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The Effectiveness of Propolis as a Natural Antibiotic against Some Skin Fungi Pathogenic to Humans

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ABSTRACT

This study was conducted to determine the extent of the inhibitory effect of propolis against some skin fungi pathogenic to humans, including T. interdigitale, T. mentagrophytes, M. cains, A. nidulans, Aspergillus flavus, C. albicans. As well as knowing the effective and biologically active substances contained in propolis through chemical analysis (gas chromatography) (GC-MS) and comparing its effect to antibiotics, the results showed: an effective inhibitory effect of aqueous and alcoholic propolis extracts against all skin fungi tested, and the alcoholic propolis extract was more effective against the fungi compared to the aqueous propolis extract, which had an effective effect against A.flavus better than alcoholic, and the propolis extract, as a natural antibiotic, was effective compared to the antibiotics used, and the highest average inhibition of the type of extracts was alcoholic against (20.10A) C.albicans and the highest average inhibition of fungi was (16.99A) for C. albicans, and the largest inhibition diameter was recorded against T. mentagrophytes (27.75 mm).

KEYWORDS: Propolis - Antifungal properties - Skin fungi - GC-MS analysis - Pathogenic fungi - Herbal medicine - C. albicans - T. mentagrophytes - Microbial resistance

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INTRODUCTION

The honey bee, Apis mellifera Linnaeus, is recognized as the most significant pollinator, vital to the success of modern agriculture and the stability of human food production (Belsky and Joshi, 2019). Belonging to the animal kingdom, the honey bee is one of the most economically and socially important insects. It produces honey by relying on the nectar of various plant flowers (Abdel-Aziz, 2019). Bee products possess diverse biological properties such as anti-microbial, anti-inflammatory, anti-cancer activities, and antioxidants (Nainu et al., 2021). Recently, fungal infections have been responsible for over a million human deaths annually and are an increasingly significant cause of mortality and morbidity (Robbin et al., 2017). This issue is attributed to the lack of effective monitoring measures and the widespread overuse of drugs. In the 1990s, there was a sharp decline in the development of new drug classes, accompanied by the emergence of drug-resistant strains of human pathogens, raising significant concerns, especially multi-drug resistant strains. Now, microbial resistance causes over 700,000 deaths annually, a number that could rise to 10 million by 2050 (Kong and Yanng, 2021). Given the scarcity of new and effective therapeutic agents, the pharmaceutical industry has discovered a new source of therapeutic compounds in natural products and herbal medicine to address current human and animal health issues (Zulhendri et al., 2021). This means that these treatments will become widely available, especially to people in less developed countries who cannot afford expensive therapies. The reduction in side effects also means increased patient tolerance and compliance, leading to the maximum therapeutic impact without negatively affecting life. Propolis is one of the most renowned honey bee products and has been used in folk medicine for its numerous health benefits since the dawn of civilization (Przybytek and Karpinski, 2019; Zulhendri et al., 2021).

Therefore, propolis is a natural, wax-like resin produced by the honey bee (Apis mellifera L.), composed of salivary secretions, wax, pollen, and various plant materials. Honey bees use propolis to seal cracks or open spaces in the hive, thus preventing parasite invasion and helping to

maintain appropriate internal temperature and humidity (Pavlovic et al., 2020).

MATERIALS AND METHODS

1. Preparation and Chemical Analysis of Sample Models:

After obtaining propolis samples directly from beehives with the help of beekeepers, the samples were cleaned of impurities and stored in sterilized, opaque, airtight 200 ml glass bottles. They were labeled with specific information regarding the geographic location, types of plants in the vicinity of the hives, and the time and date of sample collection. The samples were then refrigerated for freezing and subsequent cutting into small pieces for later use. Some samples were sent to a laboratory in Samarra for analysis and determination of active ingredient percentages using a GC-MS device, to study their inhibitory effectiveness against certain fungi under study and compare them with antifungals.

2. Preparation of Propolis Extract:

The alcoholic and aqueous extracts of propolis were prepared according to the method described by Yghobi et al. (2007). This involved grinding the frozen propolis with an electric blender and adding 99% ethanol or water in a 10:1 weight/volume ratio. The mixture was placed in a flask and left at room temperature for 48 hours with intermittent shaking using a shaker (300 pr). The solution (either ethanol or aqueous extract) was then filtered using Watman No. 1 filter paper, and the filtrate was evaporated to obtain a very dense substance, which was the alcoholic or aqueous extract of propolis. This was then mixed with 99% ethanol or water to obtain the different concentrations used in the current study.

3. Inhibitory Efficacy Test of Bee Product (Propolis):

The well diffusion method was used to test the inhibitory efficacy of bee product (propolis) on fungal growth. This involved pouring 20-25 ml of Sabouraud Dextrose Agar into each Petri dish. After the medium solidified, the dishes were incubated for 24 hours at 37°C to ensure the agar was not contaminated. The medium was then inoculated using a sterilized cotton swab, spreading the fungal suspension evenly on the Sabouraud Dextrose Agar. After 15 minutes at room temperature for absorption, five holes were made in each dish using a 6 mm cork borer. Each hole represented a specific concentration, with 100 microliters of each concentration added to the wells, and distilled water used in one of the wells instead of the product (extract), serving as a control sample to compare with the inhibitory efficacy of each extract (represented by the area of no fungal growth around each well). The dishes were incubated for

1-4 days depending on the fungus type (Mahdi, 2020). After the incubation period, the diameters of the inhibition zones around the wells were measured using a ruler in millimeters, and the average diameters of the inhibition zones were calculated (Ivancajic et al., 2010).

4. Antibiotic Sensitivity Test:

A pure culture of the fungi under study, previously identified, was placed in a test tube containing 5 ml of Nutrient broth and incubated for 1-4 days depending on the fungus type, at 37°C. A portion of the liquid medium was then spread evenly on Muller Hinton agar using a cotton swab. Antibiotic discs were placed using sterilized forceps, ensuring a distance between each disc. The plates were incubated for 24 hours at 37°C, and the diameter of the inhibition zone around each antibiotic disc was measured with a ruler (Macfaddin, 2000).

RESULTS AND DISCUSSION

1. Chemical Analysis of Propolis by GC-MS:

Analysis using Gas Chromatography/Mass Spectrometry (GC-MS) techniques was conducted. This involved a carrier gas column using helium at a flow rate of 1 ml/min. The injector temperature was set at 280°C at minimum split. Initially, the oven temperature was set at 60°C for 4 minutes before increasing to 150°C at a rate of 10°C/minute for 15 minutes. The following parameters were used to optimize the mass spectra: source temperature at 280°C, transfer temperature at 150°C, solvent delay time of two minutes, and a scan range of 35-500. Finally, the temperature was raised to 310°C, with a total operation time of 40.5GC. The results were then compared with stored mass spectra (Mohiuddin et al., 2022).

The results showed the presence of numerous biologically active substances in the propolis samples, including alkaloids, carbohydrates, aldehydes, terpenoids, alcohols, saponins, flavonoids, phenols, coumarins, amino acids, glycosides, proteins, and others, as indicated in Table (1), which shows the retention times, percentages, molecular weights, chemical formulas, and names of the separated substances in Model No. 2.

T	Retention Time Rt / minute	%	M.W. Molecular Weight	Formula	Compound Name
1	4.21	0.120	120	C_9H_{12}	1,3,5 – Trimethyl Benzene
2	6.13	0.470	156	$C_{10}H_{20}O$	Menthol
3	8.12	1.380	370	$C_{23}H_{30}O_4$	Spromesifen
4	8.18	1.020	328	$C_{19}H_{36}O_4$	Gultaric acid, di (4,4 - dimethyl pent – 2 yl) ester
5	8.35	5.367	156	$C_{10}H_{20}O$	2,2-Dimethyl-3-Octanone

6	8.40	1.823	311	$C_{20}H_{41}N_0$	Propanamide, N-Heptyl-N-Octyl, 2,2-Dimethyl
7	8.54	9.523	273	$C_{13}H_{19}N_{03}$	Beta-Dihydro Caramine
8	8.79	19.547	128	$C_{13}H_{19}N_{03}$	3,6-Heptanedione
9	8.90	18.630	128	$C_7H_{12}O_2$	2,5-Heptanedione
10	8.98	15.849	114	$C_7H_{12}O_2$	Hexane, 2.5-dimethyl
11	9.11	9.810	273	C_8H_{18}	Beta-dihydro caramine
12	9.67	0.931	298	$C16H19N_{03}$	Ethyl, 15-methyl-hezadecanoate
13	9.74	0.600	513	$C_{19}H_{38}O_2$	N-methyl-pseudotomatidine diacetate
14	9.80	0.442	506	$C_{32}H_{51}N_{04}$	Succinic acid, di (tetradec-11-ethyl) ester
15	9.90	1.244	222	$C_{32}H_{58}O_4$	Diethyl phthalate
16	10.00	2.908	366	$C_{12}H_{14}O_4$	Erucic acid ethylester
17	10.10	2.807	324	$C_{24}H_{46}O_2$	Ethyl 18-mondecoate
18	10.21	1.604	366	$C_{21}H_{40}O_2$	Ethyl - 13 - docosenoate (ethylerucate)
19	10.29	0.217	212	$C_{24}H_{46}O_2$	Decanoic acid, 2-Propenyl ester
20	10.40	0.169	254	$C_{13}H_{24}O_2$	Palmitoleic acid
21	11.10	0.296	282	$C_{16}H_{30}O_2$	Ethyl 9-hexadecenoate
22	11.22	0.052	458	$C_{18}H_{34}O_2$	Hexa siloxane, tetradeca methyl
23	11.71	5.447	284	$C_{14}H_{44}O_5Si_6$	Hexadecanoic acid, ethyl ester

2-The Inhibitory Efficacy of Propolis Against Certain Pathogenic Skin Fungi:

The study involved the use of aqueous and alcoholic extracts of propolis on various tested pathogenic fungal species. The agar well diffusion method was employed for this purpose. Consequently, the results demonstrated a clear variation in the inhibitory effect based on the concentration and solvent used. The maximum impact was observed at a concentration of 1% mg/ml of the extract. An increase in concentration corresponded to an enhanced inhibitory effect, with the best results seen in the raw propolis extract in ethanol. This exhibited a lethal and fungicidal effect, as evidenced by the absence of microbial growth around the wells after 24 hours of incubation.

Table (2): Propolis Extract / Fungi"

Average Fungal Type	Average Extract Type	Concentrations					Extract Type	Pathogenic Fungi
		0.2	0.4	0.6	0.8	1		
15.83A	16.50 B	9.75	10.5	13.125	21.375	27.75	Propolis in Ethanol	*Trichophyton mentagrophytes*
	15.15 BC	13.5	14.625	15	16.125	16.5	Propolis in Distilled Water	
		11.63 d	12.56 d	14.06 c	18.75 b	22.13 a	Average Concentration	
15.26A	16.20 B	12	15	16.875	17.625	19.5	Propolis in Ethanol	*Trichophyton interdigital*
	14.33 C	13.125	13.5	14.25	15	15.75	Propolis in Distilled Water	
		12.56 d	14.25 c	15.56bc	16.31 ab	17.63 a	Average Concentration	
12.98B	15.30 BC	12	12.75	13.5	16.5	21.75	Propolis in Ethanol	*Microspore Canis*
	10.65 D	8.25	9	11.25	12	12.75	Propolis in Distilled Water	
		10.13 d	10.88 d	12.38 c	14.25 b	17.25 a	Average Concentration	
16.99A	20.10A	13.125	16.125	21.375	24	25.875	Propolis in Ethanol	*Candida albican*

	13.88C	12	12.75	13.5	14.25	16.875	Propolis in Distilled Water	
		12.56 e	14.44 d	17.44 c	19.13 b	21.38 a	Average Concentration	
16.20A	15.60 BC	8.25	15.375	16.875	17.625	19.875	Propolis in Ethanol	*Aspergillus flavus*
	16.80 B	12.375	14.25	16.125	18.75	22.5	Propolis in Distilled Water	
		10.31 e	14.81 d	16.50 c	18.19 b	21.19 a	Average Concentration	
11.33B	16.73 B	12.75	14.25	15.375	16.875	24.375	Propolis in Ethanol	*Aspergillus nidulans*
	5.93 E	0	0	0	13.125	16.5	Propolis in Distilled Water	
		6.38 c	7.13 c	7.69 c	15.00 b	20.44 a	Average Concentration	

* Similar lowercase letters horizontally indicate no meaningful difference between them.
1** Similar uppercase letters vertically indicate no meaningful difference between them.

The current study's results demonstrated varying effects of alcoholic propolis extract against the tested pathogenic fungi, as shown in Table (2). Sensitivity results were observed for the fungus T. mentagrophytes, which was inhibited at low concentrations, specifically at a concentration of 0.2% (the minimum inhibitory concentration, MIC), where the diameter of the inhibition zone around the well was 9.375 mm. A concentration of 1% was considered fungicidal and lethal, with an inhibition zone diameter of 27.75 mm, the highest recorded diameter among the tested fungal species. The highest average inhibition for the extract type was (B16.50). However, using aqueous propolis extract, the fungus showed sensitivity at low concentrations, where the inhibition zone diameter around the well was 13.5 mm at the lowest MIC (0.2 ug/ml). At a 1% lethal and fungicidal concentration, the inhibition zone diameter was 16.5 mm, with the highest average inhibition for the extract type being (BC15.15), and the highest average inhibition for the fungal species being (A15.83). These results are consistent with those using aqueous propolis extract in (Batac et al., 2020), and with (Salatino et al., 2022) when using alcoholic propolis extract.

For the fungus T. interdigitale, it showed sensitivity to the alcoholic propolis extract, inhibiting growth at low concentrations, with an inhibition zone diameter of 12 mm at the MIC of 0.2 ug/ml. At a 1% concentration, considered fungicidal and lethal, the diameter was 19.5 mm, with the highest average inhibition for the extract type being (B16.20). However, using aqueous propolis extract, the growth was inhibited at a 0.2% concentration, with an inhibition zone diameter of 13.125 mm, larger than that using the alcoholic extract, possibly due to the content of active substances in propolis and the applied concentration and type of microorganism. A 1% concentration was considered fungicidal and lethal, with an inhibition zone diameter of 15.75 mm, and the highest average inhibition for the extract type being (C14.33), and the highest average inhibition for the fungal species being (A15.26). These results are consistent with (Batac et al., 2020) using aqueous propolis extract and with (Zulhendri et al., 2021) using alcoholic propolis extract.

For the fungus Microsporum canis, it showed sensitivity at low concentrations, inhibiting growth at the lowest inhibitory concentration (MIC = 0.2 ug/ml), with an inhibition zone diameter of 12 mm when using alcoholic propolis extract, similar to the diameter for T. interdigitale. However, at a 1% concentration, considered lethal and fungicidal, the diameter was 21.75 mm, with the highest average inhibition for the extract type being (BC15.30). But, using aqueous propolis extract, the inhibition zone diameter around the well was 8.25 mm at the MIC of 0.2 ug/ml, and at a 1% lethal and fungicidal concentration, the diameter was 12.75 mm, with the highest average inhibition for the extract type being (D10.65) and the fungal species being (B12.98). This differs from (Netikova et al., 2013), which showed a decreased fungal response at high concentrations of Czech ethanol-free propolis extract, possibly due to the solvent type used and the content of active substances in propolis, as well as the applied dose quantity. This was confirmed by (Petruzzi et al., 2020), while the results are consistent with (Salatino et al., 2022).

For the fungus C. albicans, it showed sensitivity to the alcoholic propolis extract, with growth inhibited at an MIC of 0.2 ug/ml and an inhibition zone diameter of 13.125 mm. At a 1% concentration, considered lethal and fungicidal, the diameter was 25.875 mm, with the highest average inhibition for the extract type being (A20.10). However, using aqueous propolis extract, growth was inhibited at an MIC of 0.2 ug/ml, with an inhibition zone diameter of 12 mm, similar to T. interdigitale and M. canis, possibly due to the partial solubility of active substances in water. This was confirmed by (Suran et al., 2021), while a 1% concentration was considered lethal, with an inhibition zone diameter of 16.875 mm, and the highest average inhibition for the extract type being (C13.88), and the fungal species being (A16.99). This differs from (Marquele et al., 2006), which indicated that

the fungus was more resistant to Portuguese propolis extracts, possibly because C. albicans can undergo morphological transformation, and the active substances in propolis were not sufficient to affect the fungal cell wall, causing damage and fungal death. This may be due to overexpression of membrane transporters, changes in the cell wall, biosynthesis of ergosterol (a unique component of fungal cell membranes), and acquisition of functional mutations in transcription factors regulating membrane transporters, as well as biofilm formation (Bhattacharya et al., 2020).

This differed from (Al-Ani et al., 2018), which indicated that ethanol extracts of Irish propolis were more active at (MIC = 0.3 ug/ml), whereas the current results showed activity of the ethanol propolis extract at (MIC = 0.2 ug/ml) against the fungus. This may be attributed to the extraction method and the vegetative cover affecting the efficacy of propolis, causing it to lose a significant biologically active component, as well as to seasonal changes, climate change, and the type of queen bee, all of which significantly impact the composition of propolis. This was confirmed by (Shahbaz et al., 2021), where (Stahli et al., 2018) observed that the ethanol extract of propolis caused a loss of integrity in the cell wall of white fungi and reduced metabolic activity, typically leading to the leakage of vital cellular components including proteins, nucleic acids, and inorganic ions, resulting in cell death.

The fungus A. flavus showed sensitivity to the alcoholic propolis extract, inhibiting its growth at a concentration (MIC = 0.2 ug/ml) with an inhibition zone diameter around the well of 8.25 mm. A concentration of 1% was considered fungicidal and lethal, with an inhibition diameter of 19.875 mm, and the highest average inhibition for the extract type was (BC15.60). However, using the aqueous propolis extract, the inhibition zone diameter around the well was 13.375 mm at the concentration (MIC = 0.2 ug/ml). A 1% concentration was considered lethal for the fungus, with an inhibition zone diameter of 22.5 mm, and the highest average inhibition for the extract type was (B16.80), and the highest average inhibition for the fungal species was (A16.20). This was different from (Lorini et al., 2018), who indicated that brown propolis extract was not effective in inhibiting the growth and spore formation of the fungus, possibly due to the brown propolis lacking active and biologically active substances, especially flavonoid compounds (quercetin, apigenin, kaempferol), which are the main antifungal components. This could be due to the available vegetative cover and the type of solvent used. Additionally, the presence of the toxic substance produced by Aflatoxigenic fungi aids in the resistance to propolis extract, as confirmed by (Zulhendri et al., 2021), and the results were consistent with (Salatino et al., 2022) .

Meanwhile, the fungus A. nidulans showed sensitivity against the alcoholic propolis extract, inhibiting its growth at a concentration of MIC = 0.2 ug/ml with an inhibition zone diameter around the well of 12.75 mm. A concentration of 1% was considered fungicidal and lethal, with an inhibition zone diameter of 24.375 mm, and the highest average inhibition for the extract type was (B16.73). However, the inhibition zone diameter around the well was 0.0 mm at concentrations (0.2, 0.4, 0.6%) when using aqueous propolis extract, but at a concentration of 0.8%, the inhibition zone diameter was 13.125 mm, which was considered the lowest inhibitory concentration for the fungus MIC. At a 1% concentration, the inhibition zone diameter around the well was 16.5 mm, with the highest average for the extract type being (E5.93) and the highest average inhibition for the fungal species being (B11.33).

These results differed from (Franesi, 2007), who indicated that selected propolis samples did not show antifungal activity, especially against A. nidulans, possibly due to the selection of the African bee type, which affected the quality of propolis and green propolis known for containing effective substances with a lower flavonoid content compared to red propolis. This was confirmed by (Rufatto et al., 2017), and the results were consistent with (Salatino et al., 2022) in the effect of alcoholic propolis extract on this fungus.

The current study's results showed that aqueous and alcoholic propolis extracts had an anti-activity effect on pathogenic fungi, including (C. albicans, M. canis, T. interdigitale, T. mentagrophytes, A. nidulans, and A. flavus), with the best being the alcoholic propolis extract except for the fungus A. flavus. This may be due to it being a toxic fungus capable of producing aflatoxins, which can interact with the active substances in propolis found in the alcoholic extract, limiting its effect on the fungus compared to the aqueous propolis extract. Aflatoxin is a secondary metabolite derived from polyketide, a carcinogenic compound that causes teratogenesis, is highly toxic to the liver, and is immunosuppressive. It's noteworthy that some phenylpropanoid-derived compounds such as flavonoids, stilbenes, monolignols, and numerous phenolic acids reported inhibit the production of aflatoxin by A. flavus in fungal primary and secondary metabolisms, possessing a genetic pattern more resistant than the sensitive genotype (Jayaprakash et al., 2019). This suggests that it has genes that may play a significant role in not being greatly affected by the alcoholic extract compared to the aqueous propolis extract.

3-Antibiotic sensitivity testing:

Table (3) illustrates the sensitivity test results for some pathogenic fungi adopted by this study, totaling (6), against which (4) different antibiotics were used.

Table 3. Effect of some antibiotics on fungal species

FUNGI / Antibiotic	Trichophyton mentagrophytes	Trichophyton interdigital	Microspore Canis	Candida albican	Aspergillus flavus	Aspergillus nidulans	average inhibition
Ketoconazole (KT_{50})	30	10	13	25	18	27.5	20.583 A
	15	5	11.5	12.5	9	13.75	11.125 C
Fluconazole (Flc_{10})	14	28	35	11.5	12	10	18.417 B
	7	14	17.5	5.75	6	5	9.208 D
Clotrimazole (CC_{10})	19	14	8	0	19	14	12.333 C
	9.5	7	4	0	9.5	7	6.167 E
Nystation (NS_{50})	0	12	0	0	17	12	6.833 E
	0	6	0	0	8.5	6	3.417 F
متوسط الفطريات	11.813 a	12.000 a	11.125 a	6.844 b	12.375 a	11.906 a	

*Similar lowercase letters horizontally imply no significant difference between them.

**Similar uppercase letters vertically imply no significant difference between them.

Nonetheless, the study's results were in agreement with (Ibrahim and Al-Qureshi, 2022), which found that the alcoholic propolis extract was more effective than the aqueous extract on the tested fungi. This might be due to the high phenolic content of the alcoholic propolis extract, which is attributed to its strong antioxidant and antimicrobial properties. The solubility of propolis in water ranges between 8.74% and 15.61%, indicating its weak solubility in water, which suggests the presence of fewer phenolic compounds and consequently a lower inhibitory effect of the aqueous propolis extract (Vica et al., 2023). The results also showed that the antifungal capability of propolis varies depending on the type of fungi used in the study, as confirmed by (Petruzzi et al., 2020) .

It is believed that the antifungal mechanism of action occurs through programmed cell death via Ras, metacaspase signaling, in addition to inhibiting the expression of several fungal genes involved in pathogenesis, cell adhesion, biofilm formation, and filamentous growth. Pinocembrin, a major component of propolis in temperate regions, reduces levels of phosphorylated adenosine nucleotides from Penicillium filaments and destroys the cell membrane, causing ionic leakage and loss of soluble proteins (Salatino et al., 2022). Based on this, the strongest type of extract was the alcoholic one on:

Candida albicans (A20.10) > A. nidulans (B16.73) > T. mentagrophytes (B16.50) > T. interdigitale (B16.20) > A. flavus (B15.60) > M. cains (BC15.30.(

Meanwhile, the highest average effect recorded by the aqueous propolis extract on the fungi was:

A. flavus (B16.80) > T. mentagrophytes (BC15.15) > T. interdigitalel (C14.33) > C. albicans (C13.88) > M. cains (D10.65) > A. nidulans (E5.93).

The largest inhibition diameter recorded was on fungus T. mentagrophytes, reaching 27.75 mm at a 1% concentration using the alcoholic propolis extract, while the smallest inhibition diameter recorded was 0.0 mm at concentrations of (0.2, 0.4, 0.6%) using the aqueous propolis extract on A. nidulans.

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