SC and Lopes CM. In vitro activity of propolis against bacterial and yeast pathogens isolated from human infections. J Venomous Animals Toxins. 1995, 1; 2: 63-69.
8. Lowy NE. Major virulence factors of *S. aureus* .J Med. 1998, 339: 520.
9. Ahmed MO, Abuzweda AR, Alghazali MH, Elramalli AK, Amri SG, Aghila ES and Abouzeed YM. Misidentification of methicillin resistant *Staphylococcus aureus* (MRSA) in hospitals in Tripoli, Libya. Libyan J Med. 2010, 5: 5230.
10. CLSI. Clinical and Laboratory Standards Institute. Performance Standard for Antimicrobial Disc Susceptibility Tests. Approved Standards, 10th ed. 2009, 29; 1.

11. CLSI. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standards, 8th ed. 2009, 29; 2.
12. Gonsales GZ, Orsi RO, Fernandes JA, Rodrigues P and Funari SRC. Antibacterial activity of propolis collected in different regions of Brazil. J Venomous Animals Toxins Tropical Diseases. 2006, 12; 2: 276-284.
13. Trusheva B, Todorov I, Ninova M, Najdenski H, Daneshmand A and Bankova V. Antibacterial mono and sesquiterpene esters of benzoic acids from Iranian propolis. Chem Central J. 2010, 4: 8.
14. Miorin PL, Levy NC, Custodio AR, Bretz WA and Marcucci MC. Antibacterial activity of honey and propolis from *Apis mellifera* and *Teragonisca angustula* against *Staphylococcus aureus*. J Appl Microbiol. 2003, 95; 5: 913-920.
15. Abaje E, Ogunsanya T and Aiwerioba O. Conventional use of honey as antibacterial agent. Ann Afr Med. 2006, 5; 2: 78-81.
16. Chang LL, Wenchen Y and Cheng-Chun Chou. Antibacterial activity of propolis against *Staphylococcus aureus*. Int J Food Microbiol. 2005, 102: 213-220.
17. Fernandes A, Balestrin E C, Betni JEC, Orsi RO, Cunha RS and Montelli AC. Propolis: anti *Staphylococcus aureus* activity and synergism with antimicrobial drugs. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro. 2005, 100; 5: 563-566.
18. Boyanova L, Gergova G, Nikolov R, Derejian S, Lazarova E, Katsarov N, Mitov I and Krastev Z. Activity of Bulgarian propolis against 94 *Helicobacter pylori* strains in vitro by agar well diffusion, agar dilution and disc diffusion methods. J Med Microbiol. 2005, 54; 5: 481-483.