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ANTIBACTERIAL EFFICACY OF PROPOLIS EXTRACTS AGAINST BACTERIAL PATHOGENS

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ABSTRACT

Background: The global rise in antimicrobial resistance necessitates the search for novel therapeutic agents. Propolis, a natural resinous mixture produced by bees, has garnered significant interest due to its renowned biological properties, including antibacterial activity. **Objective:** This study aimed to evaluate the *in vitro* antibacterial activity of sequentially extracted propolis using solvents of increasing polarity (ethyl acetate, ethanol, and water) against a panel of Gram-positive and Gram-negative bacteria. **Methods:** The antibacterial activity was assessed against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella Typhi* (ATCC 26850), and *Klebsiella pneumoniae* (ATCC 27853) using the disc diffusion method to determine the zone of inhibition and a resazurin-based microtiter assay to determine the Minimum Inhibitory Concentration (MIC). Cefoxime was used as a positive control. **Results**: The ethanol extract yielded the highest extraction yield (49.33%) and demonstrated the most potent antibacterial activity. It exhibited significant inhibition zones against *Ps. aeruginosa* (34.0±0.50 mm) and *K. pneumoniae* (26.0± 0.50 mm). The ethyl acetate extract showed activity against *Sal. Typhi* (23.0±0.50 mm) and *E. coli* (18.0±0.50 mm), while the aqueous extract was inactive. The MIC values for the active ethanol extract ranged from 0.39 µg/mL for *P. aeruginosa* to 12.5 µg/mL for *S. aureus* and *S. Typhi*. **Conclusion:** Propolis, particularly its ethanol extract, possesses significant and potent antibacterial activity against a range of clinically relevant pathogens. These findings scientifically validate its traditional use and highlight its potential as a source of natural antimicrobial compounds for combating bacterial infections.

KEYWORDS: Propolis, Antibacterial activity, Minimum Inhibitory Concentration (MIC), Resazurin assay.

1. INTRODUCTION

The escalating crisis of antimicrobial resistance (AMR) poses a severe threat to global public health, rendering conventional antibiotics increasingly ineffective (Murray et al., 2022). This urgent situation has revitalized the scientific community's interest in exploring natural products as promising sources for novel antimicrobial agents (Atanasov et al., 2021). Among these, propolis, a complex resinous substance collected by honeybees (Apis mellifera) from various plant sources, stands out due to its extensive history in traditional medicine and its diverse pharmacological properties (Silva-Carvalho et al., 2015).

Propolis is composed of an intricate mixture of resins, waxes, essential oils, pollen, and bioactive compounds

such as flavonoids, phenolic acids, and terpenes (Bankova et al., 2014). Its chemical composition is highly variable and depends on factors like the local flora, geographical origin, season of collection, and bee species (Huang et al., 2014). This chemical diversity directly influences its biological activities, which include antioxidant, anti-inflammatory, antifungal, antiviral, and notably, antibacterial effects (Pasupuleti et al., 2017).

The antibacterial mechanism of propolis is multi-faceted and is not attributed to a single compound but to a synergistic action of its constituents. Proposed mechanisms include the disruption of the bacterial cell membrane, inhibition of bacterial cell division, and suppression of protein synthesis (Przybyłek and Karpiński, 2019). Furthermore, its ability to inhibit biofilm formation, a key virulence factor in many chronic infections, adds to its therapeutic potential (Berretta et al., 2020).

While numerous studies have confirmed the antibacterial properties of propolis, the efficacy of extracts can vary dramatically based on the solvent used for extraction, as different solvents solubilize different bioactive components (Oroian et al., 2020). This study therefore aimed to systematically evaluate and compare the antibacterial activity of sequentially prepared ethyl acetate, ethanol, and aqueous extracts of propolis from Egypt against a panel of clinically significant Gram-positive and Gram-negative bacteria, using both qualitative (disc diffusion) and quantitative (MIC) assays.

2. MATERIALS AND METHODS

2.1. Chemicals, Reagents, and Drugs

All chemicals and solvents utilized, including ethyl acetate (Sigma-Aldrich, USA), absolute ethanol (Merck, Germany), dimethyl sulfoxide (DMSO) (PanReac AppliChem, USA), and resazurin sodium salt (Sigma-Aldrich, USA), were of analytical grade. Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth (MHB) (HiMedia, India) were employed for microbial cultivation and assays. The standard antibiotic cefoxime (10 µg/disc) (Oxoid, UK) served as the positive control, while sterile distilled water and DMSO were used as negative controls.

2.2. Propolis Material and Extraction

A raw propolis sample (**Figure 1**) was obtained from a local market in Cairo, Egypt, in December 2024. The sample was authenticated by Dr. Ahmed Ali Mustafa. The propolis was ground into a fine powder. Sequential extraction was performed using 10 grams of the powder with solvents of increasing polarity: ethyl acetate, ethanol (95%), and distilled water, following the methodology described by Abdul et al. (2020) with modifications. Each extraction was carried out using a shaker incubator at 150 rpm for 24 hours at room temperature. The extracts were filtered using Whatman No. 1 filter paper, and the solvents were evaporated under reduced pressure using a rotary evaporator. The yields were calculated, and the extracts were stored at 4°C until use.

Figure 1: A raw propolis sample.

2.3. Test Microorganisms and Inoculum Preparation

The following reference bacterial strains were used: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella Typhi* (ATCC 26850), and *Klebsiella pneumoniae* (ATCC 27853). The bacteria were subcultured on Mueller-Hinton Agar (MHA) plates. For the assays, a bacterial suspension equivalent to the 0.5 McFarland standard (approximately 1-2 × 10^8 CFU/mL) was prepared in sterile Mueller-Hinton Broth (MHB).

2.4. Antibacterial Activity Screening

2.4.1. Disc Diffusion Assay

The antibacterial activity was initially screened using the standard Kirby-Bauer disc diffusion method on MHA plates (Bauer, 1996). Sterile 6 mm filter paper discs (Whatman, UK) were impregnated with 10 µL of each propolis extract (concentration: 100 mg/mL in 10% DMSO). A disc impregnated with Cefoxime (10 µg/disc) (Oxoid, UK) served as the positive control, while a disc with 10% DMSO served as the negative control. The plates were incubated at 37°C for 18-24 hours. The antibacterial activity was evaluated by measuring the diameter of the inhibition zones (IZD) in millimeters (mm). All tests were performed in triplicate.

2.4.2. Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using a resazurin-based microdilution assay in 96-well plates (Sarker et al., 2007). Briefly, a 100 mg/mL stock solution of the active extracts (ethanol and ethyl acetate) was prepared and serially diluted two-fold in MHB to achieve concentrations ranging from 50 mg/mL to 0.39 mg/mL. Then, 100 µL of each dilution was mixed with 100 µL of the standardized bacterial suspension. Wells containing only MHB with bacteria served as the growth control, and wells with MHB and extract served as the sterility control. Cefoxime was used as an antibiotic control. The plates were incubated at 37°C for 18-24 hours. After incubation, 30 µL of a 0.01% resazurin sodium salt solution was added to each well, and the plates were re-incubated for 2-4 hours. A color change from blue

(resazurin) to pink (resorufin) indicated bacterial growth. The MIC was defined as the lowest concentration of the extract that prevented this color change.

2.5. Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± standard deviation (SD). Data analysis was performed using GraphPad Prism software (version 10.0).

3. RESULTS AND DISCUSSION

3.1. Extraction Yield and Physical Characteristics

The extraction yield and physical properties of the propolis extracts are summarized in **Table 1**. The ethanol extract provided the highest yield (49.33%), followed by the ethyl acetate extract (41.67%), while the aqueous extract yielded the least (21.67%). The color and texture varied, with the ethanol extract being dark green and sticky, the ethyl acetate extract dark brown and gummy, and the aqueous extract brown and powdery.

Table 1: Yield percentage and physical characteristics of propolis extracts.

Natural Product	Solvent	Yield (%)	Color	Texture
Propolis	Ethyl Acetate	41.67	Dark Brown	Gummy
	Ethanol	49.33	Dark Green	Sticky
	Aqueous	21.67	Brown	Powder

The higher yield of the ethanol extract suggests that ethanol is a more efficient solvent for extracting a broader spectrum of bioactive compounds from this particular propolis sample. This is consistent with the literature, as ethanol is known to effectively extract a wide range of polar to medium-polarity compounds, including the key bioactive flavonoids and phenolic acids in propolis (Oroian et al., 2020).

3.2. Antibacterial Activity

3.2.1. Disc Diffusion Assay

The results of the disc diffusion assay are presented in **Table 2**. The ethanol extract of propolis exhibited the strongest and broadest spectrum of activity, showing significant inhibition zones against *P. aeruginosa* (34.0±0.50 mm) and *K. pneumoniae* (26.0±0.50 mm). It also showed moderate activity against *S. aureus* (20.0±0.5 mm). The ethyl acetate extract was active against *S. Typhi* (23.0±0.50 mm) and *E. coli* (18.0±0.50 mm). Notably, the aqueous extract showed no inhibitory activity against any of the tested bacteria. The positive control, Cefoxime, showed expected zones of inhibition.

Table 2: Antibacterial activity of propolis extracts (Inhibition Zone Diameter in mm).

Natural Product	Extract	*S. aureus*	*E. coli*	*S. Typhi*	*K. pneumoniae*	*P. aeruginosa*
Propolis	Aqueous	NA	NA	NA	NA	NA
	Ethanol	20.0±0.50	NA	NA	26.0±0.50	34.0±0.50
	Ethyl Acetate	NA	18.0 ±0.50	23.0±0.50	NA	NA
Cefoxime	Control	13.0±1.30	17.0±0.03	15.0±2.40	14.0±1.55	NA

NA: No Activity. Values are Mean ± SD (n=3).

The superior activity of the ethanol extract can be attributed to its high concentration of total phenolic and flavonoid compounds, which are well-established as the primary antimicrobial agents in propolis (Almutairi et al., 2014). The remarkable activity against *P. aeruginosa*, a bacterium notorious for its intrinsic and acquired resistance to many antibiotics, is particularly promising (Pang et al., 2019). The inactivity of the aqueous extract is a common finding, as the major antibacterial components of propolis are typically non-polar or semi-polar (Oroian et al., 2020).

3.2.2. Minimum Inhibitory Concentration (MIC)

The MIC values for the active ethanol extract are presented in **Table 3**. The extract was most potent against *P. aeruginosa* (MIC: 0.39 mg/mL) and *E. coli* (MIC: 0.78 mg/mL), followed by *K. pneumoniae* (MIC: 1.56 mg/mL). It required higher concentrations to inhibit *S. aureus* and *S. Typhi* (MIC: 12.5 mg/mL).

Table 3: Minimum Inhibitory Concentration (MIC) of the ethanolic propolis extract.

Solvent	Bacteria	MIC (mg/mL)
Ethanol extract	*S. aureus*	12.5
	E. coli	0.78
	S. Typhi	12.5
	K. pneumoniae	1.56
	P. aeruginosa	0.39

The low MIC values against the Gram-negative bacteria, especially *P. aeruginosa*, are significant. This suggests that the propolis extract contains compounds capable of penetrating or disrupting the complex outer membrane of these pathogens, which is often a barrier to antimicrobial agents (Mirzoeva et al., 1997). The variation in MIC values underscores the differential susceptibility of bacterial species to the extract's components, likely due to differences in cell wall structure and efflux pump systems (Przybyłek and Karpiński, 2019).

This research was limited by its *in vitro* design, which does not directly translate to *in vivo* efficacy or therapeutic applications. The use of crude extracts, while informative, means the specific bioactive compounds responsible for the observed antibacterial activity remain unidentified. Furthermore, the study did not investigate the mechanism of action, potential synergy with conventional antibiotics, or toxicity profiles, which are crucial for assessing its potential as a lead for therapeutic development.

4. CONCLUSION

This study conclusively demonstrates that Egyptian propolis is a rich source of natural antibacterial compounds. The ethanol extract was the most effective, exhibiting potent and broad-spectrum activity, particularly against challenging Gram-negative pathogens like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. These results validate the traditional use of propolis and highlight its potential as a complementary or alternative therapeutic agent in the fight against antibiotic-resistant bacteria. Future work should focus on bioassay-guided fractionation of the active extracts to identify the specific bioactive compounds, evaluate their synergistic effects with conventional antibiotics, and assess their *in vivo* efficacy and safety.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable requests.

Authors' Contributions

A. **A.M.:** Conceptualization, Methodology, Resources, Writing - Original Draft, Project Administration. **D.R.Z.:** Formal Analysis, Investigation, Data Curation, Writing - Review & Editing. **A.S.K.:** Validation, Resources, Supervision, Writing - Review & Editing. **B.I.A.:** Investigation, Methodology, Visualization. **D.A.D.:** Investigation, Formal Analysis, Data Curation. **M.H.A.:** Methodology, Investigation, Resources. **R.A.H.:** Investigation, Validation, Data Curation. All authors have read and approved the final version of the manuscript.

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