

Antibacterial Activity of *Apis mellifera* Propolis Against Pathogenic Bacterial Strains Implicated in Human Skin Infections

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ABSTRACT

This study evaluates the antibacterial efficacy of *Apis mellifera* propolis against bacterial species commonly associated with human skin infections, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacter* spp., *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. The chemical composition of propolis was analyzed using gas chromatography-mass spectrometry (GC-MS) to identify its bioactive compounds. The inhibitory effects of aqueous and alcoholic propolis extracts were compared to standard antibiotics. The results demonstrated significant antibacterial activity of both extracts, with the alcoholic extract exhibiting the highest inhibitory effects. The aqueous extract, however, showed limited efficacy against Gram-negative bacteria. Among the tested bacterial species, *Staphylococcus aureus* exhibited the largest inhibition zone (31.87 mm) when exposed to the alcoholic extract. The overall highest inhibition average for Gram-positive and Gram-negative bacteria was observed with the alcoholic extract (17.44 mm), particularly against *S. aureus* (23.17 mm). These findings highlight the potential of *Apis mellifera* propolis, particularly its alcoholic extract, as a natural antimicrobial agent for managing skin infections caused by pathogenic bacteria.

Keywords: Propolis, Antibacterial, *Apis mellifera*, GC-MS, Antimicrobial Resistance

INTRODUCTION

Apis mellifera Linnaeus, commonly known as the honey bee, is acknowledged as the most significant pollinator, essential for the success of modern agriculture and the stability of human food production [1]. Belonging to the animal kingdom, honey bees are among the most economically and socially significant insects, producing honey from the nectar of various flowers [2]. They produce several products, including honey, propolis, royal jelly, bee pollen, bee bread, venom, and wax. These products have been recognized for their health, nutritional, and medicinal values since ancient times by Egyptians, Greeks, and Chinese [3]. Recently, the World Health Organization (WHO) listed twelve bacterial families resistant to antibiotics, posing significant health challenges and threats to human health [4]. This problem stems from a lack of effective surveillance measures and the widespread overuse of antibiotics. In the 1990s, there was a sharp decline in the development of new drug classes, coinciding with the emergence of pathogenic human strains resistant to multiple drugs, raising considerable concern. Currently, antimicrobial resistance causes over 700,000 deaths annually, a number projected to rise to 10 million by 2050. The need for new antibiotics has never been greater due to the emergence of drug-resistant and extensively drug-resistant microorganisms [5]. Despite the clinical demand for new antimicrobial agents, the development of such agents has been declining since the 1970s, with few new antibiotics discovered [6, 7]. Given the scarcity of new and effective therapeutic agents, the pharmaceutical industry has turned to natural products and herbal medicine to address current health issues in humans and animals [8, 9]. This means that these treatments will be widely available, especially to people in less developed countries who cannot afford costly treatments. The reduced side effects also mean greater patient tolerance and compliance, leading to maximum therapeutic impact

without negatively affecting life. Propolis is one of the most renowned products of honey bees and has been used in folk medicine for its numerous health benefits since the dawn of civilization [10, 11]. Propolis is a wax-like natural resin produced by honey bees (*Apis mellifera* L.), consisting of salivary secretions, wax, pollen, and various plant materials. Honey bees use propolis (bee glue) as cement to seal cracks or open spaces in the hive, thus preventing parasite invasion and helping maintain appropriate internal temperature and humidity [12, 13]. Dr. Petrovich notes that when any foreign creature, such as a mouse or other insects, enters the hive, the bees immediately attack and kill it with venom as a defense for themselves and their hive. To prevent decomposition of the mouse or insect, the bees completely isolate it using the gum, ensuring no contaminated air can enter or exit [14, 15]. Thus, propolis is considered an animal product because it is integrated into the hive through the honey bee's digestive system, particularly its saliva [16]. Chemical analysis has shown that propolis is composed of 300 different compounds, generally consisting of 30% wax, 50% resin and balsam, 10% essential oils, 5% pollen, and other components.

MATERIALS AND METHODS

Sample Collection

Traps were set for collecting propolis in the Balad area on September 2, 2022, and samples were obtained on December 1, 2022, with the assistance of the Head Beekeeper in Salahuddin. Additional traps were placed in the Duluiyah area with the help of a beekeeper on September 5, 2022, and samples were collected on December 5, 2022. The propolis samples were gathered after the honey collection season had concluded, cleaned of dust and pollen, and stored in dry, airtight

plastic bags away from light at a temperature of 4°C until further use [17, 18].

Sample Preparation and Chemical Analysis

Upon retrieval, the propolis samples were cleaned of any adhering impurities and stored in 200 ml sterilized, opaque, and sealed glass bottles. The bottles were labeled with geographic location, types of plants in the apiary areas, and the time and date of collection. The samples were then refrigerated for freezing and later cut into small pieces for subsequent use. Some samples were sent to a laboratory in Samarra for analysis using a GC-MS device to determine the concentrations of active substances and to study their inhibitory effects against certain pathogenic bacterial species under study compared to some antibiotics.

Preparation of Propolis Extract

The alcoholic propolis extract was prepared according to the method, where the frozen propolis was ground using an electric blender. Ethanol (99% concentration) or water was added at a 10:1 weight/volume ratio. The mixture was placed in a flask and left at room temperature for 48 hours with periodic shaking using a Shaker at 300 rpm. The solution (either ethanolic or aqueous extract) was then filtered using Whatman No. 1 filter paper, and the filtrate was evaporated to obtain a very dense substance, which formed the alcoholic or aqueous extract of propolis [19]. This was then diluted with 99% ethanol or distilled water to achieve the different concentrations used in the current study.

Minimum Inhibitory Concentration (MIC)

The MIC was determined using the dilution method in wells, as described by the NCCLS (2000). Different concentrations of the extract were added to Mueller-Hinton medium to achieve concentrations of 1%, 0.8%, 0.6%, 0.4%, and 0.2%. The prepared dishes were then allowed to dry at room temperature before being incubated at 37°C for 24 hours. Results were recorded based on the presence or absence of microbial growth, with the lowest concentration showing no growth identified as the MIC.

Inhibition Effectiveness Test of the Bee Product (Propolis)

The well diffusion method was used to test the inhibitory effectiveness of the bee product (propolis) on bacterial growth. 20-25 ml of agar medium was poured into each Petri dish. After the medium

solidified, the dishes were incubated for 24 hours at 37°C to ensure no contamination of the agar [20]. The medium was then inoculated using a sterile cotton swab by spreading a bacterial suspension evenly over the surface of Muller-Hinton agar [21-23]. After 15 minutes at room temperature for absorption, 5 wells of 6 mm diameter each were made in each dish using a cork borer, representing different concentrations. 100 microliters from each concentration, along with distilled water in one of the wells as a control, were added.

The dishes were incubated for 24 hours [24, 25], and three replicates were made for each dish. Post-incubation, the diameters of the inhibition zones around the wells were measured using a ruler, and the average diameter of the inhibition zones was calculated.

Antibiotic Sensitivity Test

A pure culture of the bacteria under study, previously characterized, was placed in a test tube containing 5 ml of Nutrient broth and incubated for 24 hours at 37°C. A portion of the liquid medium was then spread evenly across Muller-Hinton agar using a sterile cotton swab. Antibiotic disks were placed using sterile tweezers, ensuring a gap between each disk. The dishes were incubated for 24 hours at 37°C, and the diameter of the inhibition zones around each antibiotic disk was measured with a ruler [26].

Results and Discussion

Chemical Analysis of Propolis Using GC-MS: The GC-MS (Gas Chromatography-Mass Spectrometry) analysis was conducted using a helium carrier gas at a flow rate of 1 mL/min. The injector temperature was set at 280°C at minimum split mode. Initially, the oven temperature was held at 60°C for 4 minutes before it was raised to 150°C at a rate of 10°C/min for 15 minutes. The mass spectrometer's parameters were optimized with a source temperature of 280°C, a transfer line temperature of 150°C, a solvent delay of two minutes, and a scan range from 35 to 500 m/z.

Eventually, the temperature was raised to 310°C with a total run time of 40.5 minutes, after which spectra were compared with stored mass spectral databases [27, 28]. The results indicated the presence of numerous biologically active substances in the propolis samples as shown in Table 1, including alkaloids, carbohydrates, aldehydes, terpenoids, alcohols, saponins, flavonoids, phenolics, coumarins, amino acids, glycosides, proteins, and others [29].

Table 1 (A): This Table Displays the Retention Times, Percentage Compositions, Molecular Weights, Chemical Formulas, and Names of the Compounds Identified in Sample Number 2.

Rt (Min)	%	M.W.	Formula	Compound Name
4.21	0.120	120	C9H12	1,3,5 - Trimethyl benzene
6.13	0.470	156	C10H20O	Menthol
8.12	1.380	370	C23H30O4	Spmesifen
8.18	1.020	328	C19H36O4	Glutaric acid, di (4,4-dimethyl pent-2-yl) ester
8.35	5.367	156	C10H20O	2,2-Dimethyl-3-octanone
8.40	1.823	311	C20H41NO	Propanamide, N-heptyl-N-octyl, 2,2-dimethyl
8.54	9.523	273	C13H19NO3	Beta-Dihydro caramine
8.79	19.547	128	C7H12O2	3,6-Heptanedione
8.90	18.630	128	C7H12O2	2,5-Heptanedione
8.98	15.849	114	C8H18	Hexane, 2,5-dimethyl
9.11	9.810	273	C16H19NO3	Beta-dihydro caramine
9.67	0.931	298	C19H38O2	Ethyl, 15-methyl-hexadecanoate
9.74	0.600	513	C32H51NO4	N-methyl-pseudomatidine diacetate
9.80	0.442	506	C32H58O4	Succinic acid, di(tetradec-11-ethyl) ester
9.90	1.244	222	C12H14O4	Diethyl phthalate
10.00	2.908	366	C24H46O2	Erucic acid ethyl ester
10.10	2.807	324	C21H40O2	Ethyl 18-monodecoate
10.21	1.604	366	C24H46O2	Ethyl-13-docosenoate (ethyl erucate)
10.29	0.217	212	C13H24O2	Decanoic acid, 2-propenyl

Table 1(B): This Table Displays the Retention Times, Percentage Compositions, Molecular Weights, Chemical Formulas, and Names of the Compounds Identified in Sample Number 2.

Rt (Min)	%	M.W.	Formula	Compound Name
10.40	0.169	254	C16H30O2	Palmitoleic acid
11.10	0.296	282	C18H34O2	Ethyl 9-hexadecenoate
11.22	0.052	458	C14H44O5Si6	Hexa siloxane, tetradeca methyl
11.71	5.447	284	C18H36O2	Hexadecanoic acid, ethyl ester

These results contribute to understanding the complex biochemical makeup of propolis and its potential applications in pharmaceuticals, especially considering its rich diversity in bioactive compounds. Inhibitory Effectiveness of Propolis Against Certain Pathogenic Bacterial Species: This study involved the use of aqueous and ethanolic extracts of propolis on various tested pathogenic bacterial species, employing the well diffusion method [30, 31]. The

results clearly indicated a dependency of the inhibitory effect on both the concentration of the extract and the solvent used. The maximum effect was observed at a concentration of 1 mg/ml, with increasing concentrations enhancing the inhibitory impact. Notably, the ethanolic extract of raw propolis exhibited the most potent effect, acting as a bactericidal agent by completely inhibiting microbial growth around the wells after 24 hours of incubation [32].

Table 2: Antibacterial Activity of Propolis Extracts

Bacterial Species	Extract Type	Concentrations (mg/ml)					Average Type of Extract	Average Type of Bacteria
		1	0.8	0.6	0.4	0.2		
Staphylococcus aureus	Ethanol Propolis	13.87	21.37	18.75	30.0	31.87	23.17 A	17.44 A
	Distilled Water Propolis	9.0	11.25	12.0	12.75	13.5	11.70 E	
Average Concentration		11.44 c	16.31 b	15.38 b	21.38 a	22.69 a		
Pseudomonas aeruginosa	Ethanol Propolis	18.10 B	9.5	17.25	18	19.5	26.25	9.05 B
	Distilled Water Propolis	0.0 F	0	0	0	0	0	
Average Concentration		4.75 c	8.63 b	9.00 b	9.75 b	13.13 a		
Escherichia coli	Ethanol Propolis	14.40 D	11.25	13.875	14.625	15.375	16.875	7.20 C
	Distilled Water Propolis	0.0 F	0	0	0	0	0	
Average Concentration		5.63 c	6.94 bc	7.31 ab	7.69 ab	8.44 a		
Enterobacter	Ethanol Propolis	14.40 D	10.5	14.25	15	15.75	16.5	7.20 C
	Distilled Water Propolis	0.0 F	0	0	0	0	0	
Average Concentration		5.25 c	7.13 b	7.50 b	7.88 b	8.25 a		
Acinetobacter baumannii	Ethanol Propolis	18.83 B	17.25	18	19.125	19.5	20.25	9.41 B
	Distilled Water Propolis	0.0 F	0	0	0	0	0	
Average Concentration		8.63 b	9.00 ab	9.56 ab	9.75 ab	10.13 a		
Klebsiella pneumonia	Ethanol Propolis	8.36 BC	16.73 C	12	13.875	15	18.75	24
	Distilled Water Propolis		0.0 F	0	0	0	0	0
Average Concentration			6.00 c	6.94 c	7.50 c	9.38 b	12.00 a	

*Identical lowercase letters horizontally indicate no significant difference between them.

**Identical uppercase letters vertically indicate no significant difference between them. *

The results for the ethanolic propolis extract displayed varying degrees of efficacy against the studied pathogenic bacteria as detailed in Table (2). *S. aureus* bacteria demonstrated sensitivity to the ethanolic propolis extract, inhibiting growth at a 0.2% concentration (the minimum inhibitory concentration, MIC), with an inhibition zone diameter of 13.87 mm. Meanwhile, a 1% concentration was lethal, exhibiting the largest inhibition diameter recorded against all tested Gram-positive and Gram-negative bacteria, measuring 31.87 mm. The highest average inhibition for this type of bacteria was 17.44A among the bacterial types. These results differ from ethanolic and essential oil extracts of Ethiopian propolis showed no activity against *S. aureus*, possibly due to differences in chemical composition heavily influenced by geographic origin, climate, soil type, extraction methods, and solvents used.

Conversely, the results align with those by Ibrahim et al. [33], who demonstrated the sensitivity of *S. aureus* to ethanolic propolis extract. However, it showed lesser sensitivity to aqueous propolis extract, inhibiting growth at the MIC of 0.2ug/ml, with an inhibition zone diameter of 9.0 mm. At a 1% concentration, the extract was lethal, producing an inhibition zone of 13.5 mm, with the highest average extract type inhibition being 11.70E. Yet, a significant difference was

observed between the ethanolic and aqueous extracts, with the highest average extract type being the alcoholic, registering at 23.17A. These findings contrast with those of Abu Shagaleh et al. [1], who found that aqueous extracts of propolis had no antibacterial effect against these bacteria, while aligning with Mohammed [4], who noted that aqueous extracts of Chinese and Brazilian propolis had a lethal effect on microorganisms, including *S. aureus*. Perhaps these differences can be attributed to the efficacy of propolis and its content of phenolic compounds and flavonoids, given that its antimicrobial activity depends on the type and concentration of plant polyphenols present in the extract. Gram-negative species also showed sensitivity to the ethanolic propolis extract, even at low concentrations. *P. aeruginosa* growth was inhibited at the MIC of 0.2 ug/ml, with an inhibition zone of 9.5 mm. At a 1% concentration, the lethal and inhibiting diameter reached 26.25 mm, the highest recorded for the studied Gram-negative bacteria, with the highest average type of extract inhibition recorded at 18.10B [34]. This contrasts with Ibrahim et al. [33], who noted that the Turkish ethanolic propolis extract had no inhibitory activity against bacteria. However, the antibacterial effect of the green propolis extract from *Bruscella draconkulifolia*, containing chlorogenic acid derived from cinnamaldehyde and apigenin.

Table 3: Effect of Some Antibiotics on Bacteria Types

Antibiotic	Staphylococcus aureus	Pseudomonas aeruginosa	Escherichia coli	Enterobacter	Acinetobacter baumannii	Klebsiella pneumoniae	Average Antibiotic
Ciprofloxacin (Clp10)	30	35	30	32	30	0	26.167 A
Ciprofloxacin (Clp10)	15	17.5	15	16	15	0	13.083 C
Gentamycin (CN10)	20	15	17	12	20	15	16.500 B
Gentamycin (CN10)	10	7.5	8.5	6	10	7.5	8.250 D
Rifampin (RA5)	0	15	0	0	0	0	2.500 F
Rifampin (RA5)	0	7.5	0	0	0	0	1.250 G
Penicillin (P10)	0	11	12	0	0	0	3.833 EF
Penicillin (P10)	0	5.5	6	0	0	0	1.917 FG
Amoxilin (Ax25)	15	15	0	0	0	0	5.000 E
Amoxilin (Ax25)	7.5	7.5	0	0	0	0	2.500 FG
Chloramphenicol (C10)	20	22	15	10	16	8	15.167 B
Chloramphenicol (C10)	10	11	7.5	5	8	4	7.583 D
Cloxacillin (Cx10)	15	12	15	0	8	0	8.333 D
Cloxacillin (Cx10)	7.5	6	7.5	0	4	0	4.167 E
Average Bacteria	10.714 b	13.393 a	9.536 b	5.786 d	7.929 c	2.464 e	

* Lowercase letters that are similar horizontally indicate no significant difference in meaning.

** Capital letters that are similar vertically indicate no significant difference in meaning.

The results of the current study differed, which mentioned that alcoholic propolis has an inhibitory effect on Gram-positive bacteria but no effect on Gram-negative bacteria. However, the activity against both Gram-positive and Gram-negative bacteria when using alcoholic propolis extract and the synergistic data shown among different propolis compounds, particularly Galangin, Pinocembrin, and Flavonoids, when used against bacteria. It also agreed that the ability of propolis to affect the activity of Gram-positive bacterial cells more than Gram-negative bacteria. This could be due to several reasons, including the mechanism of action of propolis: Propolis and some of its derivatives could work directly on bacteria through several mechanisms or by affecting the host's immune system.

Propolis may impact the permeability of the bacterial cell membrane, leading to a decrease in membrane-associated antimicrobial activity, namely the reduction in adenosine triphosphate (ATP) production, thereby impairing bacterial motility and reducing bacterial resistance to antibiotics. This could also be due to another mechanism involving cinnamic acid, which inhibits the production of ATPase enzyme and cell division and the development of biofilms by causing damage to the bacterial cell membrane

associated with disturbances in metabolic pathways and stress on the internal pH balance within the cell [35]. The current study showed that the highest type of extract was alcoholic on *S. aureus* bacteria (23.17A) followed by *A. baumannii* (18.83B) and *P. aeruginosa* (18.10B), with no significant difference between them, then *K. pneumoniae* (16.73C) and *E. coli* (14.40D) and *Enterobacter* (14.40D), with no significant difference recorded between them during the current study. The highest concentration of bactericide and killer recorded on the tested pathogenic bacterial species was 1% on *S. aureus* bacteria, with an inhibition zone diameter around the well of (31.87 mm), and the smallest diameter of the inhibition zone around the well (9.5 mm) at a concentration of 0.2% against *P. aeruginosa* bacteria when using alcoholic propolis extract [36].

However, when using the aqueous propolis extract, the highest concentration of bactericide and killer was 1% and the diameter of the inhibition zone around the well was (13.5 mm) on Gram-positive *S. aureus* bacteria, and it recorded the smallest diameter of the inhibition zone around the well (9.0 mm) at a concentration of 0.2%. Meanwhile, all tested Gram-negative bacterial species showed resistance at all concentrations of the aqueous propolis extract.

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