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ANTIFUNGAL ACTIVITIES OF HONEY BEES PROPOLIS ETHANOLIC EXTRACT

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ABSTRACT

Two different samples of honey bees propolis collected from strong and moderate bees colonies in Dakahlia Governorate were examined for their antifungal activities. The antifungal activities were tested against five different economic fungi which cause spoilage of food and storing grains. The fungal strains were *Fusarium moniliform* DSM764, *Rhizopus arrhizus* DSM906, *Mucor miehei* DSM1330, *Aspergillus flavus* DSM1830 and *Penicillium camemberti* DSM1995. The chemical analyses of HPLC showed that both tested samples containing volatile cyclic compounds, saturated and unsaturated fatty acids, alcohol, Limonene, alkane, alkene and other compounds. Results of antifungal potentialities showed that both samples of propolis lead to remarkable inhibition of fungal mycelial growth as well as spores germination. The propolis obtained from strong colony was more effective towards the tested fungi than that one of moderate colonies. Measured MICs and MFCs values were 520, 680; 410, 530; 350, 490; 330, 450 and 390, 470 ppm for the tested fungi, respectively, data also showed that the tested propolis samples showed fungicidal effect (MFC) with higher concentration than that of MIC values.

INTRODUCTION

Propolis is used by bees as a glue, a general purpose sealer and a draught excluder in the construction of beehives. Propolis consists primarily of plant exudates gathered by bees and mixed with beeswax, which they secrete, together with small amounts of sugars. The fungal growth on cheese and other fermented dairy products is a problem for cheese manufacture during aging and for consumers during refrigerated storage. Fungi cause major economic problems and some of them are capable to produce toxic metabolites such as *Aspergillus flavus*. Therefore, many authors are looking for some active natural and readily biodegradable products having active antimicrobial activities. Therefore, the world now is returning to the use of natural products both in food processing and in the field of medicine (El-Fadaly *et al.*, 1996). Propolis is the source of the majority of the phenolic compounds present in honey. The ethanolic extract of raw propolis contains the bulk of organic constituents, which is incorporated into medicinal and health food products. It is known that propolis has antiseptic, antimycotic, bacteriostatic, astringent, choleric and anti-inflammatory effects. In addition, it produces allergic contact dermatitis. Besides, ethanolic extracts of propolis have been shown to exhibit antibacterial, antifungal, antiviral and antioxidative activities (Ricardo *et al.*, 2005 and El-Fadaly and El-Badrawy, 2001).

This paper reports on an HPLC survey of the phenolic compounds present in different propolis samples. In addition, a comparison was made of these propolis extract concerning their potentialities towards some fungi that recommended for their contamination of foods either outside or inside refrigerator.

MATERIALS

Propolis samples

Propolis samples were collected from Temey El-Amdied, Dakahlia Governorate in April and May of 2004 – 2005. Three different colonies both strong and moderate colonies were examined. The propolis samples of *Apis mellifera* L. were handly collected by using a plastic propolis trap and were kept desiccated in the dark up to their processing.

Preparation of ehtanolic extract of propolis

To prepare ethanolic extract of propolis (EEP) each samples was first cut into small pieces, air-dried at 40°C for 48 h and ground. The dried propolis samples were powdered (100 g) and exhaustively extracted with ethanol : H_2O, 9 : 1 (v/v) at room temperature by maceration. An aliquot of dried crude propolis (100 g) was dissolved with 250 ml of 80% ethanol by shaking at 150 rpm on 50°C for 3 days and protected from light. The aqueous-ethanol extract was filtered through a Whatman No. 1 filter paper and concentrated at 50°C. The resin obtained was dissolved in 80% ethanol to a final concentration of 10 mg/ml. based on the individual dry weight determined in the solution, the EEP solution was further adjusted with an appropriate amount of 80% ethanol to obtain solutions containing various amounts of EEP. This final solution was employed for the antimicrobial assays.

HPLC analysis of phenolic compounds

Analysis of phenolic compounds was carried out by using HPLC (Thermo Separation Products Inc. 4100 METRIC) with the following conditions: Flow rate, 1 ml/min., detection, UV adsorption at 265 nm, fluorescence Ex: 25 onm. EM: 400 nm, volume of injection, 20 µl, and at room temperature. The mobile phase was a system of ethanol and ammonium acetate buffer, pH 5.4 (12: 88, v/v). Phenolic compounds standards were chromatographed singly and in a mixture.

Microbiological procedure

Fungal strains

The food contaminating fungi used in this investigation were *Fusarium moniliform* DSM 764, *Rhizopus arrhizus* DSM 906, *Mucor miehei* DSM 1330, *Aspergillus flavus* DSM 1830 and *Penicillium camemberti* DSM 1995. These strains were kindly obtained from Fermentation Service Unit, Division of Biochemical Engineering, GBF, Braunshweig, Stockheim, Germany. The choice of these strains was based on their economic and hygienic roles for humanity in nature.

Cultivation media

Potato Dextrose Agar (PDA) was used for maintaining the fungal strains at 5°C till use. For the disc diffusion method linear growth of fungi and determination of minimum fungicidal concentration (MFC), PDA medium was

used. To determine the minimum inhibitory concentrations (MICs) values, a double strength of Czapek-Dox's solution was used. The composition of these media was a described in Oxoid (1982).

Inoculum preparation

The fungi used in this study were grown on slopes of PDA medium at 28°C for 10 days. The culture was then washed with 1% milk solution in water to prepare spores suspension. The latter was diluted further to obtain about $10^4 – 10^5$ spores/ml. One ml after this dilution was then added to 14 ml of melted PDA medium, poured into Petri dishes and incubated at 28°C for 10 days.

Measurements of sporulation density

Spores suspensions were prepared from 10-day-old slope cultures of the fungal strains by flooding with sterile distilled water. The suspension was centrifuged at 2000 rpm for 5 min and obtained supernatant was filtered through 2 layers of sterile cheese cloth. A drop of the resulting spores suspension was placed on a haemocytometer chamber covered with a cover slip and the number of spores/ml of sterile distilled water was estimated as the average of spores counted in 10 large squares sporulation density was calculated as described by (Fokunang *et al.*, 2000).

Determination of fungal spores germination

After holding the spores suspension containing a half value of MIC at room temperature for 10 hr, 1 ml of this suspension was cultivated in PDA medium containing Petri dishes. The plates were incubated at 28°C for 10 days. Percent germination was then determined. data obtained in case of control in which spores not treated with tested extract were adjusted to reflect percentage of viable spores (Johonson and Doyle, 1986).

Inhibition zone measurements

The plate diffusion method was used. In this method, holes with a cork borer were punched in specific cultivation plates seeded with a standard inoculum of 10 days old fungi under aseptic conditions. Five concentrations of tested extracts of 150, 300, 450, 600 and 750 ppm were separately put into the holes, left one hr at 5°C to allow diffusion, then incubated at 28°C for 72 hr. At the end of incubation period, the inhibition zones were measured and recorded. The antifungal activity was expressed in term of the diameter of inhibition zone surrounding the well (Allen *et al.*, 1991). Similarly, the control was prepared with the same solvent free of tested material.

Determination of minimum inhibitory concentrations (MICs)

The MIC value for each representative fungal strain was examined in liquid medium amended with the test extract using a step-wise broth method as described by (Fitzgerald *et al.*, 1992). After 10 days incubation, the test tube in which no growth can be recorded should contain the lowest inhibiting concentration of the tested extracts. Three replicates were prepared for each fungal strain.

Cidal or static action of propolis ethanolic extract

To verify whether the nature of the effect of tested extracts is temporary or permanent, appropriate subculturing from MIC tubes were applied on plates of PDA medium without extract. After incubation period of

10 days, it was possible to determine the minimal fungicidal and/or fungistatic concentration (MFC) (Gardner and Provine, 1984).

Effect of phenolic compounds containing extracts on linear growth of the tested fungi

Discs of 0.6 cm in diameter were cut off the edge of 10 days cultures of the tested fungi and single disc was placed in the center of a Petri dish containing milk agar medium supplemented with the tested extract (MIC/2). Three replicates were prepared for each fungal strains tested, which then incubated at 28°C. The linear growth of tested fungi was recorded after the 10^{th} day. Control devoided of the tested extract for each treatment was conducted (Bollen, 1972).

Effect of phenolic compounds containing extracts on fungal growth on liquid culture

Sterile 50% diluted milk as a natural medium amended with a half value of MIC of each extract were prepared for all fungi tested. The medium was dispensed in 250 ml Erlenmeyer flasks of 50 ml aliquots per each flask in triplicates. Medium was used without additional extracts as control. Each flask was inoculated with a standard inoculum of one disc of 0.6 cm in diameter of 10 days old fungal cultures. The flasks were then statically incubated at 28°C for 10 days after which, the produced mycelial mats were filtered and washed twice with distilled water, dried in an oven at 80°C to a constant weight (Ansari and Shrivastava, 1991).

RESULTS AND DISCUSSION

Phenolic compounds content of propolis ethanolic extract

The values of Rt (min.) and the area (%) of the phenolic compounds of the two tested propolis are shown in Table 1. The values obtained by using HPLC technique revealed that the propolis of strong colony contained 33 compounds while the propolis of the moderate one contained 21 compounds. Both of the two samples contained twelve phenolic acids, Limonene, alkane, alkene, petanoic acid, cyclohexene, Heptanoic acids but in different concentrations as shown in Table 1.

This difference in phenolic compounds between the tested propolis samples of strong and moderate colonies may be due to the botanical sources as described by El-Fadaly and El-Badrawy (2001).

Inhibition of fungal growth

Five different concentration of propolis ethanolic extract containing phenolic compounds against five tested economic fungi. Obtained results were expressed in diameter of inhibition zone as shown in Table 2. Tabulated data show that the antagonistic effect of the ethanolic extract of propolis were gradually increased with the increase of the tested extract concentration. Meanwhile, the propolis of strong colony exhibited appreciative effect higher than that of the moderate colony propolis. Date also showed that *Penicillium camemberti* DSM 1995 was the most sensitive fungus, while *Aspergillus flavus* DSM 1830 was the most resistant fungus in case of the two propolis samples. Generally, this effect proved to be in a positive correlation between the ethanolic extract concentration and the diameter of inhibition zone of fungal growth.

Table 1: Phenolic compounds found in ethanolic extract of honey bees propolis

Rt (min)	Area (%)	Name
Strong colony propolis		
3.90	2.50	Pentanoic acid
7.78	5.76	Hepatnoic acid
14.27	1.43	5-Tetradecene
18.32	7.38	7-Hexadecene
20.77	4.83	Indene-2,4-diphenyl-4-methyl
21.98	6.76	1-pentadecene, 1-octadecene
22.64	7.44	Isomeric dodecyl-benzene Naphthalene-2,5-cyclohexadiene
32.01	9.98	T-butyl perester of hepatnoic acid
25.31	2.66	5-Eicosene-Hexadecanoic acid
33.36	5.91	1,2-Benzenedicarboxylic acid
41.74	2.53	Cyclohexyl-Ethanone
44.37	2.52	3-Nonanone 1,3,2-Diioxaborinane
Moderate colony propolis		
6.29	3.60	Limonne (Cyclohexane)
10.00	3.94	Octanoic acid (Pentanoic acid)
14.38	1165	Decanoic acid
14.26	3.21	Tetradecene
18.31	4.73	Hexadecane-Eicosene
18.40	29.81	Dodecanoic acid
19.85	3.58	Octadecanoic acid
20.78	2.26	Pentene
33.02	8.58	Tetradecanoic acd
23.39	5.74	Decanoic acid
25.34	3.82	Hexadecanoic acid
20.16	2.2	Nonanedioic acid (2 COOH)

Table 2. Antifungal potentialities of ethanolic extract of honey bees propolis collected from strong and moderate colonies

EEP conc. (ppm)	Tested fungal strains Mean diameter of inhibition zone (mm)									
	***Fusarium moniliform* DSM 764**		***Rhizopus arrhizus* DSM 906**		***Mucor miehei* DSM 1330**		***Aspergillus flavus* DSM 1830**		***Penicillium camemberti* DSM 1995**	
	S	**M**	**S**	**M**	**S**	**M**	**S**	**M**	**S**	**M**
0.0	0	0	0	0	0	0	0	0	0	0
150	10.5	8.2	18.9	12.6	10.2	6.2	7.3	5.2	6.7	4.4
300	17.3	11.6	26.3	21.9	11.6	8.4	10.6	8.3	9.3	6.6
450	22.8	17.3	32.5	27.3	18.4	13.3	14.5	11.4	15.6	11.2
600	28.9	21.4	39.2	33.5	20.7	16.2	22.2	16.8	21.2	17.5
750	36.6	29.8	42.9	39.2	26.9	22.5	24.7	19.7	27.4	22.8

S = Strong colonies M = Moderate colonies

Effect of propolis extract on fungal spores germination

The same concentrations of the ethanolic extract of propolis were also used to examine their effect on the fungal spores germination. Obtained results are shown in Table 3. Negative relation was found between the ethanolic extract of propolis concentration and the fungal spores germination. Tabulated data also showed that the extract of strong colony propolis gave higher ratio of spores germination than that one of moderate colony propolis.

Table 3. Antifungal potentialities of ethanolic extract of honey bees propolis collected from strong and moderate colonies by means of spore germination.

EEP conc. (ppm)	Spore germination (%)									
	Fusarium moniliform DSM 764		*Rhizopus arrhizus* DSM 906		*Mucor miehei* DSM 1330		*Aspergillus flavus* DSM 1830		*Penicillium camemberti* DSM 1995	
	S	M	S	M	S	M	S	M	S	M
0.0	100	100	100	100	100	100	100	100	100	100
150	90	93	92	96	86	90	85	89	75	81
300	86	89	79	84	79	81	78	82	64	69
450	74	79	65	76	68	72	62	69	51	57
600	61	66	49	54	58	64	51	59	44	49
750	49	53	35	43	45	51	39	44	38	41

S = Strong colonies M = Moderate colonies

Values of the minimal inhibitory concentration (MIC)

Data in Table 4 show more precisely the inhibitory effect of propolis extract by means of MIC values expressed in ppm. Results in Table 4 represent in lowest concentration of the phenolic compounds containing extract, which is capable of totally inhibiting of fungal growth. Data exhibited that the five tested fungal strains; *Fusarium moniliform* DSM 764, *Rhizopus arrhizus* DSM 906, *Mucor miehei* DSM 1330, *Aspergillus flavus* DSM 1830 and *Penicillium camemberti* DSM 1995 have the same susceptibility towards both examined extracts. This means that the effectiveness of each extracts of the two propolis samples appear to be not similar for all fungal strains. It could be stated that the inhibitory effect of these extracts may be due to the presence of native components mainly phenolic compounds. Fleming *et al.* (1973) reported that large quantities of phenols exhibited antimicrobial activity.

Fungicidal or fungistatic effect (MFC)

Data recorded in Table 4 concerning the minimal fungicidal and/or fungistatic effect, indicate that the propolis extract show a fungistatic effect against the five tested fungi. This effect was clear up to 390, 470 ppm for the strong and moderate colonies propolis samples, respectively. The fungi exhibited growth again when recultivated on a medium containing no extract. On the other hand, fungicidal effect was observed in case of propolis and this effect was found up to concentration of 450, 550 ppm for the strong and moderate colonies samples, respectively. As can be seen from the Table 4, the fungal growth was not detected after reseeding the MIC tubes on the plates containing propolis extract-free medium.

The explanation of the first phenomenon is that the concentration of propolis extract inhibit the growth, but after that the fungi can persists and resume again the growth once the agent is removed. On the other hand, data reveal that the extract of propolis kills the fungi, therefore, they have an irreversible and permanent effect at high concentrations. Moreover, it could be noticed that the concentration of phenolic compounds needed to cause fungicidal effect is higher than that of MIC value for the same fungi. Helena and Pre-Anders (1993) reported hat the measuring of MICs and MFCs values lead to detect the reveal effect of tested material *in vivo* against the micro-organisms.

Diameter of inhibition zone of MICs

For more evaluation of the biological activities of propolis, the MICs obtained from liquid cultivation were subjected in solid culture for measuring the inhibition zones. Obtained results are also listed in Table 4. Both tested propolis extracts exhibited pronounced antagonistic effect against the five tested fungi. Meanwhile, the strong colony propolis extract containing phenolic compounds showed higher inhibition effect than that of the same extract of moderate propolis. The fungus *Aspergillus flavus* DSM 1830 exhibited more resistance against the two examined propolis extracts, since the diameter of inhibition zones were 3.3 and 1.7 mm for the strong and moderate samples, respectively. On the other hand, *Fusarium moniliform* 764 showed to be the most sensitive fungus towards both examined extracts. Paster *et al.* (1988) stated that the presence of phenolic to the extent of being non-detectable. Regarding to the infusibility of the tested extracts through the solid medium, it can cause some disruption in the permeability of the outer membrane of the bacterial cell wall (Delves *et al.*, 1992). The inhibition results of propolis extract using agar diffusion method depend on the type of microbe as well as the botanical source of propolis (Hegazi *et al.*, 1997).

Table 4. Values of minimal inhibitory concentration (MIC) with minimal fungicidal concentration (MFC) and corresponding diameter of inhibition zone (DIZ) of ethanolic extract of honey bees propolis collected from strong and moderate colonies

Tested fungal strains	values of					
	MIC (ppm)		MFC (ppm)		DIZ (mm)	
	S	M	S	M	S	M
***Fusarium moniliform* DSM 764**	520	680	730	820	5.2	3.3
***Rhizopus arrhizus* DSM 906**	410	530	620	760	4.1	2.2
***Mucor miehei* DSM 1330**	350	490	550	680	3.5	1.9
***Aspergillus flavus* DSM 1830**	330	450	480	610	3.3	1.7
***Penicillium camemberti* DSM 1995**	390	470	450	550	3.9	2.2

S = Strong colonies M = Moderate colonies
MIC = minimal inhibitory concentration

Inhibitory effect on fungal growth on solid medium

Following the linear growth of the tested fungi, the inhibitory effect of the propolis extract containing phenolic compounds on solid medium supplemented with half value of MIC after 10 days of incubation was examined. Interestingly, all the five fungi showed similar growth starting point, since they exhibited no growth up to the 3rd day of incubation. The delaying of

growth may be due to the presence of the propolis ethanolic extract, which explained as an adaptation period required for the microbial growth in its micro-environment containing antigrowth agent. After this period, poor vegetative growth was observed and normally went further up to the 10th day to give measurable growth. The treated dishes together with the control were investigated and obtained results are listed in Table 5.

The antimicrobial activity of the propolis extract is varied from microbe to another according to the different botanical origin. This is of course based on the plants from which was collected by honey bees, time, and place, consequently the constituents of obtained propolis (Kujumgiev *et al.*, 1999).

Efficiency of phenolic compounds containing extract on fungal growth in liquid medium

Great effect of the extracts was noticed on the fungi grown on liquid cultures containing half value of MIC and obtained results are shown in Table 5. Data revealed that both strong and moderate propolis extracts caused reduction in mycelial yields as well as radial growth of the five tested fungi. Results showed also that the effectiveness of these phenolic compounds containing extracts on fungal growth appear not the same with each fungus tested. With the differentiation of the reduction percentages obtained either on solid medium or in liquid one, the same order of relative sensitivity was observed.

Lastly, it could be noted that the ethanolic extracts of propolis are good inhibitors either as static factor or as fungicidal agent for different fungi, which contaminate foods. Since the risk of residues left in food stuffs limits the use of antibiotic agents, only a restricted number of chemicals (non-antibiotic drugs) have been approved now as widely accepted safe materials (Helena and Per-Anders, 1993). The same problem has been discussed in the case of storage of food especially milk and dairy products because of the need to use preservative chemicals to prolong the storage period. Consequently, efforts have been made to evaluate the preservative actions of natural substances, e.g. plant constituents or plant extracts of spices or herbs, as well as, honey bees propolis extracts.

El-Dieb and Abd El-Fattah (1997) examined the potentialities of ethanolic extract of propolis against *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremories*, *Streptococcus salvarious* subsp. *thermophilus*, *Micrococcus* sp. and *Staphylococcus aureus*. They also examined the activity against *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus versicolor*. They added that using propolis on the surface of ras cheese prevents mold and bacterial growth without affecting cheese quality. In addition, Bankova *et al.* (1995) pointed out that propolis has been extensively used in food and beverage to improve health and prevent disease, such as inflammation, heart disease, diabetes and even cancer. So, these results can suggest the possibility to sue honey bee propolis as an additive in food processing after obtaining high purity antimicrobial substances.

Table 5. Efficiency of fungal growth inhibition by ethanolic extract of honey bees propolis collected from strong and moderate colonies after 10 days of incubation at 28°C

Tested fungal strains		Radial growth diameter (mm) on PDA medium		Dry weight, mg/100 ml of Czapek-Dox medium	
		S	M	S	M
Fusarium moniliform **DSM 764**	**Control**	9.20	9.20	312	312
	Treated	4.20	5.10	148	160
	%	54.35	44.57	52.56	48.72
Rhizopus arrhizus **DSM 906**	**Control**	8.50	8.50	286	286
	Treated	6.30	6.90	205	220
	%	25.89	18.82	28.32	23.08
Mucor miehei **DSM 1330**	**Control**	7.90	7.90	274	274
	Treated	5.60	5.90	180	195
	%	29.11	25.32	34.31	28.83
Aspergillus flavus **DSM 1830**	**Control**	10.40	10.40	364	364
	Treated	7.30	7.80	250	265
	%	29.81	25.00	31.32	27.2
Penicillium camemberti **DSM 1995**	**Control**	12.40	12.40	410	410
	Treated	8.60	9.00	250	270
	%	30.65	27.42	39.02	34.15

S = Strong colonies M = Moderate colonies

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النشاط المضاد للفطريات لمستخلص الإيثانول لبروبوليس نحل العسل

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تم تجميع عينتان من البروبوليس من كل من الخلايا القوية وكذلك الخلايا المتوسطة من مركز تمى الأمديد بمحافظة الدقهلية وذلك لاختبار المقدرة التثبيطية بالنسبة للفطريات. ولقد تمت الدراسة على خمس فطريات إقتصادية والتى تسبب فساد الأغذية والحبوب المخزنة وهذه الفطريات هى:

Fusarium moniliform DSM764, *Rhizopus arrhizus* DSM906, *Mucor miehei* DSM1330, *Aspergillus flavus* DSM1830 and *Penicillium camemberti* DSM1995

ولقد أوضحت النتائج الخاصة بالتحليل الكيماوى باستخدام تكنيك HPLC أن كلا من العينيتين تحت الدراسة تحتوى على مركبات حلقية طيارة ، أحماض دهنية مشبعة وغير مشبعة ، كحولات ، ليمونين والكان والكين ومركبات أخرى. كذلك أوضحت النتائج الخاصة بالمقدرة التثبيطية أن كلا من عينات البروبوليس المستخلصة بالإيثانول قد أدت إلى تثبيط واضح سواء فى نمو الميسيليوم الفطرى أو فى إنبات الجراثيم الفطرية. كذلك أشارت النتائج أن المستخلص الإيثانولى لبروبوليس الخلايا القوية كان أكثر تاثيراً ضد الفطريات المختبرة من مثيله الناتج من الخلايا المتوسطة.

ولقد كانت قيم أقل تركيز تثبيطى وأقل تركيز قاتل هى: ٥٢٠ ، ٦٨٠-٤١٠ ، ٥٣٠-٣٥٠ ، ٤٩٠-٣٣٠ ، ٤٥٠-٣٩٠ ، ٤٧٠ جزء فى المليون للفطريات المختبرة على الترتيب. أوضحت النتائج كذلك أن مستخلص البروبوليس المختبر له تاثير قاتل على الفطريات (MFC) قيد الدراسة ولكن بتركيزات أعلى من تلك اللازمة للتركيز التثبيطى (MIC). وتتمخض هذه الدراسة عن إمكانية إستخدام البروبوليس كمادة طبيعية فالتثبيط الحيوى للفطريات الملوثة للأغذية وللحبوب المخزنة والتى ليس لها نتائج جانبية ضارة.