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Propolis an Antibacterial Agent Against Clinical Isolates of Wound Infection

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ABSTRACT

Propolis is a resinous mixture that is collected by the honeybees from the plants. The physical character of propolis generally has been used by honeybees to protect their hive but it has several beneficial properties for human beings. In the present study four different propolis samples were collected and subjected for extraction using ethanol (95%) as solvent. Their antimicrobial effect was evaluated against different bacterial strains isolated from wound infected patients at a local hospital in Islamabad including *S. aures, E. coli, P. aeruginosa* and *K. pneumonia* by agar well diffusion technique.Two strains i.e. *S. aures*(ATCC No. 25923) and *E. coli* (ATCC No. 25922) were used as control. Minimum inhibitory concentration (MIC) was also determined by agar well diffusion technique. In addition, the bioactive compounds and functional groups of the extracts were determined by paper chromatography and Spray methods. The results obtained indicated that ethanol extracts of the propolis showed antimicrobial Effect. Our finding concerning to the chemical analysis of the propolis exhibited the presence of flavonoid, tannin, steroid, alcohol and alkaloid in extracts. Overall, propolis has antimicrobial effect with different spectrum and therefore, it might consider a potent candidate for treatment of several clinical scenarios.

Keywords: Propolis, Compositions, Antimicrobial, Effect, MIC

I. INTRODUCTION

Propolis is a natural resinous mixture produced by honeybees from substances collected from parts of plants, buds, and exudates. The word propolis is derived from Greek, in which pro stands for “at the entrance to” and polis for “community” or “city,” which means this natural product is used in hive defense. Another name of propolis is bee glue. Due to its waxy nature and mechanical properties, bees use propolis in the construction and repair of their hives—for sealing openings and cracks and smoothing out the internal walls [4,6] and as a protective barrier against external invaders like snakes, lizards, and so forth, or against wind and rain. Bees gather propolis from the leaf buds of numerous tree species such as birch, poplar, pine, alder, willow, palm, *Baccharis dracunculifolia*, and *Dalbergia ecastaphyllum*means different plants in different temperate climatic zones[22,30,1].

Propolis provide beneficial effect on human health. Since ancient times propolis has been extensively employed by man, especially in folk medicine to treat several problems. Egyptians used bee glue as an antipyretic agent. Greek and Roman physicians used it as mouth disinfectant and as an antiseptic and healing product in wound treatment, prescribed for topical therapy of cutaneous and mucosal wounds [4]. Propolis was listed as an official drug in the London pharmacopoeias of the 17th century. Due to its antibacterial activity, in Europe propolis became very popular between the 17th and 20th centuries. In Italy bee glue was used as a violin varnish [19] by Stradivari. In the end of the 19th century, propolis was widely used due to its healing properties and in the Second World War it was employed in several Soviet clinics for tuberculosis treatment, due to the observed decline of lung problems and appetite recovery. In the Balkan states propolis was applied to treat wounds and burns, sore throat, and stomach ulcer [29].

Nowadays, propolis is a natural remedy found in many health food stores in different forms for topical use. It is also used in cosmetics or as popular alternative medicine

for self-treatment of various diseases (28). Current applications of propolis include formulations for cold syndrome (upper respiratory tract infections, common cold, and flu-like infections), as well as dermatological preparations useful in wound healing, treatment of burns, acne, herpes simplex and genitals' and neurodermatitis. Propolis is also used in mouthwashes and toothpastes to prevent caries and to treat gingivitis and stomatitis. It is widely used in cosmetics and in health foods and beverages. It is commercially available in the form of capsules, mouthwash solutions, creams, throat lozenges, powder, and also in many purified products from which the wax was removed. Due to its antimicrobial, antiviral, and antioxidant properties, it is widely used in human and veterinary medicine, pharmacology, and cosmetics (28).

Propolis is a complex mixture made by bee-released and plant-derived compounds. In general, raw propolis is composed of around 50% resins, 30% waxes, 10% essential oils, 5% pollen, and 5% of various organic compounds [6,21,24]. More than 300 constituents were identified in different samples [16,21,24,8]. The most significant active constituents of propolis are aromatic acids; phenolic compounds especially flavonoids (flavones, flavonols, and flavonones) and phenolic acids. The antimicrobial properties of propolis are mainly due to the flavononespinocembrin, flavonolesgalangin and the caffeic acid phenethyl ester. Studies have demonstrated that inhibitory effect of propolis on organisms depends on synergism of these compounds.

The first scientific work with propolis was published in 1908 including its chemical properties and composition which was further indexed to chemical abstract [12].Propolis is a lipophilic in nature, hard and brittle material and it becomes soft, pliable, gummy, and very sticky when heated [11]. It possesses a characteristic and pleasant aromatic smell and varies in color from yellow green to red and to dark brown depending on its source and age [4,19,29,12,11,16]. Depending on the origin of the resins, it also ranges from yellow to dark brown[13]. But even transparent propolis has been reported. Composition of propolis is varied mainly due to season of collection and the variability of plant species growing around the hive [20]. The main chemical classes present in propolis are silver, mercury, copper, manganese, iron, calcium, vanadium, silis, flavonoids, phenolics, and aromatic compounds. However, propolis contains some volatile oils, terpenes, and bee wax, but they could not be related to its antimicrobial effects [18]. Although several reports have been published on anti-inflammatory, antitumor, anti allergic, anti cancer, stimulation of Humoral and Cell Mediated Immunities[9]and anti blood pressure properties [30,27], few information is available on the antimicrobial property of propolis. Hence, the present study was conducted to investigate the antimicrobial property of Pakistani propolis on some pathogenic microorganisms.

II. METHODS AND MATERIAL

Propolis Collection

Four different varieties of *Apis mellifera* propolis; one propolis from citrus (EEPC), from Acacia (EEPA), one from Honeybee Research Institute (EEPH) and one from ber propolis (EEPB) were collected. It was observed that all of the samples were dark brown color and had hard consistency. The crude propolis was obtained in pieces. These pieces were further dehydrated at 45^{o}C for 5 minutes. The Ultrasonic Extraction (UE) was carried out using a 300 W ultrasonic bath. Propolis was placed in an Erlenmayer flask with the corresponding amount of solvent, i.e., 95% ethanol. It was treated with ultrasound at 25^{o}C for 30 minutes. After extraction, the mixture was centrifuged at 8000 rpm to obtain the supernatant. The supernatant was named the Ethanol Extracted Propolis Citrus, Acacia, HBRI and Ber(EEPC, EEPA, EEPH and EEPB) respectively. The extracts thus were stored in amber coloured bottles at 4^{o}C till use.9

Isolates Collection

Four clinical isolates of *S. aures, E. coli, P. aeruginosa and K. pneumonie* were collected from the Department of Microbiology, National Institute of Health, Islamabad. These isolates were confirmed on the basis of their morphology, cultural characteristics. Antibioticsusceptibility profile was performed using agar well diffusion technique according to Clinical Laboratory Standard Institute (CLSI) 2010 guidelines. Statistical analysis was done using SPSS 16.0.

Antimicrobial Assay of the Propolis Extracts

EEPs were screened against four clinical isolates of *S. aures, E. coli, P. aeruginosa and K. pneumonie* from

patients and two ATCC isolates i.e. *S. aureus* (ATCC No 2593) and *E.coli*(ATCC No 25922) by agar well diffusion assay. *S. aureus* (ATCC No 2593) and *E.coli*(ATCC No 25922) were used as the quality control. The isolates were adjusted to 0.5 McFarland standards and lawned on Mueller Hinton (MH) agar. The EEPs were separately diluted in ethanol to achieve concentrations of 30%, 25%, 20%, 15%, 10%, 5%, 2.5% and 1.25%.

Agar plates with 20ml of MH were prepared and wells were cut with a 9 mm sterile borer. The wells were filled with undiluted and serial dilutions in quantities of 120 µl. The plates were incubated overnight at 37°C. Clear zone ≥12 mm was considered as inhibition. Duplicate plates were prepared in this way. This procedure was performed in duplicate.

MIC was determined by agar dilution method using multi-inoculator (MAST, UK). EEPs were mixed separately in MH agar at 50°C to achieve the desired gradient concentrations from 0.5 mg/ml to 1.0mg/ml through 30 mg/ml. The grids of multi-inoculators were filled with 500 µl of each 0.5 McFarland standard bacterial suspensions. Two control plates were also set up in parallel. The positive control plate contained the inoculation of bacteria without any extract while the sterility control contained un-inoculated MH agar plate. All the plates were incubated overnight at 37°C. Triplicate plates were prepared in this way.

Chemical Screening of Propolis Extracts

To perform the experiment the crude propolis extracts was subjected for paper chromatography using ethyl acetate, acetic acid, water, with 10:50:40 proportions. The experiment was carried out by spotting the extracts on the filter paper (Whatman No. 1). The filter paper dipped into the solvents in chamber and the developing chamber was covered by watch glass to stop evaporation of eluent. When the solvent reaches the top, the filter paper pulled out, dried and placed on the cultivated Mueller Hinton Agar with sensitive bacteria. The plates were kept at 35˚C for 24 hr. afterward, the bioactive compound was recognized by exhibiting zone of inhibition interface of the filter paper. To continue the study the bioactive compound fraction of each filter paper cut out and subjected for determination of chemical composition of the bioactive compound by spraying method.

Spraying Method

To perform the experiment, the filter papers were stained with solution of 50 drops nitric acid (65%) in 100 ml ethanol and dried by heating in an oven for thirty minutes at 110 °C. The appearance of pink and yellow color zones considered positive result for detection of primary amines and alkaloids groups [2]. To evaluate the presence of alcohols, phenols and steroids groups in structure of the bioactive compound, one gram vanillin was mixed with 100ml concentrated sulphuric acid, then the filter paper was stained by the solution and dried in an oven at 110˚C till appear maximal coloured zones. Coloured zones produced on a pale background indicated positive result for detection of alcohols, phenols, and steroids compounds [7]. For detection of sugars, the filter paper was stained by a reagent prepared by 5gram urea, 20ml hydrochloric acid and 100ml ethanol. The stained filter paper heated at 110˚C till maximum coloration. The appearance of blue color indicates positive results for the presence of ketoses and oligosaccharides [11]. To determine the presence of flavonoid and tannin in the bioactive compound of propolis , the filter paper piece contain the bioactive compound was pulled in 10ml ethyl acetate and kept in water bath 40˚C for 5 minutes. The filter paper pulled out and 1ml ammonia added into the solution. Observation of yellow color considered as positive result for the presence of flavonoid. To continue the study the filter paper piece was pulled into 5ml water and boiled for 5 minutes. Afterward, the filter paper pulled out and 3 drops of ferric chloride were added to the solution. The appearance of dark brown color considered as positive results for the presence of tannin. It must be noted that the experiment was done on plain filter paper as control group [20].

III. RESULTS AND DISCUSSION

The size of Zone of inhibition was inversely proportional to the increase in the dilution of EEPs. Overall the EEPH showed a highest sensitivity as compared to other EEPs. At 30% concentration of EEPH zone of inhibition for *S. aures* and *E. coli*, was 23±0.23 mm and 23±0.22 mm while for *P. aeruginosa* and *K.*

pneumonie zone of inhibition was 24±0.23 mm. At 15% concentration it was 18.0 ±0.21 mm and 19.0±0.21 mm for *P. aeruginosa* and *K. pneumonie* while it was18.0 ±0.21 and 18.0 ±0.20 for *S. aures*and *E. coli* respectively. The EEPB inhibited different strains showing lowest sensitivity as compared to the EEPH. The zone of inhibition of EEPB at 30% concentration against *S. aures, E. coli, P. aeruginosa and K. pneumonie* were 19.5 ±0.20, 18.5 ±0.21, 17.5±0.32 and 19.5 ±0.54 respectively. At 15%EEPB 14.0 ±0.23, 13.0 ±0.23 12.0 ±0.66 and 14.0 ±0.33 inhibition zones were showed by *S. aures, E. coli, P. aeruginosa and K. pneumonie* respectinely (Table). Other two propolis samples i.e. EEPC and EEPA showed zone of inhibition and sensitivity in the range of 22.0 ±0.20 and 18.0 ±0.20 mm respectively.

Over all MIC of EEPH had better antibacterial activity as compared to EEPC, EEPA and EEPB. All the isolates of *S. aures, E. coli, P. aeruginosa and K. pneumonie* were inhibited at the concentration of 2.2 mg/ml of EEPH, 3.0 mg/ml of EEPC, 4.0 mg/ml of EEPA and 4.8 mg/ml of EEPB respectively. Table shows the MIC of all the four EEPS at MIC_{50} MIC_{90} and MIC_{100}.

Bioactive compound:

The bioactive compound of the propolis extracts were detected by paper chromatography. Furthermore, the results obtained from determination of the chemical composition of the bioactive compound illustrated the presence of alcohol, phenols, steroid and alkaloid groups in the bioactive compound of propolis extracts. In addition, observation of yellow and dark brown color after performing the spray method confirmed the presence of flavonoid and tannin in the bioactive compounds.

Table 1: Source and Place of Collection of Propolis

S.No.	Propolis Sample	Date of Collection	Place of Collection	Flora available
1	EEPH	26-7-2014----- 30-8-2014	HBRI	Mixed
2	EEPA	3-4-2014-------7-5-2014	Sitrameel	Acacia
3	EEPC	15-2-2014------30-3-2014	Sargodha	Citrus
4	EEPB	1-10-2014-------3-11-2014	Mallahal Mughlan	Ber

Table 2: Effect of Different EEPs against Different strains of Wound Infection in Agar Well Diffusion Assay

S. No	Con. of Extract %				Zone of				Inhibition				(mm)				
		EEPH	EEPH	EEPH	EEPH	EEPC	EEPC	EEPC	EEPC	EEPA	EEPA	EEPA	EEPA	EEPB	EEPB	EEPB	EEPB
		S. aures	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*S. aures*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*S. aures*	*E. coli*	*P. aeruginosa*	*K. pneumonia*	*S. aures*	*E. coli*	*P. aeruginosa*	*K. pneumonia*
1	30.0	23.0+0.23	23.0+0.22	24.0+0.23	24.0+0.23	22.0+0.20	20.0+0.23	20.0+0.25	20.0+0.27	18.0+0.20	20.0+0.22	20.0+0.21	20.0+0.20	19.5+0.21	18.5+0.21	17.5+0.32	19.5+0.54
2	25.0	21.5+0.24	21.5+0.23	22.5+0.24	22.5+0.24	19.0+0.5	18.0+0.7	18.0+0.6	18.0+0.5	17.5+0.21	18.5+0.22	17.5+0.25	18.5+0.22	17.0+0.22	16.0+0.22	15.0+0.16	16.0+0.22
3	20.0	20.0+0.23	20.0+0.21	21.0+0.23	21.0+0.23	18.0+0.6	17.0+0.5	17.0+0.5	16.0+0.6	17.0+0.17	16.0+0.15	16.0+0.16	15.0+0.17	16.0+0.21	15.0+0.21	13.0+0.34	15.0+0.43
4	15.0	18.0+0.21	18.0+0.20	19.0+0.21	19.0+0.21	17.0+0.4	16.0+0.3	17.0+0.4	15.0+0.5	16.0+0.12	15.0+0.13	15.0+0.11	14.0+0.12	14.0+0.23	13.0+0.23	12.0+0.66	14.0+0.33
5	10.0	16.0+0.17	16.0+0.12	17.0+0.17	17.0+0.17	16.0+0.2	15.0+0.22	16.0+0.2	14.0+0.22	15.0+0.23	14.0+0.21	14.0+0.20	13.0+0.23	13.0+0.25	12.0+0.25	11.0+0.38	12.0+0.23
6	5.0	15.0+0.18	15.0+0.13	15.0+0.18	16.0+0.18	15.0+0.22	14.0+0.21	15.0+0.23	13.0+0.25	14.0+0.20	13.0+0.23	13.0+0.22	12.0+0.25	12.0+0.24	11.0+0.24	11.0+0.28	12.0+0.22
7	2.5	14.0+0.17	12.0+0.16	12.0+0.15	13.0+0.17	14.0+0.3	13.0+0.4	14.0+0.5	12.0+0.4	13.0+0.24	12.0+0.23	12.0+0.2	11.0+0.24	11.0+0.22	10.0+0.21	10.0+0.33	11.0+0.45
8	1.25	13.0+0.15	11.0+0.13	11.0+0.15	12.0+0.12	13.9+0.7	12.0+0.5	12.5.0+0.5	11.0+0.5	12.0+0.20	11.0+0.22	11.0+0.12	10.0+0.2	11.0+0.21	10.0+0.23	10.0+0.21	11.0+0.15

Table 3: MIC of Different EEPs Against Different Strains

	EEPH MIC Range	1.6----2.2	
	$MIC_{50\ (mg/ml)}$	$MIC_{90\ (mg/ml)}$	$MIC_{100\ (mg/ml)}$
S. aures	1.7	2.1	2.1
E. coli	1.9	2.1	2.1
P. aeruginosa	1.6	2.2	2.2
K. pneumonia	1.8	2.1	2.2
S. aures(ATCC No. 25923)	1.7	1.7	1.7
E. coli (ATCC No. 25922)	1.9	1.9	1.9
	EEPC MIC Range	2.2----3.0	
	$MIC_{50\ (mg/ml)}$	$MIC_{90\ (mg/ml)}$	$MIC_{100\ (mg/ml)}$
S. aures	2.2	2.9	3.0
E. coli	2.5	3.0	3.0
P. aeruginosa	2.2	3.0	3.0
K. pneumonia	2.4	2.8	3.0
S. aures(ATCC No. 25923)	2.2	2.2	2.2
E. coli (ATCC No. 25922)	2.5	2.5	2.5
	EEPA MIC Range	3.0------4.0	
	$MIC_{50\ (mg/ml)}$	$MIC_{90\ (mg/ml)}$	$MIC_{100\ (mg/ml)}$
S. aures	3.0	3.6	4.0
E. coli	3.2	3.9	4.0
P. aeruginosa	3.5	4.0	4.0
K. pneumonia	3.3	3.9	4.0
S. aures(ATCC No. 25923)	3.0	3.0	3.0
E. coli (ATCC No. 25922)	3.2	3.2	3.2
	EEPB MIC Range	3.5-----4.8	
	$MIC_{50\ (mg/ml)}$	$MIC_{90\ (mg/ml)}$	$MIC_{100\ (mg/ml)}$
S. aures	3.5	4.5	4.7
E. coli	3.8	4.6	4.8
P. aeruginosa	3.8	4.6	4.8
K. pneumonia	3.9	4.5	4.7
S. aures(ATCC No. 25923)	3.5	3.5	3.5
E. coli (ATCC No. 25922)	3.8	3.8	3.8

IV. CONCLUSION

Propolis is a resinous substance collected by honey bees (*Apis mellifera*) from various species of trees. This compound is usually used by bees to coat hives, seal cracks and protect the hive against different contaminations The composition of propolis and its properties depended on the kind of the plants and geographical area. The present study was conducted to evaluate the antimicrobial property of Pakistani propolis collected from different areas and sources to be used against the pathogenic microorganisms of wound infection. These extracts showed potent activity against *S. aures, E. coli, P. aeruginosa and K. pneumonie*. All the four type of strains have shown sensitivity against all four EEPs but the concentration of the EEPs inhibited the growth were different. Therefore, it can be concluded that all propolis extracts have similar antimicrobial compounds but the concentration of these compounds is different which differ the potency of the propolis. In addition, chemical analysis of the propolis samples was carried out by TLC and spray methods. The results obtained exhibited the presence of flavonoid, tanin, steroid, alcohol and alkaloid in the extracts. On the other hand the bioactive property of propolis may be related to flavonoid follow by tannin and steroid. Many scientists believe that antimicrobial property of the propolis is related to the geographical areas. It might be interpreted that the antimicrobial property of different propolis is not identical in our study may be due to different geographical sources of the samples. However, our finding suggests that the antimicrobial action of the propolis as an adjuvant to therapy and it might be considered a potent candidate for treatment of several clinical scenarios.

Therefore, our study believes that propolis can be used as an natural alternative to antibiotics. The pharmaceutical industries have introduced the new antimicrobial components with potent activity against

pathogenic bacteria. Therefore, the new sources of remedy such as propolis might be considered valuable component to be used as medicine.

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