Study on the antimicrobial activity of honey products and some Saudi Folkloric substances

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Abstract

Alcoholic and aqueous extracts from 17 Saudi Arabia folkloric substances were screened for antimicrobial against some tested pathogenic microorganisms by zone of inhibition assay and minimal inhibitory concentration (MIC). Compared to honey and bee wax, ethanolic extract of propolis showed the highest zone of inhibition (23mm) against S. aureus ATCC255923. Aqueous extract of alum (Ming Fan) was highly effective against all tested strains with inhibition zones ranging from 25 to 29 mm followed by Juglans regia (28mm) with S. epidermis ATCC12228, Rhazya stricta (24mm) with Str. pyogenes ATCC19615, and Commiphora myrrha (24mm) with S. aureus ATCC255923. The combined effect of equimixture of ethanolic extracts of propolis and bee wax was more effective than a single extract showing 1.5 fold increase of inhibition zone against S. aureus ATCC255923 and C. albicans NCTC2708. Substances with the most consistent action against microorganisms were tested to determine their minimum inhibitory concentration (MIC). Alum (Ming Fan) was found to have the greatest activity with MIC mean value of 0.29% (w/v) followed by Rhazya stricta, Juglans regia and propolis with mean MIC values of 0.4, 2.66 and 3.75 respectively.

Keyword: Minimal inhibitory concentration (MIC), folkloric substances, propolis, alcoholic extract, aqueous extract.

Introduction

In developed countries, traditional, complementary and alternative medicine is becoming more popular. For example, the percentage of the population that has used such medicine at least once is 48% in Australia, 31% in Belgium, 70% in Canada, 49% in France and 42% in the United States of America [42]. In Saudi Arabia different kinds of herbs are available; many species of these herbs are used directly in human food or as medicine, such as *Zingiber officinale, Thymus capitatus, Crocus stiva, Nigella sativa, Zizyphus spina Christi, Coriandrum sativum, Mentha piperita, Commiphora myrrha* etc. In addition, honey products, natural gums and alum are known as folkloric medicines[1,2,3,8].

Honey is the natural sweet substance produced by honey bees from nectar or blossoms from the secretion of living parts of plants or excretions of plants which honey bees collect, transform and combine with specific substances of their own to ripen and mature [13]. One of the most important features of honey is that it can be kept for a long period of time without becoming spoiled [40]. Honey is usually contaminated with numerous microorganisms [26]. In honey, aerobic *Bacillus* as well anaerobic *Clostridium* spores and small fragments of moulds may appear [12]. Osmophilic yeast such as *Saccharomyces, Schizosasaccharomyces* and *Torula* predominate. This reservoir for microbes status however does not diminish the many important uses that honey is known for. In fact the antimicrobial status of honey is giving it a continued place in the management of wounds and injuries [29].

Propolis is a resinous hive product collected by bees from tree buds and mixed with secreted bees wax. The propolis is used by the bees as a glue to seal the opening of the hives [23]. Propolis known in folk medicine since ancient times, has attracted much attention in recent years as a useful ingredient applied in medicine and food products [9]. It is known that the ethanolic extract of propolis exhibits various pharmacological activities such as antimicrobial, antiviral, antifungal and anti inflammatory properties [9, 31].

Bee wax is also a product of bees, secreted from the wax gland of bee workers. It is a mixture of esters, fatty acids, higher alcohols and saturated hydrocarbons in addition to aromatic substances and pigments. Zanoschi et al[42] reported the use of bee wax together with other products for burns treatment. Since ancient time herbs and oleo-gum resins such as gum myrrh were widely used in unprocessed form for fragrance and in folk medicines. They have been used in a number of medicinal contexts since long time and still today in several countries across Europe, India, Africa, China and Middle East. Furthermore, they continued to find modern pharmacological applications most of them as claimed by traditional therapies [25, 28].

Material and Methods

Test Microorganisms: The pathogenic standard strains used as test organisms were *Staphylococcus aureus* (ATCC25923), *Staphylococcus epidermidis* (ATCC12228), *Streptococcus pyogenes* (ATCC19615), *Escherichia coli* (ATCC25922), *Proteus mirabilis* (ATCC14153), *Salmonella typhimurium* (ATCC14028), *Pseudomonas aeruginosa* (ATCC27853), *Bacillus subtilus* (ATCC27853) *and Candida albicans* (NCTC2708). They were obtained from the Department of Microbiology, Faculty of Science, Alexandria University, Egypt. Bacterial strains were maintained on nutrient broth and nutrient agar (Oxiod Ltd.)

while *Candida albicans* was cultivated on Sabouraud provided with 2% (ml /v) glucose agar under aerobic conditions for 48 hr at 37˚C.

Tested substances: *Rhazya stricta, Nigella sativa, Sambucus nigra, Commiphora myrrh, Zingiber officinale, Zizyphus spina Christi, Ferula asafoetida, Eugenia caryophyllus, Cuminum cyminum,, Juglans regia*, Arabic gum, Alum (Ming Fan), *Ricinus communis* and *Nasturtium officinails*.

Preparation of tested Folkloric substances extracts: Tested Folkloric materials were collected from Al AHSA local market , air-dried and ground into fine powder by a Braun Multi – Mill and passed through a sieve (24-mesh)[22]. 5 g of finely ground dried samples were extracted with adequate amount of water to a concentration of 12.5% (w/v) and then mixed in a blender. The extracts were filtered through Whatmann filter paper no. 1 to remove large particles and the extracts were passed through a 0.2 µm filter at room temperature and stored at 4°C until used in microbial assay.

Honey sample: Honey samples collected from Al AHSA local market were stored in tightly closed glass containers wrapped in aluminum foil and kept at room temperature. The honey solutions were prepared just before use to ensure that there was no loss of hydrogen peroxide. Sample of 10 g of honey was added to 10 ml distilled water and mixed to achieve 50% (w/v) solution [5].

Propolis sample: The propolis samples were kindly provided from a local apiary in Al AHSA region, Saudi Arabia. Propolis specimens were further dehydrated with a low vacuum pump and the extracts of dried propolis were prepared as described by Koo and Park[21]. The dried propolis were ground into a fine powder and 2g of the propolis powder was mixed with 25 ml of 80% ethanol in plastic centrifuge tube (LXG-50-C) and shaken at 70˚C for 30 min. After extraction, the mixtures were centrifuged to obtain the supernatants which were designated as ethanolic extracts of propolis (EEP).

Bee wax: Sample of crude bee wax was thoroughly washed using distilled water, dried in open air, then broken down, and extracted with 70% ethanol for 48hr at 37˚C using a shaker (150 rpm). The ethanol sample was filtered through Whatmann no.1 filter paper. The solvent was removed under reduced pressure at 40˚C using rotary evaporator [6].

Microbiological analysis of honey, propolis and bee wax: A volume of Seder honey sample was diluted with distilled water and stirred to achieve 50% (w/v) concentration. Serial decimal dilutions were prepared in duplicate, then 1 or 0.1 ml samples of appropriate dilutions were poured on agar plates. Mesophilic aerobic bacteria were counted on plate count agar (Merck) incubated for 48h at 35-37 °C. Coli forms were enumerated after plating on Violet Red Bile (Merck) agar plates with a cover layer of the same medium and incubated for 20-24h at 37°C. Yeast colonies were inoculated on Sabouraud agar medium (Merck) and incubated for 24-48 h at 30 °C.

Determination of antimicrobial activity: Antimicrobial activity of honey products and herbs extracts was evaluated by agar diffusion method [20]. 100 µl of diluted bacterial suspension (5x106 CFU/ ml) were spread onto the surface of plate count agar medium (PCA). Wells (0.6 mm in diameter) were cut from the agar with a sterile cork borer. Then 100 µl of honey products and/ or herbs extract were added to each well. Ethyl alcohol and water were used as negative control in all experiments. Plates were then incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the clear inhibition zone [expressed in millimeters (mm)] formed around each tested substance. All tests were performed in triplicate and the mean of three readings was calculated and used in the analysis. Measurement of the antimicrobial activity of honey products mixture was tested.[6] The interaction between propolis and bee wax mixture was calculated by subtracting the expected value of inhibition zone from the measured one.

Determination of the minimum inhibitory concentration (MIC): Extracts which exhibited high activities against one or several pathogenic organisms were further assayed for their minimum inhibitory concentration (MIC). This was carried out by the two fold serial dilution of the tested extracts in nutrient broth or Sabouraud broth for *Candida albicans* (2 ml volumes), then inoculated with 100 µl inoculum size with the test organisms. The alcoholic and aqueous crude extracts were prepared at concentrations of 5, 2.5; 1.3; 0.6; 0.3 and 0.2 % (w/v). The MIC was determined by the broth dilution method [14]. Nutrient broth samples (10 ml) were inoculated with different concentrations of the crude extracts and with 100 µl of active inocula of microorganisms (approximately 108 CFU/ml) for 24h at 37°C for bacteria and at 30 °C for yeast. The MIC was determined as the lowest concentration of the extract which inhibited the organism [11].

Statistical analysis: The results of antimicrobial activity were expressed as the mean obtained upon three independent analyses.

Results and Discussion

Normal honey must lack pathogenic microorganisms or microorganisms that produce enteric illnesses. The aerobic mesophilic bacteria counts of Seder honey sample examined in this study are 220 ± 14.14a CFU/g (Table 1). The absence of other microbial groups may be attributed to the diluted honey with water that supports the growth of nonpathogenic bacterial strains and kills dangerous strains. According to published data total aerobic viable count values for honeys can range from 0 to several thousand per gram. This variation in bacterial

counts may be due to the type of sample (raw, finished or retailed), the freshness of the honey, the time of harvest and the analytical techniques used [36]. The presence of a certain type of bacteria indicates the contamination from secondary sources during manipulations and previous processes. The contamination with fungi and bacteria indicate inadequate hygiene conditions during collections, manipulation, processing and storage [39]. On the other hand, samples of propolis and bee wax were free from microbial growth. This may be due to the chemical composition of propolis which inhibited the presence of microbes [37, 38].

Honey has been shown to be bactericidal to many pathogenic microorganisms. This is as a result of its osmotic effect, acidity hydrogen peroxide and photochemical factors [18, 29]. Unexpected data were recorded in this study using Seder honey which showed slight inhibitory antibacterial effect on *B.subtilus* and *P. mirabilis* (Table 2). These results are in contrast with those recorded by numerous reports[7,17,27,32] which concern about the antimicrobial activities of honey. This negative effect may be attributed to heat processing of honey.

The inhibitory effect of ethanolic extract of propolis (EEP) sample was pronounced on most tested microorganisms, it is worth noting that gram positive bacteria were generally more sensitive to EEP extract than gram negative. *Proteus mirabilis* ATCC14153 and *Salmonella typhimurium* ATCC14028 seemed to be the least inhibited by EEP extract compared to the other tested organisms (Table 2). The maximum inhibition zone (23 mm) was recorded against *Staphylococcus aureus*, followed by *Staphylococcus epidermidis* (20 mm) and *Bacillus subtilus* (14 mm). It is concluded that EEP could be used as antiphytopathogenic, antidermatophytic and antibacterial agent[4,16,24,30].

Table 1
Microbial contents of Seder Honey, Propolis and Bee wax

Sample	Total aerobic bacteria (CFU/ml)	Coliforms (CFU/ml)	Yeast and moulds (CFU/ml)
Seder honey	220 ± 14.14[a]	ND	ND
Propolis	ND	ND	ND
Bee Wax	ND	ND	ND

(a) mean ± standard deviation (n=2)
ND: viable colony not detected ≤ 10-1 CFU/g at detection limit.

Table 2
Antimicrobial activity of Honey and Bees products extracts against some pathogens expressed as zone of inhibition (mm)

Microorganism	Honey Bees products		
	Zone of Inhibition in (mm)		
	Honey Seder	Propolis	Bee wax
S. aureus ATCC25923	0	23	7
S. epidermis ATCC12228	0	20	6.5
B. subtilus ATCC27853	1	14	7
P. auruginosa ATCC27853	0	6	4
E. coli ATCC25922	0	2.0	3
Strept. pyogenes ATCC19615	0	8	6.5
S.typhimurium ATCC14028	0	0	0
P. mirabilis ATCC14153	1	0	0
C. albicans NCTC2708	0	10	20.0

Table 3
Antimicrobial activity of equimixture of Propolis and Bees wax against some pathogens expressed as zone of inhibition (mm)

Tested organism	Propolis and Bee wax mixture		
	Zone of Inhibition in (mm)		
	Measured	Expected	Interaction
S. aureus ATCC25923	20	15	5
S. epidermis ATCC12228	14	13.5	1.5
B. subtilus ATCC27853	12	10.5	1.5
C. albicans NCTC270	22	15	7

Interaction:(measured inhibition zone of propolis and Bee wax - measured inhibition zone of propolis and Bee wax)

Table 4
Antimicrobial activity of Folkloric Herbs extracts against some pathogens expressed as zone of inhibition (mm)

Microorganism	Folkloric Herbs													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
S. aureus ATCC25923	-	-	-	24	-	15	-	19	-	20	-	25	-	-
S. epidermis ATCC12228	17	-	-	-	-	20	--	20	-	28	-	29	-	-
B. subtilus ATCC27853	-	-	19	15	-	-	-	-	-	-	-	28	-	-
P. auruginosa ATCC27853	-	-	-	-	-	-	-	-	-	17	-	26	-	-
E. coli ATCC25922	-	-	-	-	-	-	-	-	-	23	-	28	-	-
Strept. Pyogenes ATCC19615	24	14	-	-	-	-	-	-	-	19	17	25	-	-
S.typhimurium ATCC14028	-	20	-	-	-	20	20	-	23	-	-	27	14	-
P. mirabilis ATCC14153	-	-	-	-	-	-	-	17	-	20	-	25	-	-
C. albicans NCTC2708	-	-	-	-	-	-	-	-	-	20	-	25	-	-

1- *Rhazya stricta,* 2- *Nigella sativa,* 3- *Sambucus nigra,* 4- *Commiphora myrrha* 5- *Zingiber officinale,* 6-*Ziziphus spina – Christi,* 7- *Ferula asafoetida,* 8- *Eugenia caryophyllus*, 9-*Cuminum cyminum,* 10- *Juglans regia*, 11- Arabic gum, 12- Alum (Ming Fan*),* 13- *Ricinus communis,* 14- *Nasturtium officinails*

Table 5
Minimum inhibitory concentration (MIC) of EEP extract and some aqueous herb extracts

Microorganisms	EEP and herb extracts MIC					
	1	**2**	**3**	**4**	**5**	**6**
S. aureus ATCC25923	2.5	--	5	2.5	2.5	0.3
S. epidermis ATCC12228	--	0.4	0.2	--	--	0.2
P. auruginosa ATCC27853	--	--	0.6	--	--	0.3
E. coli ATCC25922	--	--	--	--	--	0.3
Strept. Pyogenes ATCC19615	--	0.4	2.5	--	--	0.3
S.typhimurium ATCC14028	--	--	--	--	--	0.3
P. mirabilis ATCC14153	--	--	--	--	--	0.3
C. albicans NCTC2708	5	--	5	--	--	0.3
Overall mean MIC	3.75	0.4	2.66	2.5	2.5	0.29

1- Propolis, 2- *Rhazya stricta,* 3- *Juglans regia,* 4- *Commiphora myrrha,* 5- *Eugenia caryophyllus*
6- Alum (Ming Fan).

Crude bee wax, unlike propolis is more known and is easier to obtain. It is well know in revealing therapy for gastric ulcer [10]. Zanoschi et al[42] reported on the use of bee wax for the treatment of burns. Bee wax sample used in this study was found effective against the studied gram positive and gram negative bacteria and showed pronounced inhibitory effect with *Candida albicans* NCTC2708 (20 mm) as well (Table 2). This is not in agreement with data obtained by Hasanain.[19]

The possible synergistic interactions existing between propolis and Bee wax provided useful antimicrobial activity. Zone diameters were then compared to those developed around control wells receiving single samples, one at a time. The combined effect of equimixtures of propolis and Bee wax revealed highest positive interaction[7] on *Candida albicans* followed by *Staphylococcus aureus*[5] (Table 3). Moreover, the zone of inhibition recorded showed 1.5 fold increase than that found with propolis alone. This experiment confirms the possibility of synergistic as well as antagonistic interactions between natural mixtures of Bee products [35].

The antimicrobial activities of some herbs and gum extracts were evaluated; the results indicated various degrees of growth inhibition on the test microorganisms. Crude extract of Juglans regia and alum exhibited inhibitory effects against almost all tested strains. In table 4 extract of *Commiphora myrrha* showed a high degree of antibacterial activity against *Staphylococcus aureus* (24mm). *Commiphora* species have a considerable antimicrobial activity against some gram positive and gram negative bacteria as recently reported [33]. Moreover, the extracts of *Rhazya stricta* could inhibit *Streptococcus. pyogenes* ATCC19615 with zone of inhibition of 24 mm. *Nigella sativa, Ziziphus spina, Ferula asafoetida,* and *Cuminum cyminum* inhibited the growth of *Salmonella typhimurium* ATCC14028. On the other hand *Zingiber officinale* and *Nasturtium officinails* showed no inhibitory effect on all the tested microorganisms [34].

Table 5 shows the MIC of selected samples propolis, *Rhazya stricta, Juglans regia, Commiphora myrrha, Eugenia caryophyllus* and Alum (Ming Fan) extracts on the inhibition of eight test strains. A wide range

of MIC values was recorded depending on the microbial strain. The ethanolic extract of propolis (EEP) showed MIC value of 2.5% (w/v) against *S. aureus* ATCC25923 and MIC of 5% (w/v) for *C. albicans* NCTC2708. So it can be useful for preventing candidal infections [30]. *Rhazya stricta* is effective against both strains of *Staphylococcus epidermis* ATCC12228 and *Streptococcus pyogenes* ATCC19615 with MIC (0.4%) (w/v). Similar result was obtained by Salamah et al[34]. Alum (Ming Fan) extract showed a pronounced inhibitory activity against all tested strains with lower minimum inhibitory concentration of 0.3% (w/v) This may be due to the chemical composition of hydrated potassium aluminum sulfate $\{KAl(SO_4)_2 \cdot 12 H_2O\}$. Potassium alum is an astringent and antiseptic[15]. For this reason, it can be used as a natural deodorant by inhibiting the growth of the bacteria responsible for body odor.

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