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ANALYTICAL STUDIES AND BIOLOGICAL ACTIVITIES OF PROPOLIS OIL FROM NORTH PUNJAB, PAKISTAN

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Abstract

Propolis is a resinous material prepared by honeybees, has an extensive range of biological properties, and is an important part of the herbal medicinal system. This is the first study to investigate the chemical composition and biological potential of oil extracted from brown propolis collected from the hives of *Apis mellifera* on the trees of Populus from Northern Punjab, Pakistan. The extraction of oil was carried out using the microwave oven. Forty five phytochemical constituents were identified by gas chromatography-mass spectrometry (GC-MS). Sesquiterpenoid (α-Eudesmol and (-)-β-Copaene) and sesquiterpenes including τ-Cadinol, L-Calamenene and α-Humulene, were the most prominent natural products. The results showed two unknown compounds (5%) which remained unidentified due to unavailability of data on NIST-2011. The volatile oil derived from brown propolis displayed remarkable efficacy in terms of its antioxidant (IC_{50} 0.54 ± 0.07mg/mL), anti-inflammatory (IC_{50} 51.0239± 0.01mg/mL), antitumor (against the HeLa-human cervical cancer cell line) (IC_{50} 1.96± 1.52 mg/mL), antifungal against (*A. niger* (MIC > 3mg/mL), *A. flavus* (MIC > 5mg/mL) and antibacterial properties against (*Bacillus mojavensis* (MIC 0.421mg/mL), *Staphylococcus aureus* (MIC 0.501mg/mL), *Pseudomonas aeruginosa* (MIC 0.829mg/mL), *Escherichia coli* (MIC 0.943mg/mL) and *Salmonella typhimurium* (MIC > 2mg/mL). Therefore, propolis oil is suggested as a potential therapeutic agent and can be used as a readily available indigenous source for treating microbial infections.

Key words: *Apis mellifera*, Propolis, Antioxidant, Antibacterial, Anti-inflammatory, Antitumor.

Introduction

Over the last three decades, researchers have been fascinated by the amalgamation of thick bee saliva with resins, exudates from buds, and other botanical components (Kurek-Górecka *et al*., 2023). Propolis serves the purpose of sealing fractures and cavities inside beehives. One hypothesis suggests that it enhances the immune system of honeybees, thereby reducing the probability of illness and parasite transmission within the colony (Turcatto *et al*., 2018). Propolis is being progressively used in pharmaceutical sector due to its diverse array of biological capabilities, including antimicrobial, antioxidant, anti-inflammatory, antiviral, antiulcer, immunostimulating, hypotensive, and cytostatic effects (Shehata *et al*., 2020; Pelvan *et al*., 2022; Son *et al*., 2023).

The composition of this complex blend of natural substances is mostly influenced by the surrounding flora; therefore, plant diversity topography and climatic conditions of the area (Ruiz Ruiz *et al*., 2023) are of great interest as all these conditions greatly affect the chemical composition of propolis. Propolis comprises of approximately 40-70% resin, primarily consisting of phenolic compounds, and 3-5% essential oils. Additionally, a non-balsamic component includes approximately 20-35% wax, 5% pollen, and 5% other substances such as minerals, polysaccharides, and proteins (Ahangari *et al*., 2018; Karagecili *et al*., 2023). In contrast to the extensive research done on the non-volatile chemicals of propolis, the volatile components have been relatively understudied. Volatile chemicals, albeit present in lower amounts, have a substantial influence on the characterization of propolis with notable biological activities (Bankova *et al*., 1994). These phytochemicals include terpenoids, alcohols, aldehydes, hydrocarbons, and aliphatic ketones (de Oliveira *et al*., 2021). Qualitative and quantitative variations are observed because of the impact of regional soil conditions of plants where bees build their hives and the seasonal extraction period of propolis (Borčić *et al*., 1996).

Propolis extract is comparatively safe to use as herbal medicine due to the presence of bioactive compounds. Pakistan is an agricultural country; therefore, the diversity of the plant species particularly of phenerogams – about 5800 species (Ali, 2008) found in Pakistan may show various compositions of propolis. As previously reported in the literature of Brazil, Croatia, and Greece, propolis oil has a dynamic phytochemical composition (Bankova *et al*., 1999; Melliou *et al*., 2007; Huong, 2023). Encouraged by the limited literature reported on propolis oil composition particularly from Pakistan, herein, we first report the phytochemical components and the biological activity of oil derived from brown propolis collected from Northern Punjab, Pakistan.

Material and Methods

Sample collection: In summer 2021, a sample of brown propolis was collected from *Apis mellifera* colonies, located in Rawalpindi (Latitude 33.5651°N, Longitude 73.0169°E) Punjab, Pakistan present on *Populus euphratica* L. (Populus) trees. The sample was kept in a light-free environment at -20°C.

Microwave-assisted extraction (MAE) of oil: The sample (10 g) was ground and mixed in 100 mL of distilled water and irradiated for 30 min in a 600 W household microwave oven (Nobel-OM46SS, input voltage: 230V, output voltage: 1000 V, output frequency: 2450 MHz). The propolis oil-water mixture was separated by solvent extraction using n-hexane, and the organic layer was dried with sodium sulfate (1:5), evaporated under *vacuum,* and stored in a refrigerator (Cardoso-Ugarte *et al*., 2013).

Gas chromatography-mass spectrometry (GC-MS) analysis: The GC-MS analysis was conducted using an Agilent 5977 A series GC-MSD instrument. The DB5-MS column of 30 m length, 0.25 mm inner diameter, and 0.25 µm film thickness, was used with a split ratio of 5:1. The mobile phase consisted of helium gas injected into the system at a volume of 1 mL and flow rate of 1 mL/min. The initial temperature was recorded as 50°C, for two minutes with a consistent increase of 7°C/min until it reached 290°C. The temperatures of the transfer line, detector, and ion source were recorded as 230 and 250°C, respectively. The ionization energy (EI) with 70 electron volts (eV) was used to measure the mass-to-charge ratio (m/z) at 35-450. To determine the linear retention indices (RI), a set of saturated alkanes with equal carbon chain lengths ranging from C7 to C30 were analyzed using consistent experimental settings. The identification approach included comparing the mass spectra of the samples to those in the NIST-2011 collection, and comparing the findings to recognized standards (NIST. 2011; Ikeda *et al*., 2021).

Biological activities of propolis oil: *In vitro* biological activities were performed to investigate the therapeutic potential of propolis oil. All experiments were performed in triplicate, and the results were expressed in terms of IC_{50}.

DPPH-free radical scavenging potential: Blois method (Blois *et al*., 1958) with slight modifications was used to evaluate the free radical scavenging potential. Propolis oil (0.5 mL, 0.1-0.3 mg/mL,) was mixed with methanolic DPPH solution (2.5mL). The control blank was prepared using only methanol (0.5 mL) with DPPH solution (2.5mL). The test and blank samples were incubated in dark at 30°C for 30 minutes. Absorbance of samples was measured at 517 nm on spectrophotometer. The percent antioxidant index was calculated using the formula given below and the results are mentioned as IC_{50} values.

$$\text{Percent antioxidant index} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}$$

Anti-inflammatory activity: An albumin denaturation assay was used to determine the anti-inflammatory potential of propolis oil (Mizushima *et al*., 1969). Bovine serum albumin (BSA, 5%) was mixed with propolis oil (0.5, 1.0, 2.0 mg/mL), and the pH was adjusted by adding HCl (1N). The mixture was incubated at 37°C for 20 min, and then at 57°C for 3 min. After cooling to room temperature, phosphate buffered saline (pH 6.3, 2.5 mL) was added, and turbidity was measured at 660 nm. A control experiment was performed without the bovine serum.

Antibacterial activity: Agar well diffusion method (Perez *et al*., 1990) was used to assess the antibacterial effect of propolis oil against two gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Bacillus mojavensis* (ATCC 51516), and three gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Salmonella typhimurium* (ATCC 700931). Each petri plate was filled with sterile agar solution (25 ml) and inoculated with fresh bacterial culture (105 CFU/mL, 50 µL), grown in Mueller Hinton medium, left until solidified, and wells were made using a sterile cork borer. Different dilutions of propolis oil (0.5, 1.0, and 2.0 mL), dissolved in n-hexane (96%) and reference drug Ciprofloxacin (0.01 mg/mL) were added to the wells, the petri dishes were kept for 30 min to facilitate the diffusion of samples and finally incubated for 24 h at 37°C, and the zone of inhibition was recorded and measured in mm for each well. The minimum inhibitory concentration (MIC) of each bacterial strain was calculated using the macro-broth dilution method. In 48-well plates, each well contained a known quantity of bacteria (105 CFU/mL) and propolis oil (2–0.01 mg/mL). Control wells do not contain any test sample. After 24 h of incubation at 37°C, the MIC was determined by observing turbidity (Wiegand *et al*., 2008).

Antifungal activity: Agar diffusion method (Magaldi *et al*., 2004) was used to estimate the antifungal potential of propolis oil against two fungal strains, *Aspergillus flavus* (ATTC 9643) and *Aspergillus niger* (ATTC 9642). The aqueous solution of each fungal spore (10^6 spore mL^{-1}) was spread onto solid media (Sabouraud dextrose agar (SDA) plates. A sterilized cork borer was used for cutting wells, and propolis oil (0.5mL) was poured, while DMSO was used as a negative control followed by incubation for 24 hrs. at 30°C, separately. The zone of inhibition was measured in mm for each strain and the results were expressed in terms of the MIC value.

Antitumor activity: Cytotoxicity of propolis oil was evaluated in 96-well plates (flat bottom) using a standard colorimetric MTT assay (Mosmann *et al*., 1983). HeLa cells (cervical cancer) were cultured in MEM-eagle medium supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin in a 75 cm^2 flask. The cells were maintained in a CO_2 incubator (5%) at 37°C. Exponentially growing cells were harvested and counted with a hemocytometer, and 100 µL/well of medium containing 6 × 104 cells was poured into each well. After overnight incubation, the medium was removed, and 200 µL of fresh medium containing different concentrations of extracts (1-30µM) was added to the wells. After 48 h of incubation, 200 µL of MTT (0.5 mg/mL) was added to each well and further incubated for 4 h. Finally, 100 µL of DMSO was added to each well and the extent of formazan production was calculated by measuring the absorbance at 570 nm using a microplate reader. Cytotoxicity was recorded as a concentration affecting 50% growth inhibition (LC_{50}) for HeLa.

Table 1. Composition of the propolis oil.

Sr. No.	Compounds	RI exp.	RI lit.	Rt.	%RA
1.	Ethyl-3-phenylpropionate	854	852	3.835	1.245
2.	α-pinene	932	928	5.48	1.346
3.	Linalool	958	955	7.1	0.607
4.	Undecane	974	971	7.27	3.959
5.	Acetophenone	1072	1078	10.206	0.475
6.	Guaiacol	1093	1092	10.631	0.634
7.	Limonene	1103	1101	10.7	1.649
8.	Nonanal	1112	1112	11.052	3.596
9.	Methyl salicylate	1203	1202	13.026	1.154
10.	Decanal	1213	1212	13.255	1.631
11.	β-Cyclocitral	1231	1226.1	13.61	1.834
12.	3-Phenylpropanol	1240	1238	13.796	0.362
13.	Tridecane	1246	1241	13.82	3.376
14.	4-phenyl-2-Butanone	1252	1251	14.046	2.754
15.	(E)-Cinnamaldehyde	1283	1283	14.701	2.52
16.	2-Propen-1-ol, 3-phenyl-	1317	1312	15.372	1.154
17.	p-Vinylguaiacol	1319	1317	15.421	0.204
18.	Capric acid	1369	1371	16.381	0.075
19.	(-)-β-Copaene	1389	1385	16.78	6.039
20.	Pulegone	1394	1391	17.642	0.49
21.	α-Humulene	1471	1465	18.291	7.34
22.	Alloaromadendrene	1475	1477	18.373	0.379
23.	γ-Muurolene	1487	1486	18.596	0.173
24.	α-Curcumene;	1492	1500	18.667	2.945
25.	α-Muurolene	1511	1506	19.005	0.112
26.	α-Amorphene	1528	1519	19.3	1.489
27.	Δ-Amorphene	1532	1533	19.36	3.312
28.	L-calamenene	1536	1534	19.442	9.23
29.	Cadinadiene-1,4	1547	1546	19.633	0.634
30.	L-phenylalanine	1565	1561	19.91	0.077
31.	Eudesmol	1649	1646	21.346	0.188
32.	τ--Cadinol	1658	1649	21.52	6.039
33.	α-Eudesmol	1675	1677	21.777	15.154
34.	Cadalin	1688	1682	21.995	0.26
35.	Heptadecane	1708	1700	22.311	2.002
36.	Pentadecane	1735	1732	24.21	1.157
37.	Hexadecanoic acid	1965	1963	26.168	1.154
38.	Eicosane	2008	2000	26.768	1.204
39.	Unknown	-	-	27.197	0.122
40.	Heneicosane	2108	2100	28.116	0.179
41.	Scillarenin	2125	2122	28.272	1.727
42.	Allyl malonic acid	2246	2244	29.125	1.178
43.	Cyclodecanol	2258	2256	29.17	0.120
44.	Unknown	-	-	27.197	4.062
45.	Unknown	-	-	29.430	0.942
46.	Tetracosane	2409	2405	31.853	0.042

RI exp: Retention index relative to n-alkanes (C 8 –C 20), RI lit: Retention index literature, Rt: Retention time, Total percentage = 96.353 %

Results and Discussion

The microwave assisted extraction of brown propolis resulted in the production of a transparent oil, with a yield of 0.615% (Zheljazkov *et al*., 2013). This extraction method was found to be more efficient and economical in terms of time, cost, and energy consumption (Golmakani & Rezaei, 2008; Nitthiyah *et al*., 2017; Ghazanfari *et al*., 2020) than the conventional method.

The chromatographic analysis by GC-MS has been interpreted based on retention time and retention index and compared with the literature (Table 1). The major constituents of the extracted oil were sesquiterpenoid and sesquiterpenes, including α-Eudesmol (15.154%), τ-Cadinol (6.039%), (-)-β-Copaene (6.039), L-Calamenene (9.23%) and α-Humulene (7.34%) (Claeson *et al*., 1991; K Asakura *et al*., 2000; M Salmoun *et al*., 2007) (Fig. 1). Two unknown compounds were also present (5%) with other identified compounds, which have never been reported before as data was missing on NIST-2011.

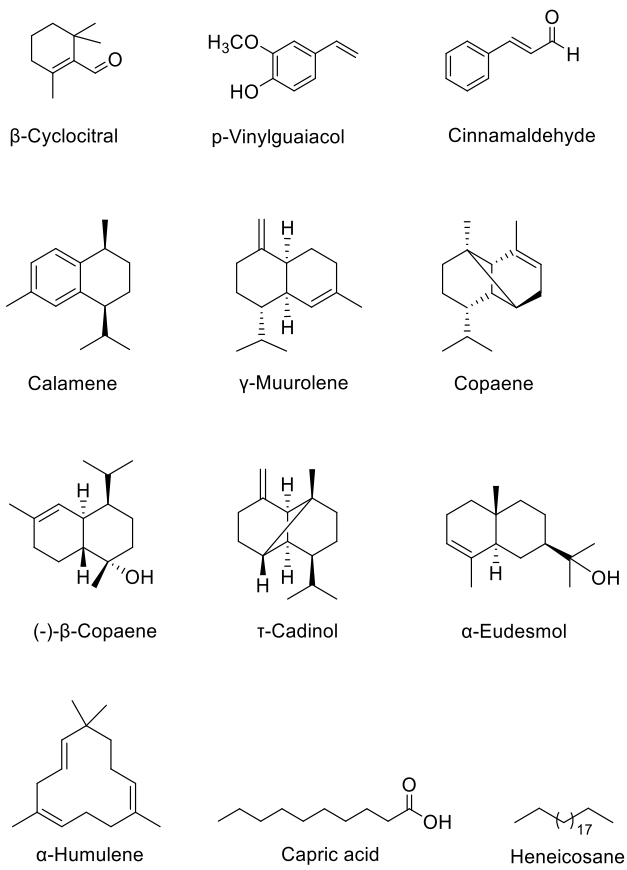

Fig. 1. Some significant Sesquiterpenes and sesquiterpenoid isolated from propolis oil.

The extracted oil was then subjected to biological studies and it showed noteworthy DPPH radical scavenging activity (IC_{50} = 0.54± 0.07 mg/mL) compared to related work (Adefegha *et al*., 2017; Ghahari *et al*., 2017; Yang *et al*., 2020).

The oil extracted from brown propolis also exhibited good anti-inflammatory inhibition (IC_{50} 51.0239 ± 0.01 mg/mL) and was comparable with the findings reported by Bankova in 1994. Thus, it has been used as an anti-inflammatory agent in wound healing (Liu *et al*., 2017; da Rosa *et al*., 2022; Merecz-Sadowska *et al*., 2023; AlKandari *et al*., 2024).

The colorimetric MTT test employs the mitochondrial dehydrogenase enzyme found in healthy cells (Sarvmeili *et al*., 2016). The cytotoxicity of propolis oil toward HeLa cell lines was observed, and it showed a dose-dependent relationship. The results revealed that volatile oil (5 mg/mL) from brown propolis resulted in a cell death rate above 50% in HeLa cell lines. These findings further indicated that there was a decline in the viability of HeLa cells when oil concentration was increased. Previous

studies have provided compelling evidence that the volatile oil derived from brown propolis has cytotoxic properties against cancer cells, (Döll-Boscardin *et al*., 2012; de Andrade *et al*., 2020).

The antibacterial activity of propolis oil was observed using Ciprofloxacin as a reference drug due to its wide range against both Gram-positive and Gram-negative bacteria (MIC<0.001 mg/mL) as shown in (Table 2). Propolis oil (5 mg/mL) showed maximum antibacterial activity when tested against different strains: *Bacillus mojavensis* (MIC 0.421 mg/mL)*, Staphylococcus aureus* (MIC 0.501 mg/mL)*, Pseudomonas aeruginosa* (MIC 0.829 mg/mL)*, Escherichia coli* (MIC 0.943 mg/mL), and *Salmonella typhimurium* (MIC > 5 mg/mL).

These bacteria were selected because of the common diseases caused by them in the local population, such as foodborne illness, diarrhea, skin infections, urinary tract infections, respiratory system infections, vomiting, and fever (Kim *et al*., 1995; Hsouna *et al*., 2013; Son *et al*., 2023). The results showed that propolis oil was more active against gram-positive bacteria than gram-negative strains. *Bacillus mojavensis* and *Staphylococcus aureus* are the most susceptible microorganisms, whereas *Salmonella typhimurium* is the most resistant (Claeson *et al*., 1992). The antifungal properties of propolis oil against two filamentous fungal species, *Aspergillus niger,* and *A. flavus*, showed that propolis oil had moderate efficacy against *A. niger* (MIC > 3 mg/mL) compared to *A. flavus* (MIC > 5 mg/mL). The *In vitro* biological potential of propolis oil established it as a good candidate for herbal treatment. Propolis facilitates skin regeneration and inhibits the disruption of the healing process (Süntar *et al*., 2012; Perveen *et al*., 2018; Huanbutta *et al*., 2020; Stojko *et al*., 2020). Propolis oil is also used as a coating material for fruits and vegetables to enhance their shelf life (Zahid *et al*., 2013; Ali *et al*., 2014; Urrea *et al*., 2022).

Table 2. Antioxidant, Anti-inflammatory, antitumor, antifungal and antibacterial activities of propolis oil.

DPPH	Anti-inflammatory	Antitumor	Antifungal		Antibacterial				
(IC_{50} mg/mL)	(IC_{50} mg/mL)	(LC_{50} mg/mL)	MIC (mg/ml)		MIC (mg/ml)				
			A.niger	*A.flavus*	*Bs.*	*Sa.*	*Pa.*	*Ec.*	*St.*
00.54 ± 0.07	51.0239 ± 0.01	1.96 ± 1.52	> 3	> 5	0.421	0.501	0.829	0.943	>5

Note: Results are expressed as mean ± standard deviation of triplicate

(*A. niger: Aspergillus niger, A. flavus: Aspergillus flavus, Bs*: *Bacillus mojavensis, Sa*: *Staphylococcus aureus, Pa*: *Pseudomonas aeruginosa, Ec*: *Escherichia coli, St*: *Salmonella typhimurium*)

Conclusion

Analysis of the oil extracted from brown propolis, collected from Punjab, Pakistan, involved the identification of 46 components with diverse aromas and medicinal properties. These findings indicated that sesquiterpenes were the predominant class of natural products present in this oil sample. Moreover, two unknown compounds were identified which may be of interest to the researchers. Propolis oil has exhibited significant anti-inflammatory, anticancer, antibacterial, and antifungal effects, thereby identifying it as a promising option for potential utilization in pharmaceutical and food-related contexts owing to the presence of bioactive chemicals. Further research is needed to fully understand the mechanism of action and determine the appropriate dosages for specific diseases.

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