

Physico-chemical and microbiological studies on Jordanian Honey and propolis as potential self-preserving pharmaceutical systems

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Abstract

Introduction: The aim of this project was to study the physico-chemical and antimicrobial properties of Jordanian honey and propolis in order to determine their potential as pharmaceutical preservation systems.

Methods: Physico-chemical analysis included, pH and free acidity, moisture content, ash content and HydroxyMethylFurfural content in three honey samples, and total flavonoid content in the propolis sample. The antimicrobial activity of honey and propolis samples was then evaluated by determining the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231. Subsequently, Honey 1 (H1) was selected for further study and combined with propolis to test their potential synergistic activity. Finally, a preservative effectiveness test was conducted in order to assess the possibility of using honey and propolis as natural preservatives in aqueous dosage forms.

Results: The results of this study showed that all the tested honey samples and propolis possessed significant antimicrobial activity against the standard test microorganisms, and that honey with propolis exhibited synergistic activity that enhanced their antimicrobial activity and resulted in up to 90% reduction in their MIC values.

Conclusion: Our results reveal the possibility of using honey-propolis mixtures as natural preservatives in oral aqueous pharmaceutical dosage forms and other local application products.

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Introduction

The increasing resistance of pathogens to many commonly used antibiotics has resulted in a considerable international effort to develop novel and alternative antimicrobials and antimicrobial combinations. Historically, honey has been widely used for both its nutritional and medicinal properties. Clinically, honey has been used in wound dressings [1]. Honey has been shown to possess antimicrobial activity [2], but that this effect is highly dependent on the type of honey, geographical origin, and the flowers from which the final product is derived from, as well as harvesting, processing and storage conditions. The difference in antimicrobial potency of different honeys can vary greatly [3]. According to Kwakman and Zaat [4], the antimicrobial activity of honey is due to its high osmotic pressure, and the presence of agents such as hydrogen peroxide, methylglyoxal, bee defensin-1, and other undescribed components.

In order to understand the properties of honey, its potential medical applications and due to the high diversity of bee forage flora, it is important to investigate the physico-chemical characteristics of the honey prior to the evaluation of its antimicrobial activity [5,6]. Whereas there are large amounts of data on the characterization of honey from North America, Europe, Australia, India and South Africa, there is a paucity of data on Middle Eastern, and particularly Jordanian honeys. According to Sodre *et al.* [6] hydroxymethylfurfural (HMF, CAS Number 67-47-0. Also known as 5-(Hydroxymethyl)-2-furaldehyde and 5-(Hydroxymethyl)furfural) is the most studied of honey's components. HMF is formed by the reaction of some sugars and acids, and more particularly by the decomposition of fructose in the presence of various organic acids. Finding large amounts of HMF in honey may indicate overheating, age or the addition of inverted sugar. HMF content of honey can also be affected by the acidity, pH, water and mineral content of the honey [6]. Fallico *et al.* [7] stated that HMF is, to some degree,

produced in honey all the time and is a breakdown product arising from the action of normal honey acidity on sugars (glucose and fructose) at ambient temperature. Regarding the physico-chemical characterization of honey, colour, pH, refractive index, moisture and ash contents are the most important parameters studied [5].

Propolis (<https://www.specializedbeeproducts.com/bee-propolis/>; accessed 27th December 2021) is another bee-product that bees use in the construction of their hives, mainly to fill out cracks and to seal the internal walls of the beehive. Propolis is sticky in nature (sometimes called 'bee glue'), dark-coloured and resinous, bees collect it from various plant materials (including tree exudates and leaf resins, [8]) and then mix it with wax. Just like honey, the chemical composition of propolis, along with its antibacterial activity varies widely according to the plant species, geographical region and time of collection [9]. Propolis is not solely used in the construction of beehives, its high antimicrobial activity allows it to be used to sterilize the beehive in order to prevent bacterial and fungal infection of the pupae [9]. It has been reported in previous literature that the active principles in propolis responsible for its antimicrobial action mainly include, flavonoids (e.g. flavone), phenolic acids and esters [10]. However, in general its composition includes flavonoids, aromatic acids, esters, aldehydes, ketones, fatty acids, terpenes, steroids, amino acids, hydrocarbons, alcohols, hydroxybenzene, and several other compounds in trace amounts [9]. However, the estimation of flavonoids remains crucial in the process of characterization of propolis.

Previous studies have shown that honey and propolis possess potent antimicrobial activity. Tan *et al.* [1] studied the Malaysian Tualang and Manuka honeys against 13 species of bacteria. Their results showed that the MICs for Tualang honey ranged from 8.75% (w/v) to 25% (w/v), while those for Manuka honey ranged between 8.75% (w/v) and 20% (w/v). Pakistani honey has been found to

have broad-spectrum antimicrobial activity against Gram-positive cocci, Gram-positive bacilli (including three species of *Corynebacterium* (*C. diphtheriae*, *C. hoffmani*, and *C. xerosis*), Gram-negative bacteria (e.g. *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi*) and has a prominent antifungal activity [11]. The antifungal activity of honey was examined in different Nigerian honey samples by Anyanwu [12]. They studied its effects against *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum*, *Microsporium gypseum*, *Candida albicans* and *Saccharomyces* sp. and found MIC values ranging between 12.5% (v/v) to 50% (v/v).

Miorin *et al.* [13] evaluated the antimicrobial activity of honey and propolis produced by two species, *Apis mellifera* and *Tetragonisca angustula* against *Staphylococcus aureus*. They reported that propolis produced by *Apis mellifera* exhibited MICs ranging from 0.36 to 3.65 mg/ml, MICs of propolis produced by *Tetragonisca angustula* were 0.44 to 2.01 mg/ml, the MICs for honey produced by *Apis mellifera* were 126.23 to 185.70 mg/ml and for honey produced by *Tetragonisca angustula* they were 142.87 to 214.33 mg/ml. Darwish *et al.* [10] studied Jordanian propolis obtained from two botanical sources against standard strains of Methicillin-resistant *Staphylococcus aureus* (MRSA) and multi drug-resistant (MDR) *Escherichia coli*. They found that both types of propolis exerted considerable antibacterial activity against the tested species of bacteria.

However, further detailed studies are required on Jordanian honey and propolis. The objective of this study was to physico-chemically characterize Jordanian honey by determining pH, ash content, moisture content and hydroxymethylfurfural content in all honey samples. Additionally, propolis was chemically characterized by the determination of flavonoid concentration. The second goal in this study was to evaluate the individual and combined antimicrobial activity of honey and propolis by determining the minimum inhibitory concentration (MIC) for both agents separately and their potential as synergistic

agents against the test microorganisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*). Ultimately, we aimed to determine the efficacy of honey and propolis as a natural preservative system in aqueous dosage forms using the preservative effectiveness test in the European Pharmacopoeia.

Materials and Methods

Test microorganisms and culture maintenance

Pseudomonas aeruginosa ATCC 9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538, and *Aspergillus niger* ATCC 16404 spores, were from the kind gift of Dar al Dawa Group (Na'ur, Jordan). All test strains were selected for use as general purpose antimicrobials sensitivity test microorganisms. None of the strains exhibits antibiotic resistance, but all are related to MDR strains within the same species. Cultures of *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027) and *S. aureus* (ATCC 6538) were maintained and stored on Nutrient Agar plates in a darkened cool place at $20\pm 2^{\circ}\text{C}$ after being incubated at 37°C for 48 hours. Cultures of *C. albicans* (ATCC 10231) were maintained and stored on Sabouraud Dextrose Agar plates and kept in darkened cool place at temperature of $20\pm 2^{\circ}\text{C}$ after being incubated at 20°C for 48 hours. For the preparation of spores, an aliquot (0.2ml) of a suspension of *A. niger* (ATCC 16404) spores was spread onto the surface of solidified Sabouraud Dextrose Agar plates. The plates were then inverted and incubated at 20°C for five days. Subsequently, the culture was harvested by washing the surface of incubated plates with sterile distilled water and centrifuged at 4000xg for 15 minutes, washed twice (with sterile distilled water; centrifugation at 4000xg for 15 minutes) and resuspended in sterile distilled water. The final spore count was determined by viable counts of cells after

morphological characteristics were examined and confirmed via microscopic examination. All culture media were sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

Preparation of bacterial cells for the challenge test

Preparatory to the preservative effectiveness test, an overnight culture of each test microorganism was prepared using the appropriate sterile solidified agar medium. These cultures were incubated at 37°C for bacteria and at 20°C for fungi. Afterwards, the cultures were harvested by washing the surfaces of the agar plates with sterile saline TS solution (0.05% w/v Tween-80, 0.9% w/v NaCl) and diluting the broth to give a final cell concentration of 1.0×10^6 CFU/ml. In all cases, the final concentration was determined by viable cell counts.

Preparation of fungal spores for the challenge Test

Broth cultures of *A. niger* (0.2ml) were spread onto the surface of solidified Sabouraud Dextrose Agar plates. The plates were then inverted and incubated at 20°C for five days. Finally, the culture was harvested by washing the surfaces of incubated plates with sterile saline TS solution (0.05% w/v Tween-80, 0.9% w/v NaCl) and the broth was diluted to give a final concentration of 1.0×10^6 spores/ml. The final spore concentration was determined by total viable cell counts.

Honey and propolis samples

Three honey samples (H1, H2 and H3) and one propolis sample were obtained from two different beekeepers, one beekeeper located in Amman and the other beekeeper located in Irbid (North Jordan). The three honey samples (H1, H2, H3) and one propolis sample were collected from honey bees (*Apis mellifera*) at various locations in Jordan (Table 1). All honey and propolis samples were stored in glass containers in a dark cool place.

Ethanol extraction of propolis

Propolis was extracted using the classical maceration method, where raw propolis was ground into a fine powder and extracted using ethanol (70% v/v) in a ratio of 1:30 (w/v). The subsequent extract was kept in a dark cool place until required. After seven days, the extract was directly filtered through Whatman filter paper (CAT# 001-12.50; size 125mm), and the residue was submitted for a second extraction using the same conditions as the first one. Finally, the two filtered extracts were united and the volume was adjusted to 100 ml in a volumetric flask in order to give a final concentration of 1% w/v in alcohol [14].

Honey and Propolis Physico-Chemical Characterization

pH and free acidity

The pH and acidity of the honey and propolis samples were determined by the methods of AOAC (2000).

Table 1. The sources and nature for the honey and propolis samples used in this study .

Sample	Tree species	Bee species	Time of collection	Geographical region
Honey 1 (Sidr)	<i>Ziziphus spina-christi</i> L.	<i>Apis mellifera</i> L.	December 2011	Southern Shouna
	<i>Frangula alnus</i> Mill			
Honey 2 (Wildflower)	Numerous local wildflower species	<i>Apis mellifera</i> L.	May 2012	Um Qais
Honey 3 (Mountain)	Numerous local mountain flower species	<i>Apis mellifera</i> L.	May 2012	Jerash
Propolis (Pine & Poplar)	<i>Pinus halepensis</i> Mill.	<i>Apis mellifera</i> L.	April 2012	Jerash
	<i>Populus nigra</i> L.			

Moisture content

The moisture content of the honey and propolis samples was determined by the methods of Mărghitaş *et al.* [15]. Two measurements were made for each sample and the mean value was calculated. The corresponding moisture content was determined by reference to the Standard Table and was recorded [15].

Ash content

The ash content of all samples was determined by the methods of Mărghitaş *et al.* [15]. The ashing process was repeated until a constant weight was reached [15].

The proportion of ash W_A in g/100g honey was calculated using the following formula:

$$W_A = \frac{(m_1 - m_2)}{m_0} \cdot 100 \quad 1$$

Where W_A = proportion of ash in g/100g of honey; M_1 = weight of dish + ash; M_2 = weight of dish; M_0 = weight of honey taken.

Hydroxymethylfurfural (HMF) content

In order to calculate the amount of HMF in the three honey samples a standard curve was created as follows. Four HMF standard aqueous solutions were prepared with the following concentrations: 1, 2, 5, and 10 mg/l. Subsequently, sample solutions were prepared by separately dissolving 10 g of each honey sample in 50 ml water and then filtering the solutions through 0.45 µm membrane filter. This process provided a sample solution that was ready for chromatography. The conditions for chromatography were as follows: Flow rate, 1.0 ml/min; Quantity injected, 20 µl of sample or standard solution; Detection, UV 285 nm; Range, 0.2 AUFS. A calibration curve was constructed using the five standard solutions of HMF.

Total protein content

The total protein content of the honey and propolis samples was spectrophotometrically determined using Bovine Serum Albumin (BSA) to plot a standard curve according to Lowry *et al.* [16].

Sugar content

Total sugar content was determined spectrophotometrically according to the Dubois *et al.* [17] method, using sucrose to plot a calibration curve. Reducing sugar was determined according to the dinitrosalicylic acid (DNS) method [18], using glucose as a standard to plot a calibration curve. The non-reducing sugar, as sucrose, was calculated by subtracting the reducing sugar amount from the total sugar value as demonstrated in **Table 3**.

Determination of Flavone/Flavonol (FF) content for propolis only

Previous studies have shown that flavone/flavonols (FF) react better and form more stable, coloured complexes with $AlCl_3$ [14]. Samples were prepared according to the method of Mărghitaş *et al.* [14] and afterwards the absorbance was measured against a blank at 425 nm. Galangin was chosen as the internal standard, using five different concentrations ranging from 4 to 40 µg/ml diluted in methanol [14].

Determination of Flavanone/Dihydroflavonol (FD) content for propolis only

This method is based on the interaction between Flavanone/Dihydroflavonol and 2,4 Dinitrophenylhydrazine (2,4DNP) in an acidic environment to form coloured phenylhydrazones [14]. Samples were prepared according to the method of Mărghitaş *et al.* [14]. The absorbance was read immediately at 486 nm against a reagent blank. In this trial, pinocembrin was chosen as the internal standard and was prepared in five different concentrations that ranged between 0.14 to 1 mg/ml diluted in methanol [14].

Antimicrobial Assessment

Minimum Inhibitory Concentration (MIC)

Given the complex chemical nature, viscosity and physico-chemical properties of honey and propolis, and the fact that their antimicrobial action is the result of this physico-chemical complexity as opposed to a single antimicrobial active, they do not lend themselves to normal means of antimicrobial testing (e.g. Clinical and Laboratory Standards Institute (CLSI) or antimicrobial synergy testing [19]. Hence, alternative methods are required as follows.

In order to determine the MIC of honey samples and propolis against the four test microorganisms, an inoculum (10^5 CFU/ml) of each microorganism was prepared as per **Table 2**. Honey and propolis samples were used in concentrations ranging from 30% to 80% (w/v). Mueller Hinton Broth was added accordingly to achieve the desired final concentration in each test tube as shown in **Table 2**. The final volume of honey or propolis along with the medium in each test tube was 5 ml. The test tubes were incubated at 37°C for 24 hours. The MIC was determined by finding the test tube with the lowest concentration of honey or propolis in which no turbidity was observed. Positive controls were prepared as four test tubes each containing 1 ml of each microbial broth and 4 ml of sterile broth that were used in the test. Test tubes that contained honey or propolis and sterile broth to replace the bacterial broth were used to act as the negative control.

Potential synergy

Typically, the potential synergy of antimicrobial agents and antibiotics is determined by an experimental approach based upon the work of Berenbaum [19] and the calculation of the fractional inhibitory concentration index (FICI, [20]). However, and as mentioned above, the physico-chemical and complex antimicrobial nature of honey and propolis do not lend themselves to these approaches. Hence, other workers have developed protocols which attempt to address these problems [21, 22, 23]. In these latter studies it has been possible to determine the FICI of honey or propolis when tested in combination with a single chemically defined antimicrobial agent. However, these workers have also shown that it is impossible, as yet, to determine the FICI for honey and propolis in combination. Hence, in our study we use the widely accepted alternative of determining the *additive MIC* [24, 25], which is commonly used where the antimicrobials tested are of a complex, multi-factorial nature. The additive MIC is useful in that it allows the estimation of potential synergistic effect, but lacks the accuracy of the FICI method.

In this stage, concentrations of H1 less than the MIC were added to sub-MIC concentrations of propolis, which were then incorporated into Mueller Hinton Broth (MHB) in order to determine the minimum additive MIC against the four test species. Similarly, the final volume in each test tube was 5ml.

Table 2. The amount of honey or propolis, sterile broth and microbial suspension placed in each test tube to estimate the MIC values.

Test tube number	Contents				Honey/Propolis
	honey (g)	propolis (ml)	microbial suspension (ml)	sterile broth (ml)	Concentration (%)
1	4.0	4.0	1.0		80
2	3.5	3.5	1.0	0.5	70
3	3.0	3.0	1.0	1.0	60
4	2.5	2.5	1.0	1.5	50
5	2.0	2.0	1.0	2.0	40
6	1.5	1.5	1.0	2.5	30

After incubation at 37°C for 24 hours, sterile swabs were dipped into each tube and inoculated onto Mueller Hinton Agar (MHA) plates. The plates were incubated for 24 hours at 37°C. When honey and propolis were mixed together, the mixture was opaque and thus, the visual inspection of the turbidity was not possible. As a result, growth inhibition was detected by sub-culturing a volume (0.1 ml) from each tube onto Mueller Hinton Agar and comparing the subsequent number of colonies with the number of colonies observed in positive growth controls. The additive MIC was determined by finding the plate with the lowest concentration of both honey and propolis on which the microorganisms had the least growth. The minimal biocidal concentration (MBC) was determined by finding the concentration at which no microbial growth was observed.

Antimicrobial activity against *Aspergillus niger* spores

In this test, we aimed to study the additive sporicidal activity of honey and propolis using 20% w/v and 20% v/v of each respectively, against 10⁶ CFU/ml spores of *A. niger*. A blank was prepared replacing the same amounts of honey and propolis with distilled water, two samples were taken at zero time and after two hours. Prior to culturing aliquots (0.1 ml) of the samples on Sabaraoud Dextrose Agar for counting purposes, a neutralization step was done (a 1 in 10 dilution in sterile normal saline), followed by four serial 1 in 10 dilutions. Afterwards, the plates were incubated at 20°C and the colonies were counted after 72 hours.

The Preservative Effectiveness Test

In preparation for this test, five different microorganisms (*E. coli*, *S. aureus*, *P. aeruginosa*, *C. albicans* and *A. niger* spores) were inoculated on Casein Soya Bean Digest Agar for bacteria and Sabouraud Dextrose Agar for fungi. The bacteria were incubated at 37°C for 18 to 24 hours, the culture of *C. albicans* at 20 to 25°C for 48 hours, and the

culture of *A. niger* spores at 20 to 25°C for one week or until good sporulation was obtained. The microorganisms were then harvested and centrifuged at 4000xg for 15 minutes, washed three times with sterile normal saline and the pellets were re-suspended in sterile normal saline. An aliquot (0.1 ml) was removed from each suspension in order to determine the number of colony-forming units per milliliter (CFU/ml) in each suspension by the plate count method. A count of 10⁷ CFU/ml was used in a final volume of 20 ml in each container for the preservative effectiveness test.

An aqueous pharmaceutical formulation provided by the Dar al Dawa Group (Na'ur, Jordan) was added to a series of containers, each with a suspension of one of the microorganisms to be tested (in a final inoculum size of 10⁵ microorganisms per milliliter of the preparation) along with 4 g of honey (H1) and 4 ml of propolis. The inoculated containers were stored at 20 to 25°C and protected from light. Aliquots (1 ml) were collected at zero time, 14 days and 28 days. Prior to culturing the samples on suitable agar media, any residual antimicrobial activity of honey and propolis was neutralized by a three-fold serial dilution. A microbial count was done by spreading an aliquot (0.1 ml) from each test tube on the surface of suitable agar media and incubated for 30 to 35°C for 18 to 24 hours for bacteria, at 20 to 25°C for 48 hours for *C. albicans* and at 20 to 25°C for 3 days for *A. niger*.

The European Pharmacopeia (2011) recommended procedure for evaluating the preservative activity requires three sample points for oral preparations (at 0 time, at 14 days and at 28 days). It also requires 3 log cycle reductions in the count of bacteria and one log cycle reduction for fungal spore count at the 14 days interval with no increase in count at the 28 days interval (<https://www.drugfuture.com/Pharmacopoeia/EP7/DATA/50103E.PDF>; accessed 27th December 2021).

Results

Honey and Propolis Physico-Chemical Characterization

The physico-chemical characteristics of the three honey samples (H1, H2 and H3) are given in **Table 3**. The pHs of the three samples were fairly similar at between 4.09 (H1) and 4.62 (H2), indicating that all three samples were slightly acidic. This was also supported by high free acidity values of 19 (H3), 31 (H2) and 39 millequivalents/kg (H1). The water content of all three samples was constant at approximately 14% (14.2% (H3) to 14.9% (H1 & H2). There was distinct variability in the ash content of the three samples, with H1 having the highest value of 11.147% and H2 and H3 being at 1.244% and 2.017% respectively.

The three honey samples exhibited considerable variation in the chemical characteristics we observed (**Table 3**). Total protein content varied between 0.2 ± 0.49 mg/kg (H3) and 0.47 ± 0.03 mg/kg (H1). Total sugar content was fairly constant at between 79.15 ± 1.5 mg/kg (H1) and 82 ± 1.1 mg/kg (H3). However, H1 exhibited the lowest reducing sugar content of 57.7 ± 1.9 mg/kg and the highest non-reducing sugar content of 21.3 ± 0.9 mg/kg. Previous studies suggested we examine hydroxymethylfurfural (HMF) content, where we found that H1 had the largest HMF content by far at 632.53 ± 1.7 mg/kg, H3 the next at 297.64 ± 1.2 mg/kg and H2 the lowest

at 11.26 ± 0.8 mg/kg. Interestingly, H2 exhibited the highest reducing sugar content at 70.5 ± 1.4 mg/kg and lowest non-reducing sugar content at 9.5 ± 0.7 mg/kg.

Flavone/Flavonol (FF) content

The two propolis extracts contained relatively similar amounts of Flavone/Flavonol, where extract A contained 0.1560% (w/w) and extract B contained 0.1626% (w/w).

Flavanone/Dihydroflavonol (FD) Content

Propolis extract A contained 8.3348% (w/w) while extract B contained 6.7013% (w/w) Flavanone/Dihydroflavanol.

Antimicrobial Assessment

Minimum Inhibitory Concentration (MIC)

In the majority of published *in vitro* studies to determine the antimicrobial activity of honey, variations of the Kirby-Bauer agar diffusion test or impregnated disc test are used [26]. However, we had difficulty in using these methods, as the discs often became dry due to evaporation of fluid during the incubation period at 37°C for 24 h. This is a problem which was previously reported by others [21, 22]. Therefore we used a modification of the 'macro-dilution in broth method' [27] for the determination of the MIC of H1 and of Propolis against the microbial panel.

Table 3. Honey physico-chemical characterization, including pH value, free acid content, water content, ash content, HMF concentration, total protein content, total sugar content, reducing and non-reducing sugar content in the three honey samples (H1, H2 and H3). Values are the means of triplicate readings \pm standard deviation, where shown.

Honey Sample	pH	Free acidity	Water content	Ash content	Amount of HMF	Total Protein content	Total sugar content	Reducing sugar content	Non-reducing sugar content
		millequivalents/kg	%	%	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
H1	4.09	39	14.9	11.147	632.53 ± 1.7	0.47 ± 0.03	79 ± 1.5	57.7 ± 1.9	21.3 ± 0.9
H2	4.62	31	14.9	1.244	11.26 ± 0.8	0.33 ± 0.06	80 ± 1.2	70.5 ± 1.4	9.5 ± 0.7
H3	4.61	19	14.2	2.017	297.64 ± 1.2	$0.2\pm 0.49\%$	82 ± 1.1	64.9 ± 1.4	17.1 ± 0.9

HMF: Hydroxy Methyl Furfural.

The MIC results show that the three samples possessed considerable antimicrobial/antifungal activity, with H1 being the most potent and H2 the least potent (**Table 4**). Propolis has the lowest MIC against the Gram-negative facultative anaerobic *E. coli* (2 ml), H1 and H3 had similar activity against *P. aeruginosa* and *C. albicans* (MIC values 2 g and 1.5 g respectively). H1 possessed considerably better antimicrobial activity than H2 and has a much

Table 4. The MIC values of the three tested Jordanian honey samples and propolis against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Values are the means of triplicate readings.

Test Species	MIC of Treatments							
	H1		H2		H3		Propolis	
	g	%	g	%	g	%	ml	%
<i>S. aureus</i> ATCC 6538	2.0	40	2.5	50	2.5	50	2.0	40
<i>E. coli</i> ATCC 8739	3.0	60	3.0	60	3.0	60	2.0	40
<i>P. aeruginosa</i> ATCC 9027	2.0	60	2.5	50	2.0	40	2.5	50
<i>C. albicans</i> ATCC 10231	1.5	30	2.5	50	1.5	30	2.5	50

higher HMF content (H1 HMF = 632.535 mg/kg and H2 HMF = 11.26 mg/kg).

Potential synergy

Results illustrated in **Table 5** show the additive MIC values for H1 and propolis when combined together (the MIC of H1 and Propolis against *S. aureus* is 1.0 ml and 1.0 g respectively, against *E. coli* 1.0 ml of propolis and 0.5 g of H1, against *P. aeruginosa* 0.5 ml of propolis and 0.5 g of H1, and against *C. albicans* 0.5 ml of propolis and 1.0 g H1). **Table 5** compares the results of H1 and propolis when used separately and when combined. It also summarizes the percentage of MIC reduction in H1 and propolis, suggesting the presence of a potent synergistic activity between H1 and propolis (the percentage of reduction ranged from 50% to 90%), which is consistent with the results of Al-Waili *et al.* [22].

Antimicrobial activity against *Aspergillus niger* spores

The sporicidal activity of H1 sample and propolis against *Aspergillus niger* spores is shown in **Table 6**, in which a concentration of 20% w/v of H1 and 20% v/v of propolis was sufficient to produce a sporicidal action against a count of 10^6 spores/ml of *A. niger* spores within two hours.

Table 5. The additive MIC values of H1 and propolis against the control test microorganisms, the MIC values of H1 and propolis when used separately, and the total amount of reduction in the quantities of honey and propolis used, expressed as a percentage. Values are the means of triplicate readings.

Test Species	MIC					
	Additive MIC		H1 MIC	Propolis MIC	Reduction in Honey MIC	Reduction in Propolis MIC
	propolis	H1				
	ml	g	g	ml	%	%
<i>S. aureus</i> ATCC 6538	0.25	1.0	2.5	2.5	60	90
<i>E. coli</i> ATCC 8739	1.0	0.5	3.5	2.5	85.7	60
<i>P. aeruginosa</i> ATCC 9027	0.5	0.5	2.5	3.0	80	83.3
<i>C. albicans</i> ATCC 10231	0.5	1.0	2.0	3.0	50	83.3

Table 6. The sporicidal activity of the combination of H1 and propolis, showing the difference in the numbers of colonies of *Aspergillus niger* when treated with H1 and propolis in comparison to a test reagent blank (without H1 and propolis) at zero time and after two hours. Values are the means of triplicate readings.

Time	Number of colonies			
	H1 (20% w/v) Propolis 20% v/v 10 ⁶ CFU/ml of <i>Aspergillus niger</i>		Test Reagent Blank	
	dilution	colonies	dilution	colonies
At Zero time	1 st	52	1 st	9
	2 nd	12	2 nd	18
	3 rd	0	3 rd	0
	4 th	0	4 th	0
After two hours	1 st	0	1 st	92
	2 nd	0	2 nd	23
	3 rd	0	3 rd	4
	4 th	0	4 th	1

The Preservative Effectiveness Test

In this study, our ultimate goal was to assess the possibility of using honey and propolis as an antimicrobial preservative in an unidentified aqueous pharmaceutical formulation supplied to us by Dar al Dawa Group, Na'ur, Jordan. We were informed that the final pH of the given pharmaceutical preparation would not cause any degradation of the honey and propolis upon addition (Dar al Dawa Group Personal Communication; Honey and propolis pH was 4.64). The efficacy of honey and propolis as an antimicrobial preservative was evaluated using the European Pharmacopoeia procedure (<https://www.drugfuture.com/Pharmacopoeia/EP7/DATA/50103E.PDF>; accessed 27th December 2021). The results of the preservative effectiveness test summarized in **Table 7** show that these requirements were met for the rest of the microorganisms, in that no growth was observed through all sampling intervals. While in the control samples (without the addition of honey and propolis), the microbial count was not reduced and in fact there was an increase in the counts.

Table 7. The number of colonies at time interval for the preservative effectiveness test, for samples treated with H1 and propolis as a preservative and for blank samples (without honey and propolis), the number of colonies in blank samples at zero time serves as the baseline to be used all through the test. Values are the means of triplicate readings.

Test Species	Number of Microorganisms			
	Blank Samples	Tested Samples	Tested Samples (oral preparation)	Tested Samples (oral preparation)
	without H1 and Propolis CFU/ml	At 0 time CFU/ml	after 14 days CFU/ml	after 28 days CFU/ml
<i>S. aureus</i> ATCC 6538	8.5x10 ⁵	No growth	No growth	No growth
<i>E. coli</i> ATCC 8739	8.9x10 ⁵	No growth	No growth	No growth
<i>P. aeruginosa</i> ATCC 9027	9.9x10 ⁵	No growth	No growth	No growth
<i>C. albicans</i> ATCC 10231	9.2x10 ⁵	No growth	No growth	No growth
<i>A. niger</i> (spores) ATCC 16404	9.1x10 ⁵	2.0x10 ⁵ CFU/ml in original sample	4.0x10 ⁴ CFU/ml in stock sample	3.0x10 ⁴ CFU/ml in stock sample

Discussion

The physico-chemical characterization results indicate that honey samples from different geographical locations and raised on different botanical sources may exhibit distinct physico-chemical characteristics. The importance of such differences is open to debate, but must be considered in the light of subsequent antimicrobial action findings. The physico-chemical characterization results indicated that high HMF value for H1 honey may explain some of the higher antimicrobial activity we observed in the subsequent antimicrobial studies. The data in **Table 3** shows that H1 is an outlier sample, in that it exhibits the highest protein content (0.47 mg/kg), the lowest reducing sugar content (57.7 mg/kg) and the highest non-reducing sugar content (21.3 mg/kg) which, coupled with the highest HMF content may partially explain its better antimicrobial action.

The variation seen in the Minimum Inhibitory Concentration (MIC) measurements between the three honey samples (H1, H2 & H3) indicates that the antimicrobial activity of Jordanian honey may be associated with variations in HMF content. The antimicrobial activity of the honey samples appears to be independent of their sugar content, but could be partially attributed to their low pH (H1 pH was 4.09, while H2 pH was 4.63). H1 and H3 (pH 4.09 and pH 4.61 respectively) had approximately equal activity against *P. aeruginosa* and *C. albicans*, in spite of the difference in their pH values. This may indicate that the potent activity of H1 is due to other unmeasured factors, e.g.: high osmotic pressure and low acidity, hydrogen peroxide or methylglyoxal content. However, and based upon its better MIC results, the H1 sample was chosen along with propolis for the determination of additive MIC measurement.

The observed mechanism of antimicrobial action of the honey and propolis samples is complex, and may be due to their biological active ingredients, such as phenols and flavonoids as reported by

others [28, 29, 30]. It has been reported that propolis affects the plasma membrane, inhibits bacterial motility, and inhibits microbial enzyme activity [22, 31]. In this study Jordanian H1 honey and propolis showed good antimicrobial activity against *Staphylococcus aureus* (40%), and the antifungal activity of H1 was higher than that of propolis (30% and 50% respectively). Boukraa and Bouchehrane [24] studied the possibility of combining honey from Algeria with bee royal jelly, and found that this increases antibacterial activity against *P. aeruginosa*. The percentage of reduction in honey and royal jelly MIC ranged from 25% to 94.4%, while in this study, adding honey to propolis resulted in a reduction of 50% to 90%. The observed antibacterial activity of H1 against *P. aeruginosa* and *E. coli* was lower than the activity against *S. aureus*. After mixing H1 with propolis the reduction was 85.7% for H1 and 60% for propolis. The antibacterial activity of H1 and propolis against *P. aeruginosa* were 60% and 50% respectively, and after mixing together, the reduction in H1 was 80% and for propolis was 83.3%. Hence, propolis exhibits a greater antimicrobial activity against Gram-negative bacteria than H1, which is an observation also reported by Gur *et al.* [32].

The antifungal activity of honey and propolis has been well established in previous literature [24, 33], where both honey and propolis were shown to possess antifungal activity against non-spore-forming and spore-forming fungi. As was the case with antibacterial activity, the antifungal potency of honey and propolis varies widely according to parameters such as botanical source and geographical origin [33]. Thus, we deemed it essential to assess the sporicidal activity of our Jordanian honey sample H1 and propolis against a spore-forming fungus, *Aspergillus niger*. The results are given in **Table 6**. Our results are comparable to those obtained by Anupama [34] who studied Nigerian honey samples, and found that the sporicidal concentration of studied honey samples against *A. niger* spores (1.5×10^5 spores/ml) ranged from 20 to 40% (v/v).

An antimicrobial preservative is an essential component of all pharmaceutical preparations, especially if the pharmaceutical preparation does not possess the ability to preserve itself adequately. The antimicrobial preservative works to suppress the growth of microorganisms and prevent microbial contamination during normal conditions of storage and use, which could otherwise create a threat to the patient's well-being and spoil the preparation (<https://www.drugfuture.com/Pharmacopoeia/EP7/DATA/50103E.PDF>; accessed 27th December 2021). The results of our preservative effectiveness test show that these requirements were met for the H1/propolis combination we chose, in that no growth was observed through all sampling intervals. While in the control samples (without the addition of honey and propolis) the microbial count increased.

Collectively, our findings indicate that different honey samples possessed differing profiles of physico-chemical properties dependent upon geographical and botanical origin. The results of this study suggest that Jordanian honey and propolis possess a wide spectrum of antimicrobial activity, in that all microorganisms were susceptible at some level to the tested honey and propolis samples. Enhancement of the antibacterial activity of honey and propolis was achieved by combining the two agents together, suggesting the potential for a potent synergistic activity. Our results reveal the possibility of using honey-propolis mixtures as natural preservatives in oral aqueous pharmaceutical dosage forms and other local application products (such as wounds dressings).

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Authors Contributions

This work was the result of the contribution of all authors, they participated in the study design, clinical testing and in writing the manuscript. All authors contributed to reading, amending and approving the final manuscript.

Availability of Data

All data is available on request from the authors.

Conflict of interest

The authors have no conflict of interest to declare.

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