Determination of the antibacterial effect of some natural products against some gram-positive and gram-negative bacteria

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Objective
This study evaluates the antibacterial activity of five natural substances (hot water extract of cinnamon sticks, peppermint, and lemon leaves, Egyptian local packed honey, and Yemeni Sidr honey) against some gram-positive and gram-negative bacteria.

Materials and methods
The well diffusion method was first used to evaluate the antibacterial effect of each of these tested natural products on the tested organisms. The minimal inhibitory concentration and the minimal bactericidal concentration were detected for the effective substances. The highly effective antimicrobial ones were chosen to investigate their effect on the tested organisms by graphing the bacterial growth curve of each bacterium before and after treatment.

Results and conclusion
The findings indicated lower antibacterial effect of the three plant extracts compared with both bee honey samples. Yemeni Sidr honey, local honey, and cinnamon extract were the more potent antibacterial agents, respectively. Minimal inhibitory concentration and minimal bactericidal concentration of these three natural substances ranged between 10 and 80% for the tested organisms. Bacterial growth curve indicated that honey had powerful antimicrobial activity that did not allow bacteria to grow, especially after treatment with Yemeni Sidr honey. The study recommends that herbal extracts and honeys could potentially be used as therapeutic agents against bacterial infection particularly on the tested microorganisms.

Keywords:
bee honey, extracts, growth curve, minimal inhibitory concentration, pathogenic bacteria

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Introduction

Herbal products have been used since ancient times in folk medicine, involving both eastern and western medical traditions [1]. Many plants and plant-derived antimicrobial components are used in folk lore therapeutics for oral hygiene [2]. Some have been evaluated for possible use in modern medicine, whereas thousands of other potentially useful plants have not been tested [2]. During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics have led to the search for new antimicrobial agents mainly among plant extracts with the goal of discovering new chemical structures that can overcome the above disadvantages [3–6]. A wide range of antimicrobial agents and herbal products are added to dentifrice and mouth-rinsing solutions and sanitizers with the aim of preventing biofilms' formation [1].

The fact that bee honey has antibacterial properties was recognized more than a century ago because it cures infections [7]. Honey resistance has never been reported; the absence of toxicity or side effects, low cost of maintenance, and local availability represent valuable advantages to the use of honey as an alternative antimicrobial therapy [8]. There are numerous reports of the antimicrobial activity of honey against a wide range of bacterial and fungal species [9,10]. The antimicrobial activity could be attributed to the osmotic effect of honey, the low pH of honey being between 3.2 and 4.5 [11], hydrogen peroxide, defensin-1, as well as the presence of phytochemical factors [12].

Several types of bacteria, commonly involved in wound infections such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas mirabilis*, *Klebsiella* spp., *Streptococcus faecalis*, and *Pseudomonas aeruginosa*, are susceptible to the antibacterial activity of bee honey irrespective of their resistance to antibiotics [13–15].

This study aimed to investigate the antibacterial activities of some plant extracts (cinnamon sticks, peppermint, and lemon leaves) compared with two

types of honey (Yemeni Sidr and local bee honey) against some pathogenic microorganisms, and also to comparing the growth curves of some tested gram-positive and gram-negative bacteria before and after treatment with the most effective tested materials.

Materials and methods

Bacterial strains

The following control bacterial strains were used (ATCC, USA): *Neisseria meningitides* (ATCC: 13090), *E. coli* (ATCC: 25922), *S. aureus* (ATCC: 25923), and *P. aeruginosa* (ATCC: 27853). Bacterial strains were subcultured on nutrient agar (Lab M, UK) and incubated aerobically at 37°C for 24 h and stored in a refrigerator at 4°C [16].

Plant extract

Four grams of each of dry plant material (peppermint leaves, cinnamon sticks, and lemon leaves) was extracted with 20 ml sterile boiled distilled water. The suspensions were stored at room temperature for 24 h and then centrifuged (3000 rpm, 15 min). The extracts obtained were filtered through a Seitz filter and stored in a refrigerator at 4°C until use [17].

Honey samples

Two bee honey samples were used in this study, one obtained from the local market in Egypt and the other from Saudi Arabia (Yemeni Sidr bee honey), and stored in the dark at room temperature.

Antibacterial activity

Different concentrations of honey and plant extracts constituting, 10–100% were prepared using sterile distilled water. This was done by dissolving the respective volumes: 1–8 ml of each bee honey type into the corresponding volumes of sterile distilled water to yield a 10 ml preparation [18].

The well diffusion technique was used as described previously by Bauer *et al*. [19]. McFarland standard inoculums was prepared using the method of Koneman *et al*. [20] as follows: the turbidity was adjusted to 1.5×10^8 CFU/ml (corresponding to 0.5 McFarland standards) and then a sterile cotton swab was dipped into the standardized bacterial suspension and used to inoculate the nutrient agar plates evenly.

The plates were left to dry for 3–5 min. Thereafter, 0.45 µl of each tested natural product was placed in a well prepared in the nutrient agar plate by a sterile borer. Plates were incubated for 24 h at 37°C, and then the mean diameter of the inhibition zone was measured in mm. The experiment was repeated in triplicate for each isolate.

Minimal inhibitory and minimal bactericidal concentration

The minimal inhibitory concentration (MIC) was determined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in a broth dilution susceptibility test. The minimal bactericidal concentration (MBC) was determined, after determining the results for the MIC, as the lowest concentration that achieved a 99.9% decrease in viable bacteria. The MBC can be determined from broth dilution MIC tests by subculturing on agar medium without a disinfectant and incubating at 35°C for 16–20 h according to the macrodilution method described by the National Committee of Clinical Laboratory Standards [21]. The experiments were conducted in triplicate.

Determination of the growth curves of bacterial cells

The four tested bacterial strains were cultured on Mueller–Hinton (MH) broth and the bacterial cell concentration was adjusted to 0.5 McFarland standards. Tubes were prepared as follows: 0.45 µl of the McFarland standard inoculums of each organism was added to 1 ml MH broth media and then inoculated with 0.45 µl of each highly effective inhibitor product. The control tube contained only MH broth media and was inoculated with each strain without the inhibitor product.

Each culture was incubated in a shaking incubator at 37°C for 17 h. Growth curves of bacterial cell cultures were determined through repeated measures of the optical density (OD) at 600 nm each hour using a spectrophotometer (humalyzer junior GmbH ser.# 72333; EEA) [22].

Heterotrophic plate count detection

Heterotrophic plate count was performed in parallel to OD measurements each hour up to 17 h to detect the number of viable bacterial cell count for the control and treated isolates using the heterotrophic plate count standard protocol and reported as colony-forming units (CFU/ml) [23].

Results

The inhibition zone diameter of the five natural products tested was determined for, *N. meningitides*, *E. coli, S. aureus*, and *P. aeruginosa*. All of them were

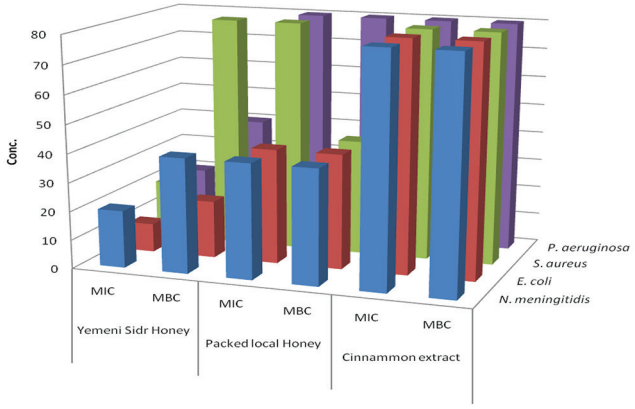
effective against the four bacterial strains, except the peppermint extract, which was only effective against *S. aureus*, and lemon leaves extract, which was effective against *E. coli* (Table 1).

Table 1 indicates that the highly effective antibacterial products for all bacterial strains were Yemeni Sidr honey, packed local honey, and cinnamon sticks extracts, respectively. They were examined for the MIC and MBC value of each of them (Fig. 1).

Bacterial growth curves for control and treated bacterial strains were constructed over all 18 h after growing bacteria lonely (control) on MH broth and with each of cinnamon extract, packed local honey, and Yemeni Sidr honey to determine the effect of each of these on these bacterial strains (Figs. 2–5).

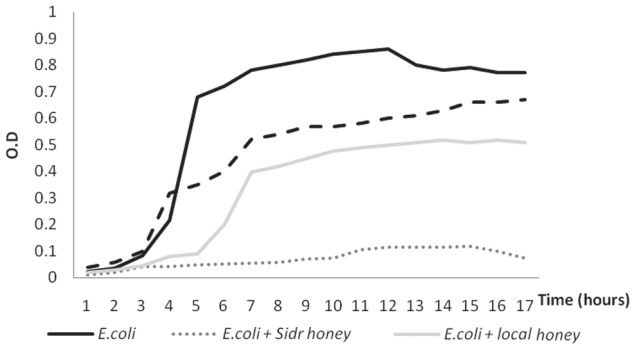
Figure 2 shows the growth curve of *E. coli* before and after treatment with the MIC of Yemeni Sidr honey, packed local honey, and cinnamon extract (10, 30, and 80%, respectively), and it was obvious that in the control one after the first 2 h, *E. coli* growth increased until the sixth hour (log phase) and then it remained in the stationary phase, whereas after treatment with Yemeni Sidr honey, there was a slight growth all over the 17s hours. The treatment with Yemeni Sidr honey was the most effective one.

Figure 1



Detection of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of Yemeni Sidr honey, packed local honey, and cinnamon extract against the tested bacterial strains.

Figure 2

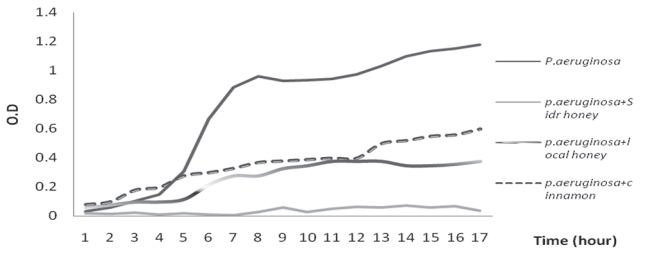


Growth pattern of *E. coli* before and after treatment with the minimum inhibitory concentrations of Yemeni Sidr honey, packed local honey, and cinnamon extract.

Table 1 Determination of the mean inhibition zone diameter (mm) of the five tested natural products against the four bacterial strains examined

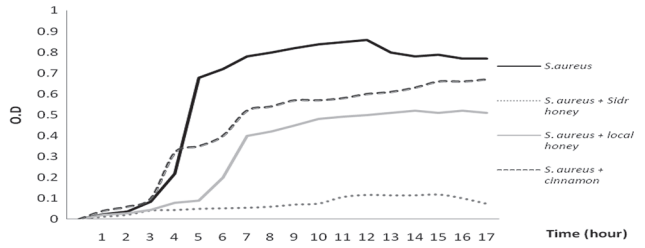
Concentration	10	20	30	40	50	60	70	80	90	100
Bacterial treatment										
E. coli + peppermint	0	0	0	0	0	0	0	0	0	0
E. coli + cinnamon	0	0	0	0	12	14	16	16	16	16
E. coli + lemon	12	14	14	16	18	22	22	22	21	22
E. coli + local honey	38	38	38	36	36	38	37	37	38	37
E. coli + Yemeni Sidr honey	38	38	39	38	38	39	38	38	36	37
S. aureus + peppermint	0	0	0	18	19	19	20	20	19	19
S. aureus + cinnamon	0	0	0	14	16	18	18	20	19	19
S. aureus + lemon	0	0	0	0	0	0	0	0	0	0
S. aureus + local honey	10	12	12	11	13	10	10	11	10	11
S. aureus + Yemeni Sidr honey	28	30	32	32	34	31	35	36	32	35
P. aeruginosa + peppermint	0	0	0	0	0	0	0	0	0	0
P. aeruginosa + cinnamon	0	0	12	15	16	19	21	22	20	20
P. aeruginosa + lemon	0	0	0	0	0	0	0	0	0	0
P. aeruginosa + local honey	11	11	12	12	10	11	10	12	11	11
P. aeruginosa + Yemeni Sidr honey	28	28	30	31	32	33	33	36	36	35
N. meningitidis + peppermint	0	0	0	0	0	0	0	0	0	0
N. meningitidis + cinnamon	0	12	14	14	14	18	19	22	22	22
N. meningitidis + lemon	0	0	0	0	0	0	0	0	0	0
N. meningitidis + local honey	37	38	37	38	35	35	33	36	37	37
N. meningitidis + Yemeni Sidr honey	39	39	39	38	38	37	38	39	39	39

Figure 3



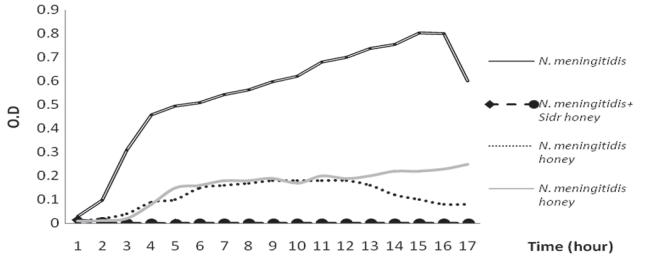
Growth pattern of *P. aeruginosa* before and after treatment with the minimum inhibitory concentrations of Yemeni Sidr honey, packed local honey, and cinnamon extract.

Figure 4



Growth pattern of *S. aureus* before and after treatment with the minimum inhibitory concentrations of Yemeni Sidr honey, packed local honey, and cinnamon extract.

Figure 5



Growth pattern of *E. coli* before and after treatment with the minimum inhibitory concentrations of Yemeni Sidr honey, packed local honey, and cinnamon extract.

In Fig. 3, there were low bacterial growth in the first four hours then growth increased until the eighth hour (log phase for the control one) and then it remained in the stationary phase, whereas regrowth was observed after 13 and 17 h. Treatment with Yemeni Sidr honey, packed local honey, and cinnamon was effective, but Yemeni Sidr honey was the most effective.

Figure 4 shows the increased growth of *S. aureus* after the second hour to the sixth hour (log phase for the control one); then, it remained stable up to the 17th hour, but after treatment with Yemeni Sidr honey, the growth was more inhibited than that with local honey or cinnamon extract.

Figure 5 showed that there was an increase in bacterial growth from the first to the 15th hour and then it as found to nearly stable for an hour; then, the death phase was observed, but after treatment with Yemeni Sidr honey, there were no growth all over the 17 h, whereas there was a slight growth nearly the same after treatment with both local honey and cinnamon.

Table 2 shows the heterotrophic plate counts for *E. coli*, *P. aeruginosa*, *S. aureus*, and *N. meningitides* before and after treatment. It was found that the highest colony count number before treatment was for *P. aeruginosa* compared with the other three strains tested, whereas the treated *N. meningitides* showed the least colony count number, indicating the powerful effect of the three products. It was also found that the lowest HPC was after treatment with Yemeni Sidr honey for the four tested strains.

Discussion

The inhibitory activity caused by the osmotic effect of honey dilutions obviously depends on the species of bacteria. Hydrogen peroxide is the major contributor to the antimicrobial activity of bee honey and the different concentrations of this compound in different bee honeys result in their varying antimicrobial effects [24].

In-vitro studies support the antimicrobial effect of bee honey against a wide range of pathogens including β-*haemolytic streptococci*, methicillin-resistant *S. aureus*, and *Pseudomonas* spp. [25]. In-vivo studies are less conclusive, but bee honey has been used to treat burns [26] and meningococcal lesions [26,27]. Subrahmanyam [28] compared honey and silver sulfadiazine for the treatment of patients with burns and found less inflammation, lower infection rates, and faster healing in patients treated with honey.

In the present study, local and Yemeni Sidr bee honey, cinnamon, lemon leaves, and peppermint extract samples were tested for their antimicrobial activity on *N. meningitides*, *E. coli*, *S. aureus*, and *P. aeruginosa*. There were varying degrees of *in-vitro* growth-inhibition activity of these natural products against the tested organisms. The highly effective products were Yemeni Sidr honey, packed local honey, and cinnamon extract, respectively. Some authors found that the antibacterial effect of honey might be because of the osmotic effect, the effect of pH, and the sensitivity of these organisms to hydrogen peroxide, which are unsuitable for bacterial growth, represented as an inhibition factor in honey [26,29].

Table 2 Heterotrophic plate count of *E. coli*, *P. aeruginosa*, *N. meningitides*, and *S. aureus* before and after treatment with minimal inhibitory concentration of Yemeni Sidr honey, packed local honey, and cinnamon extract

Time	Mean heterotrophic plate count/ml (CFU/ml)													
	E. coli control	*E. coli* + Sidr honey	*E. coli* + local honey	*E. coli* + cinnamon	*P. aeruginosa* control	*P. aeruginosa* + Sidr honey	*P. aeruginosa* + local honey	*P. aeruginosa* + cinnamon	*N. meningitis* control	*N. meningitis* + Sidr honey	*N. meningitis* + local honey	*N. meningitis* + cinnamon	*S. aureus* control	*S. aureus* + Sidr honey	*S. aureus* + local honey	*S. aureus* + cinnamon
1 h	1.4×10^8	1×10^2	1×10^2	1×10^4	1×10^8	1×10^2	2×10^2	1.5×10^4	1.5×10^6	1×10^2	3×10^2	1×10^2	2.5×10^7	2×10^2	2×10^2	4×10^3
2 h	1.5×10^8	1.5×10^2	1.7×10^2	1.5×10^4	1.9×10^8	1.2×10^2	3×10^2	1.5×10^4	1.5×10^6	1.5×10^2	3×10^2	1.7×10^2	5×10^7	3×10^2	3×10^2	6×10^3
3 h	1.5×10^8	3×10^2	3.2×10^2	2×10^4	2×10^8	2.2×10^2	3.2×10^2	2.5×10^4	25×10^7	3×10^3	3×10^2	3×10^2	1.4×10^8	$3. \times 10^2$	3.2×10^2	1.4×10^4
4 h	1.6×10^8	3.5×10^2	3.5×10^2	2.5×10^4	8×10^8	3.2×10^2	3.5×10^2	6×10^4	2.8×10^7	3.5×10^3	3.5×10^2	3.4×10^2	2×10^8	3.1×10^2	3.5×10^2	3×10^4
5 h	1.7×10^8	3×10^3	4×10^3	5×10^3	8×10^8	4×10^3	4×10^3	7.5×10^4	2.9×10^7	0	2×10^3	3×10^3	3×10^8	4×10^3	6×10^3	3.2×10^4
6 h	2×10^8	5×10^2	8×10^2	4×10^3	1×10^9	6×10^2	8×10^2	1.2×10^4	4×10^7	0	1×10^2	5×10^2	4×10^8	7×10^2	8×10^2	3.5×10^3
7 h	2×10^8	3×10^2	7×10^2	4×10^3	1.5×10^9	7×10^2	9×10^2	9×10^3	8×10^7	0	1×10^2	3×10^2	6×10^8	9×10^2	9×10^2	3.5×10^3
8 h	2×10^8	1×10^2	5×10^2	4×10^3	1.3×10^9	5×10^2	9×10^2	9×10^3	3×10^7	0	9 × 10	1×10^2	8.5×10^8	8×10^2	9×10^2	3.2×10^3
9 h	2.5×10^8	1×10^2	3.2×10^2	4×10^3	1×10^9	3×10^2	6×10^2	5×10^3	2.5×10^7	0	7 × 10	1×10^2	5×10^8	5×10^2	6×10^2	3×10^3
10 h	2.7×10^8	1×10^2	1×10^2	4×10^3	9.5×10^8	2.2×10^2	6×10^2	3.5×10^3	2×10^7	0	7 × 10	1×10^2	4×10^8	6×10^2	6×10^2	2.5×10^3
11 h	2.7×10^8	1×10^2	2×10^2	3.5×10^3	9.3×10^8	2×10^2	5×10^2	2.5×10^3	1×10^7	0	7 × 10	1×10^2	2.5×10^8	5×10^2	5.6×10^2	2×10^3
12 h	3×10^8	5×10	9 × 10	3.5×10^3	7.5×10^8	9.5 × 10	9.8 × 10	2×10^3	5×10^6	0	2 × 10	9 × 10	2×10^8	8 × 10	4 × 10^2	1.7×10^3
13 h	7×10^8	4 × 10	9 × 10	3.5×10^3	6×10^8	9.2 × 10	9.9 × 10	1.6×10^3	4×10^6	0	1 × 10	7 × 10	2×10^8	9 × 10	2×10^2	1.5×10^3
14 h	8×10^8	2×10	6 × 10	3.5×10^3	5.9×10^8	7 × 10	9.9 × 10	1×10^3	3.5×10^6	0	1 × 10	6 × 10	2×10^8	9 × 10	2×10^2	1.2×10^3

Our result was supported by a number of previous studies that have reported that various honeys have antibacterial activity. Nzeako and Hamdi [30] studied six commercial honeys and found inhibition of *S. aureus*, *E. coli*, and *P. aeruginosa* occur at all honey concentrations, except 40%. Ceyhan and Ugar [31] tested 84 honeys against eight bacteria and two fungi, showing that honey has broad-spectrum activity. In addition, the antibacterial activity of honey was greater than that attributed to the sugar content of the honey. The antibacterial activity of honey has also been investigated for its potential use in reducing food-borne pathogens [32], preventing catheter exit/entry site infection [33], for the treatment of colitis [34], or even to protect against gastric mucousin *Helicobacter pylori*-induced inflammation [35–37].

All the different concentrations of both honey samples (10–80%) showed growth-inhibitory activity against *E. coli* more than other bacteria tested using the well diffusion method. This was in contrast with the result reported by Hegazi [38] and Hegazi and Fyrouz [39], who reported that the different types of Saudi honey were less inhibitory against *E. coli* than other bacteria. All the bacteria tested were sensitive to local, Yemeni Sidr bee honeys, and cinnamon extract at 40–80% concentrations. The antibacterial activity of Yemeni Sidr bee honey was higher than that obtained by Egyptian local honey. This was discussed by other authors, who reported that variations in honey antibacterial activity were because of changes in the level of hydrogen peroxide achieved and in some cases the level of nonperoxide factors, which are related to the floral source [18]. Molan and Cooper [40] reported that the difference in antimicrobial potency among the different honeys can be more than 100-fold depending on its geographical, seasonal, and botanical source.

The poor activity of the three plant extracts examined in this study may be because of what was mentioned in the literature data as differences in the extract preparation methods. Most often, ethanolic extracts are positioned as more active than aqueous extracts [41].

In the present findings, the MBC value of Sidr honey, local honey, and cinnamon extract samples were in the range of 20–80%. Hern *et al.* [42], Kwakman *et al.* [43], and Lusby *et al.* [44] showed that all honeys tested had some antibacterial action from concentrations as low as 5%; however, the greatest inhibition is observed at 20%.

The present study of bacterial growth patterns and heterotrophic plate counts of *E. coli*, *P. aeruginosa*, *N. meningitides*, and *S. aureus* before and after treatment with Yemeni Sidr bee honey showed that the high

effectiveness of Sidr honey was found to be as follows in descending order: *N. meningitides*, *P. aeruginosa*, *S. aureus*, and *E. coli*. The results in this study were also supported by a similar *in-vitro* antimicrobial study on the activity of honey carried out by Coates *et al*. [45] and Mohapatra *et al.* [46], who observed that honey stopped the growth of *P. aeruginosa* and *E. coli*. Honey has a potent antibacterial activity and is very effective in preventing wound infection [46].

In light of the enormous potential applications of honey within a clinical environment, it is important that research continues not only into those honeys recognized as antibacterial but also into other locally produced, as yet untested, honeys.

Conclusion

Egyptian local and Yemeni Sidr bee honeys and cinnamon extract were effective in inhibiting the *in-vitro* growth of *N. meningitides*, *E. coli*, *S. aureus*, and *P. aeruginosa*, which means that using natural products to overcome pathogenic bacteria is a more safe and valuable way nowadays since the development of the multidrug-resistant phenomenon among pathogenic bacteria.

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Conflicts of interest

There are no conflicts of interest.

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