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Flavonoids of Propolis and Their Antibacterial Activities

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Abstract: Honey bees propolis is a sticky amalgamation of plant resins collected by *Apis mellifera* L. and is used for filling cracks and repairing combs in hives. Propolis contains a diversity of compounds of plant origin and is reported to have medicinal antimicrobial insecticidal and phytotoxic properties. Flavonoids and other phenolic compounds were determined in two samples of propolis originating from Egypt and Yemen. Flavonoids were identified using mainly HPLC and a few other analytical methods. The most abundant flavonoids found were chrysin, galangiu, pinocembrin and pinobanksin. The bacterial strains used are *E. coli, Staphylococcus aureus, Brucella abortus, Shigella dysenteriae* and *Salmonella typhimurium.* The obtained results show different reactions of examined propolis extracts against tested bacterial strains. Gram negative bacteria were more resistant against the examined flavonoids containing extracts than the bacterial strain belonging to Gram positive group.

Key words: Phenolic acids, Flavonoids, Honey bees propolis, Pathostenic bacteria, antibacterial activities

Introduction

Propolis is a natural product, made and used by bees to tighten and seal their hives to protect against diseases. Propolis possesses a pleasant aromatic smell and varies in color depending on its source and age. The composition of the propolis depends on the place and time of collection. As a consequence, more than 160 constituents have been identified so far, among which phenolic compounds, including flavonoids are major constituents (Bankova *et al*., 1996). Various biological activities such as. anticancer, antioxidant, antiinflammatory, antibiotic and antifungal effects have been reported for propolis and its constituents (Marcucci, 1995).

The activity of ethyl alcohol extract of Egyptian propolis was examined against *Staphylococcus aureus* by Ahmed *et al*. (1997). They found that the mixture of methyl alcohol, ether, acetone and chloroform had the highest strength of extracting biologically active substances from propolis. Ethanolic extract of Egyptian propolis was investigated against *Staphylococcus aureus, Streptococcus faecalis, Corynebacterium* sp., *Pseudo-tuberculosis* sp., *Aeromonas hydrophila, E. coil, Salmonella typhimurium, Aspergillus niger* and *Candida albicans* (Hegazi *et al*., 1997). El-Dieb (1998) examined the ethanolic extract of propolis against *E. coli* and *Staphylococcus aureus.* Bankova *et al*. (1995) investigated the chemical composition and antibacterial activity by some of the propolis fractions. Propolis samples from different geographic origin were investigated for their antimicrobial activities, *Staphylococcus aureus, E. coli* and *Candida albicans* (Kujumgiev *et al*., 1999). The aim of the present investigation is to examine the effect of phenolics extract of the two different types of bees propolis against food-containing bacteria which have medical importance for humans.

Materials and Methods

Propolis samples: Two types of natural beehive product were used as sources for antibacterial agents. The first is the Egyptian propolis collected in Ctalubyia province (*Apis mellifera* L.). The second is the Yemeni propolis (*Acacia mellifera benth.*) provided by Dr. Fayza A. Saleh, Plant Protection Dept., Naser Faculty of Agriculture, Aden University, Yemen. The samples were kept desiccated pending its processing.

Preparation of propolis extract: Propolis was diluted in 2 M NaOH under nitrogen to obtain the flavonoid extract, as previously described by Berahia *et al*. (1993). Alkaline treatment was carried out for 4 hours to hydrolyze cinnamic esters. The reaction was stopped by acidification to pH 2 with 4 M Hp. Phenolic acids and flavonoids were extracted with ethyl acetate (v/v, 1/1) three times. The three organic phases were collected and ethyl acetate was evaporated under vacuum and 40°C. The flavonoid extracts were made up to 100 ml with ethanol (grade HPLC).

HPLC Analysis of phenolics were carried out by using a diode array detector according to Sabatier *et al*. (1992), in the Institute of Animal Nutrition, Federal Agriculture Research Center, Braunschweig, Germany.

Separation of phenolic compounds by using paper chromatography: The ethanol extract was concentrated and aliquots chromatographed one dimensionally on two sheets of Whatman No. 3 paper chromatography, alongside authentic markers in the solvent system, BAW (n-butanol-acetic acid-water, 4:1:5, top layer). After development, the chromatogram was air-dried, exposed to ammonia vapour and detected by UV light (Markham and Chari, 1982).

Mass spectrometry: The analysis of phenolic acids was carried out by using EI + DIMS LMR UP LR apparatus in central service laboratory, National research center, Cairo, Egypt.

Bacterial strains and cultivation media: The bacterial strains were from the Hungarian National Collection of Medical Bacteria, OKI, BP, Hungary. These strains were selected for their economic and medical importance.

For maintaining and counting the bacterial strains used in this study, MacConkey's agar medium was used for *E. coli* at 37°C for 24 hr Brilliant green agar medium was used for *Shigella dysenteriae* and *Salmonella typhimurium* at 37°C for 24 hr. For *Brucella abortus,* serum dextrose agar with pH 7.5 at 37°C for 24 hr was used. *Staphylococcus* medium No. 110 was used for *Staphylococcus aureus.* Tryptone Soya Broth (TSB) was used for determination of the minimum inhibitory concentration (MIC) and Tryptone

Soya Agar (TSA) for the agar diffusion experiments. The composition of these media were as described in Oxoid Ltd (1982).

Assessment of antibacterial activity:

Determination of minimum inhibitory concentrations (MICs): The MIC value for each representative bacterial strain was examined in a specific liquid medium amended with the test extract using a tube dilution technique as described by Simpson and Smith (1992). After the incubation period, the test tube in which no growth can be recorded contained the lowest inhibiting concentration of the tested extract. Three replicates were prepared for each bacterial strain.

Measurement of inhibition zone: Holes were punched with a cork borer (6 mm) in plates of TSA freshly seeded with 1 ml of 24 hr old bacterial suspension cultured in specific media mentioned above. The holes were then filled with the tested extracts. The plates were kept at 5°C for one hr to allow diffusion of the extract through the agar media. The incubation was carried out for 24 hr at 37°C. The diameter of clear zones surrounding the wells were measured and recorded expressing the antibacterial activity (Toda *et al*., 1989). Similarly, the control prepared with the same solvent (free of tested material).

Detection of the nature of the effect: Appropriate subculturing from MIC tubes were applied on plates of the same used media without addition of tested extract. After the incubation period, it was possible to determine the minimal bactericidal and/or bacteriostatic concentration (MBC) as mentioned by Gardner and Provino (1984).

Effect of propolis extract on bacterial growth pattern in liquid culture: Specific liquid media amended with half value of MIC of each extract were prepared for each bacterial strain. Each cultivation medium was dispensed in 250 ml Erlenmeyer flasks of 50 ml aliquots per each flask. Cultivation media were used without addition extract as control. Each flask was inoculated with 1 ml of 24 hr old culture. The incubation was statically carried out at 37°C. During 20 hr incubation, the growth was measured by plating appropriate dilution of each flask using a specific culture medium for counting the viable cells expressed in colony forming unit (cfu/ml) after 4, 8, 12, 16 and 20 hr.

Results and Discussion

Identification of phenolic compounds of propolis samples: Phenolic compounds extracted from Egyptian and Yemeni propolis were 9.8 and 13.6%, respectively. The results revealed that the Yemeni propolis contains phenolic acids more than the Egyptian one where it contains caffeic acid as a major component followed by coumaric and cinnamic acids. The Egyptian propolis contains only caffeic and coumaric acids. Their Rf values (×100) were 95, 79 and 93 for cinnamic, caffeic and coumaric acids, respectively. Identification of these acids was also confirmed by mass spectrometry and mass spectrometric data are recorded in Table 1.

Table 1: Mass spectroscopic data for phenolic acids of propolis

m/e (relative abundance %)	Compound
148 (M 65.1); 104 (64.8); 78 (27.3)	Cinnamic
180 (M 100) ;163 (16.2); 134 (32.5); 107 (8.4); 77(23.7)	Caffeic
164 (M14.1)1; 121 (34.8); 93 (25.5); 74 (14.9)	Coumaric

The flavonoids identified by HPLC are listed in Table 2. These are chrysin, pinocembrin and acacetin in the Yemeni propolis, where chrysin, galangin, pinobanksin and quercetin were found in the Egyptian propolis.

Table 2: Phenolic compounds extracted from propolis samples

Phenolic compounds	Extract of	
	Egyptian propolis	Yemeni propolis
Cinnamic acid	-	+
Caffeic acid	+	+
Coumaric acid	+	+
Chrysin	+	+
Galangin	+	-
Pinocembrin	-	+
Pinobanksin	+	-
Acacetin	-	+
Duercetin	+	-

MIC values and MBC of propolis extract: Results in Table 3 show the values of minimum inhibitory concentrations (MICs), the diameter of growth inhibition zones (DIZs) as well as the minimum bactericidal concentrations (MBCs). More precisely the inhibiting effect of propolis extracts shown in Table 3 by means of MICs values expressed in pg/ml. Recorded values are the lowest concentrations of honey-bees propolis extracts which capable to inhibit the growth of food-contaminating bacteria. Different responses are observed from tested bacteria towards the extract of propolis samples.

The values of MIC show that the Gram-positive bacterium *Staphylococcus aureus* shows to be more sensitive towards the tested extracts of both two types of honey-bees propolis than the others of Gram-negative bacteria. On the other hand, Gram-negative bacteria exhibit different behaviour against the extract of examined propolis. *Brucella abortus* is most sensitive (800 μg/ml) than the others which show the same tolerance degree against the tested extract and 1000 μg/ml for Egyptian and Yemeni propolis, respectively. Ahmed *et al*. (1997) found that Gram-positive bacterium *Staphylococcus aureus* was the most sensitive against propolis extract amongst Gram-positive, Gram-negative and examined yeast strains. They found that MIC value of Egyptian propolis was 500 μg/ml.

For more evaluation, the biological activities of propolis, the MICs obtained from liquid cultivation were subjected in solid culture for measuring the inhibition zones. The diameter of growth inhibition zones caused by tested propolis extract were measured and obtained results are also recorded in Table 3. Data show that both extracts of the Egyptian and Yemeni propolis have marked antagonistic action against all the bacterial strains tested. Tabulated results of inhibition zone illustrate the superiority of the extract of Egyptian propolis over the sample of Yemeni propolis. Based on the bacterial sensitivity, *Staphylococcus aureus* comes in the first order followed by *Brucella Salmonella typhimurium* come last. According to the diffusibility 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-Broughton *et al*., 1992). The inhibition results of propolis extract using agar diffusion method depend on the type of pathogen as well as the geographic zone (Hegazi *et al*., 1997).

The minimum bactericidal concentrations (MBCs) were determined to show whether the observed inhibition of bacterial growth is bactericidal or bacteriostatic. However, each strain was tested and subcultured again on free of

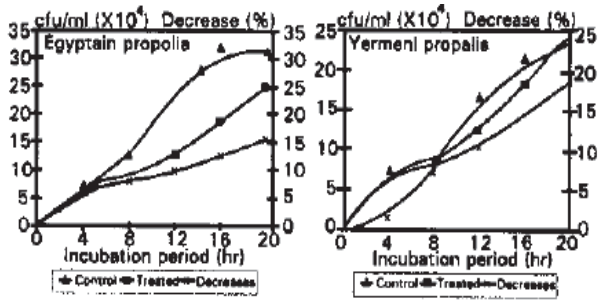

Fig. 1: Effect of the extract of honey bees propolis on the growth pattern of *Escherichia coil*

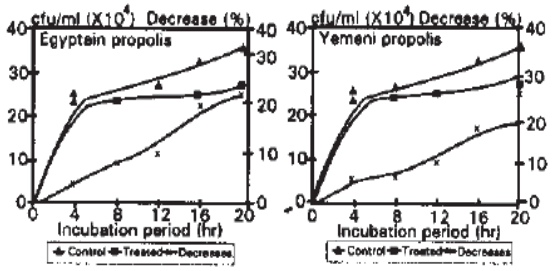

Fig. 2: Effect of the extract of honey bees propolis on the growth pattern of *Staphylococcus aureus*

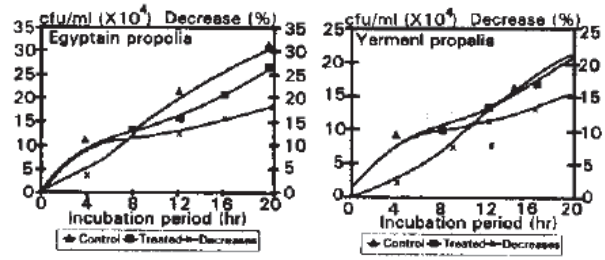

Fig. 3: Effect of the extract of honey bees propolis on the growth pattern of *Brucella abortus*

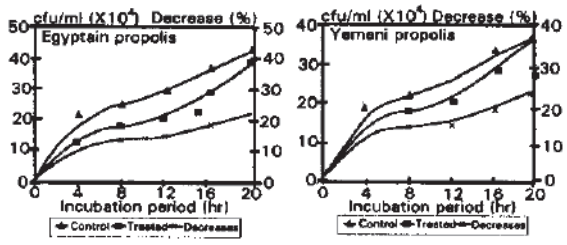

Fig. 4: Effect of the extract of honey bees propolis on the growth pattern of *Shigella dysenteriae*

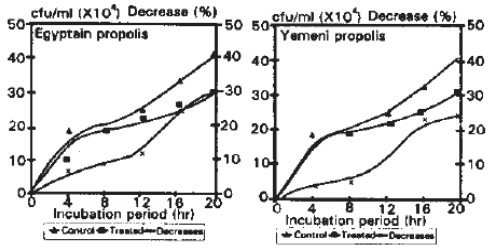

Fig. 5: Effect of the extract of honey bees propolis on the growth pattern of *Salmonella typhimurium*

propolis extract medium and obtained results recorded in *abortus, E. call* then both *Shigella dysenteriae* and Table 3. From the sign of MBC, the extract of Egyptian propolis exhibit bactericidal action against all tested bacteria. Meanwhile, the bacteriostatic effect was observed in case of the extract of Yemeni propolis. The static action of the extract against the microorganisms could be explained by the inhibition at MIC level and after that the microorganisms can persist and resume the growth again once the agent is removed in spite of the value of MIC of each strain tested. Data revealed that the extract, in case of microbicidal effect, kill the microorganisms and therefore, have an irreversible and permanent effect. The philosophy of measuring both MICs and MBCs values that are expressed in numbers, lead to detect the real effect of the extract *in vivo* against the bacterial cells as reported by Cederlund and Mardh (1993). The values of MIC required for killing G^+ are less than that of Gram-negative. Hegazi *et al.* (1997) found that the extract of propolis showed pronounced antimicrobial activity against G^+ bacteria more than G^+ bacteria. The bactericidal effect of Egyptian propolis extract may ascribed to the presence of galangin, pinobanksin and quercetin as well. Toda *et al*. (1989) observed inhibition activity of Caffeic acid of tea and cofee against bacterial growth of 24 strains of pathogens causing diarrhoeal diseases.

Effect of propolis extract on bacterial growth pattern: The effect of propolis extract on food-contaminating bacteria was examined on specific liquid culture medium amended with half value of MIC of each bacterial strain. The same cultivation media were used without supplementation as control and obtained results were plotted in (Fig. 1-5). Appreciative effect was found on tested bacteria considering the colony forming unit (cfu/ml) compared to the value of corresponding control. Figure 1 show the results of the effect of propolis extract on growth pattern of *Escherichia tali* along the incubation period of 20 hr. Figured data illustrate that the decrease percent was 31.1 and 23.8% after 20 hr by Egyptian and Yemeni propolis, respectively. The inhibition percent of *Staphylococcus aureus* was 25.4 and 18.9% (Fig. 2) in case of Egyptian and Yemeni propolis, respectively. For *Brucella abortus,* these values were 31.3% caused by the Egyptian propolis, while it was 27.2% in case of Yemeni propolis after 20 hr incubation period (Fig. 3). *Shigella dysenteriae* exhibited 42.6% decrease percent with Egyptian propolis, while it was 37.8 caused by Yemeni propolis extract (Fig. 4). After 20 hr incubation, results in Fig. 5 show 29.9% decrease percent by Egyptian propolis extract for *Salmonella typhimurium* while this value was 24.3% in case of Yemeni propolis extract. From plotted data in Fig. 1-5 it could be concluded that the order of relative resistance of bacterial strains against the extract of Egyptian propolis was as follows, *Shigella dysenteriae, E. coil, Brucella abortus, Salmonella typhimurium* and *Staphylococcus aureus.* The relative sensitivity of tested bacteria towards the extract of Yemeni propolis was *Staphylococcus aureus, E. coil, Salmonella typhimurium, Brucella abortus* and *Shigella dysenteriae.*

Staphylococcus aureus is the commonest cause of pyogenic infections of man and food-poisoning as well. *Salmonella, E. coil* and *Shigella* cause progressively mounting fever, headache and severe malaise and diarrhea.

Table 3: Measured values of minimum inhibitory concentrations and corresponding values of diameter of growth inhibition zones of food-contaminating bacteria as affected by propolis extract

Tested Bacterial Strains	Phenolic compotimds of extract of					
	Egyptian propolis			Yemeni propolis		
	MIC	MBC	DIZ	MIC	MBC	DIZ
Escherichia coli	850	-	19.20	1000	+	17.60
Staphylococcus aureus	750	-	25.50	850	+	20.30
Brucella abortus	800	-	22.90	950	+	18.40
Shigeo dysenteriae	850	-	18.50	1000	+	16.50
Salmonella typhimurium	850	-	18.60	1000	+	16.60

MIC: Minimum inhibitory concentration (µg/ml), = MBC: Minimum bactericIdal concentraction (+: growth, -: No growth) = DIZ: Diameter of growth inhibition zone (mm)

Brucella abortus is well known by Brucellosis and abortion. The appreciative effect of Egyptian propolis extract may be due to the presence of phenolic compounds such as galangin, pinobanksin and quercetin. These compounds which not detected in the Yemeni propolis samples. The importance of these natural'compounds is the possibility to use it as preservative in different foodstuff and in prolongation of shelf-life period. Food contamination by some pathogens, which have the potential to cause some problems, such as diarrhea! diseases have been, documented (Kornacki and Marth, 1982). The same health symptoms can be caused by different contaminated foods by enteric *E. coil* like meat, fish, milk and dairy products. Lower incidence of enteric pathogens in soft ripened cheese sample associated with diarrheal symptoms was detected by Fantasia *et al*. (1975). Such these natural compounds are of interest as a source of safer or more effective substitution for synthetically produced materials as reported by Heisey and Gorham (1992).

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