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Susceptibility of multidrug-resistant enteric pathogenic diarrheal bacteria to Saudi Honey

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Abstract. Infectious diarrhea remains the second leading cause of death in children. Initially, the antibacterial activity of saudi honey and Manuka honey was screened using the agar diffusion assay and the minimum inhibitory concentrations using the micro broth dilutions assay in the presence and absence of catalase. Moreover, the physicochemical properties of all tested honey were evaluated and included: density, pH, carbohydrates, moisture content, and total phenols were determined. Manuka honey diluted in sterile distilled water in the absence of catalase inhibited the growth of bacteria tested at a concentration of 8.3±0.4% v/v. While, the Saudi Talh honey inhibit the bacterial growth at 15.3±0.57% v/v. In the presence of a catalase, the bacterial growth was completely inhibited by Manuka honey at 11.3±0.4% v/v dilutions, whereas Talh honey inhibited bacterial growth at 22.3±0.5% v/v. In the other hand, an artificial honey solution inhibited bacterial growth at 30±0.0% dilutions. The results showed that different types of Saudi honey had antibacterial activities against common pathogenic bacteria causing diarrhea. Further studies are needed to elucidate the underlying mechanism of action and to identify active components in Saudi honey, which are responsible for antibacterial activity.

Keywords: Antibacterial activity, Saudi honey, diarrhea, Talh, Manuka

Introduction

Escherichia coli, Shigella spp, and *Salmonella* are the major causative agents responsible for about one and a half million deaths each year worldwide [1, 2]. In developing countries, shigellosis is one of the major causes of diarrheal diseases. Around 165 million cases of shigellosis are reported worldwide, with the majority of those cases occurring in the developing countries [3] despite global success in its management over the past 30 years[4, 5]. Such bacteria are exclusively human pathogens, spread through the fecal-oral route, and are strongly linked to poor sanitation, lack of clean drinking water, and inadequate hygienic conditions. Nevertheless, occasional cases of diarrhea continue to occur in developed countries due to foreign travel and imported foods [6]. However, the disease's highest burden is in the developing world, particularly in Southeast Asia, including Saudi Arabia, where it has reached endemic levels and resulted in epidemics [3]. Saudi Arabia, especially Jeddah and Mecca regions, is more prone to such diarrheal disease because of the large influx of people from other countries for Hajj and Ummrah. Pilgrims continue to suffer

from gastrointestinal diseases and food-poisoning outbreaks. The prevalence of diarrhea among Hajj pilgrims is around 2% with the highest prevalence of 23% reported among a group of French pilgrims in 2013[7]. In 2017, El Ghany confirmed that bacteria were the key agents identified during the 2011-2013 Hajj seasons when 544 fecal specimens were obtained from pilgrims with clinically identified diarrheal disease from 40 countries. *Salmonella spp, Escherichia coli*, and *Shigella spp*, were the major pathogens involved [8]. In 2018, Sow reported that bacteria were causing about 13.7% of diarrhea during Hajj [9]. So, Multidrug-resistant (MDR) strains of bacteria are rapidly expanding on account of their potential for growth and transmission of exogenous genes linked to mobile genetic components such as transposons, R-plasmids, integrons, and genomic islands associated with bacterial chromosome(s) [10]. It is noted that bacteria is always evolving through mutations to increase their resistance to antibiotics and chemicals to be able to survive [11]. As a result of increased resistance to commonly used antimicrobial agents, other natural products have been sought, such as honey. Where honey has been used throughout the centuries in the treatment of many diseases [12]. In addition, Honey has also been used since ancient times, as a natural sweetener [13, 14], and is recognized for its antibacterial potential [15, 16]. Also, it is known for its ability to be used for topical treatment of burns and wounds [17, 18], gastritis [19, 20], intestinal ulcer [21], and liver illness [22]. Honey has an antimicrobial function that has an inhibitory effect on about 60 bacterial species including anaerobic, aerobic, gram-negative, and gram-positives including multi-drug resistant strains [23, 24]. However, honey hides many wonders with its components and properties. There are many studies in the world where the antibacterial activity of honey was evaluated by multiple *in vitro* assays against pathogenic bacteria [11, 24-26]. However, there is a lack of evidence regarding of honey against bacteria causing diarrhea. Therefore, in this study, the antibacterial activity of seven indigenous honeys collected from different geographical areas of Saudi Arabia was investigated using agar well diffusion assay against selected bacteria in order to screen for the most efficient honey against pathogenic bacteria. The type of honey, which exhibited highest antibacterial efficacy evaluated by the agar well diffusion assay, was further evaluated for minimum inhibitory concentrations against bacteria by micro-broth dilutions assay in standard media with and without the addition of catalase for comparison purposes. Medically graded Manuka honey was used as standard. The clinical isolates were obtained from the Microbiology laboratory, King Abdulaziz University Hospital, Jeddah, Saudi Arabia and identified by 165 rRNA. This is followed by the determination of the susceptibility of twenty bacterial isolates to commonly used antimicrobial agents.

2. Material and methods

Folin-Ciocalteu reagent, phenol, sulfuric acid, Catalase (C1345-10G 2950 units/mg), sucrose, maltose, fructose, and glucose were purchased from Sigma-Aldrich. All chemicals used were of analytical grade.

2.1. Bacterial strains

Twenty stool culture isolates of *Escherichia coli, Shigella sonnei,* and *Salmonella typhimurium* were obtained from King Abdulaziz University Hospital, Jeddah, Saudi Arabia. Re-identification of the isolate was performed via colony morphology, culture characteristics, and biochemical profile. Final identification of organisms was carried out

by16s rRNA using GN ID cards using macrogen system. The isolates were kept at -80 °C in BHI broth (Difco) containing 16% glycerol. A sterile loop was utilized to transfer culture in a glass tube containing 10 mL of BHI broth before one day of the experiment. The culture was incubated for 4 h at 37 °C and then transferred to another fresh BHI broth and incubated at 37 °C without agitation for approximately 18 h.

the methods, adopted by previous studies [28-30]. Medically graded Manuka (UMF18+) honey was purchased from a pharmacy in Jeddah and was used for comparison.

2.3. Simulated honey (Artificial honey)

To assess whether the efficacy of honey is due to its sugar content, we used a simulated honey solution to compare its efficacy with natural honey. This was made by dissolving 1.5 g sucrose, 7.5 g maltose, 40.5 g fructose, and 33.5 g glucose in 17 mL distilled water as

Table 1. Floral source, geographical location, and harvesting season of Saudi honey.

No. of samples	Floral Source	Common name	Botanical name	Geographical location	Harvesting season
Manuka	Manuka	Manuka	*Leptospermum*	New zeland	2018
SI -1	Sidr	Sidr	*Ziziphus*	Rotht Krame . Riyadh	Jun 2018
AC -1	Talh	Talh	*Acacia*	Hail	Jun 2018
SA - 1	Sifi	Sifi	*Bassia scoparia*	Rotht Krame. Riyadh	Jun 2018
BA - 1	Multi-flowers	Al- Bahha	*Multi-flowers*	Al Bahha	Jun 2018
SI - 2	Sidr	Sidr	*Ziziphus*	South of Saudi Arabia	Jun 2018
ACT - 1	Sommra	Sommra	*Acacia tortilis*	South of Saudi Arabia	Jun 2018
AC - 2	Talh	Talh	*Acacia*	Abha	Jun 2018

SI; Sidr, AC; Acacia (Talh), SA; Saffy.BA; Baahah, ACT; Acacia tortilis

2.2. Sample collection

Seven types of local honey were bought directly from beekeepers from different regions of Saudi Arabia produced by *Apis mellifera jemenitica* as shown in Table 1. The *Apis mellifera dementia* is the local bee that has adapted to withstand the dry and hot weather[27]. Identification of the botanical origin of honey samples was done based on geographical areas, blossoming plants, season, color, and aroma of each honey according to described by French *et al* [31].

2.4. Susceptibility testing

The antibacterial susceptibility was assessed by Kirby-Bauer disk diffusion assay in conformity with the Clinical and Laboratory Standards Institute (CLSI) Guidelines using antimicrobial discs (Oxoid, Basingstoke, UK) (CLSI, 2010) [32]. Next antibiotics were used: Amikacin(AK) (30µg), Gentamicin (GM) (10µg), Cefepime (CPM) (30µg), Ticarcillin (TC) (75µg), Piperacillin (PRL) (100µg),

Imipenem (IMI) (10µg), Norfloxacin (NOR) (10µg), Tobramycin (TM) (10µg) Cephalothin (CEF) (30µg), Cefoxitin (FOX) (30µg), Ciprofloxacin(CIP) (5µg), Cefotaxime(CTX) (Mast Diagnostics, Merseyside, UK) (30µg) (Table 2). *E. coli* (ATCC,25922) was used as control.

2.5.Assay of antibacterial activity of honey

2.5.1. Agar well diffusion method

The antibacterial effect of the honey was assessed using the agar well diffusion method according to Smania, *et al*., [33]. A primary honey solution was made by adding 2 g of honey thoroughly mixed honey in standard bottles with 2 mL of sterile water and incubated at 37 ºC for half an hour to help mix by stirring intermittently. A 25% (w/v) of the solution was prepared in sterile water or catalase solution considering that the density of honey is 1.37g to prepare secondary solutions. Catalase solution was made by adding 20 mg catalase to 10 mL of sterile distilled water [28]. Honey's antibacterial activity was determined by agar well diffusion assay as described by Hussain *et al* (2015)[34]. Seven local kinds of honey and one medical graded honey Manuka honey (UMF-18+) were evaluated against twenty bacteria. Overnight culture of target bacteria was inoculated at 10^7 CFU onto Muller Hinton agar plates (20 mL/plate). The inoculated agar plates were punched to produce a 7 mm hole. One hundred microliters of honey's different concentrations (50 and 25 % in catalase solution and sterile water, respectively) were transferred into each allotted well. Sterilized water and catalase solution were used as negative controls and 6% phenol was used as a positive control. The plates were incubated for 24 h at 37 ± 1 ºC under aerobic conditions. Inhibition zones were measured in mm after incubation. The tests were carried out in triplicate.

2.5.2. Micro broth dilution assay

Determination of minimum inhibitory concentrations (MICs) Micro-dilution method as described by NCCLS was used to determine the MICs of each honey sample against the bacteria tested [35]**.** 10 mg of Augmentin was used as control. Sterile distilled water (SDW), used as a negative control. The tests were carried out on the same day using three similar wells in triplicates.

2.6. Physico-chemical characteristic of honey

2.6.1. Determination of density

Using the expression below, the pycnometer ap proach was used to determine the density of the samples [32];

$$P = \frac{(W_2 - W_1)}{V}$$

Where: W_1= Pycnometer mass when empty (g); W_2= Pycnometer mass loaded with the extract of ho ney (g); V = volume of the pycnometer (cm^3).

2.6.2. Determination of pH

The pH was calculated in conjunction with the Swiss Food Guide Briefly, 30% w/v honey was created in 10 mL of sterile distilled water b y the dissolving 3 g of the honey. The pH is registered using a pH meter (Jenway 3510 pH) [36].

2.6.3. Total carbohydrate contents

The phenol-sulfuric acid assay is the most reliable method among all the quantitative assays for total carbohydrate estimation [37, 38]. To carry out the carbohydrate assay, a total of 10 µL of 80% phenol and 1.5 mL concentrated H_2SO_4 were mixed with the honey sample. The reaction mixture was incubated at 95 °C for 5 min then leave to stand for 30 min at room temperature. The absorbance was recorded against a blank at 485 nm. The amount of sugar was calculated from a typical D-glucose curve.

2.6.4. Moisture content determination

The moisture content of all honey samples has been measured by weighing 10 g of the honey in a pre-weighted aluminum drying dish. The sample was dried for six hours under vacuum in an oven at 110 °C [39, 40].

$$\text{Moisture content} = \frac{M1-M2}{M1-M0}$$

Where: M_0 = weight of aluminum dish; M_1 = weight of the fresh sample + dish; M_2 = weight of the dried sample + dish

Table 2. Analyses of bacterial genomes through 165 rRNA sequencing

No.	Description	Max score	Total score	Query cover	E value	Ident	Accession
1	*Escherichia coli -1*	1871	1871	99%	0	99%	MH782077.1
2	*Escherichia coli -2*	1873	1873	100%	0	99%	MF179683.1
3	*Escherichia coli -3*	1694	1694	100%	0	97%	NR_114042.1
4	*Escherichia coli -4*	1718	1718	100%	0	98%	NR_114042.1
5	*Escherichia coli -5*	1334	1334	71%	0	94%	NR_114042.1
6	*Escherichia coli -6*	1692	1692	100%	0	97%	NR_024570.1
7	*Escherichia coli-7*	1792	1792	99%	0	99%	NR_114042.1
8	*Escherichia coli-8*	1727	1727	99%	0	98%	NR_114042.1
9	*Escherichia coli-9*	1483	1483	99%	0	98%	NR_114042.1
10	*Escherichia coli-10*	1842	1842	99%	0	99%	NR_114042.1
11	*Escherichia coli -11*	1157	1157	99%	0	99%	NR_114042.1
12	*Escherichia coli -12*	1762	1762	98%	0	97%	NR_114042.1
13	*Shigella sonnei -1*	712	712	37%	0	94%	NR_104826.1
14	*Shigella sonnei-2*	1886	1886	99%	0	98%	NR_104826.1
15	*Shigella sonnei -3*	1517	1517	100%	0	100%	NR_104826.1
16	*Shigella sonnei-4*	1871	1871	99%	0	98%	NR_114042.1
17	*Salmonella enterica.-1*	1784	1784	99%	0	99%	NR_104709.1
18	*Salmonella enterica.-2*	1784	1784	99%	0	99%	NR_104709.1
19	*Salmonella sp. -3*	946	946	100%	0	97%	MH985331.1
20	*Salmonella Typhimurium-4*	1818	12432	100%	0	97%	CP034968.1

2.6.5. Determination of the total phenolic contents

Ten g of honey was extracted by shaking at 150 rpm for 24 h at 25 ºC with 10 mL of 80% methanol. The Singleton method was adapted to evaluate the total phenolic content [41].

Fifty µL of the methanol extract was mixed with 100 µL Folin-Ciocalteu reagent, 850 µL of distilled water, and allowed to stand for 5 min at ambient temperature. The reaction mixture was then added to 500 µL of 20% sodium carbonate. The absorbance was recorded at 750 nm after 30 min. The total phenolic content was expressed as mg gallic acid equivalent/100 g honey.

Statistical analysis

The IBM Statistical Package for Social Sciences software SPSS (Version 19.0. Armonk, NY: IBM Corp). was used to analyze the data. The mean values of the MICs and the inhibition zone of each honey sample and were calculated. The statistical analyses had been performed using a one-way ANOVA and Kruskal-Wallis test. The difference is considered significant when $P < 0.05$.

3. Results and discussion

3.1. Characteristics of Saudi honey

Seven types of local honey are collected from different geographical locations of the Kingdom of Saudi Arabia. Types of honey and geographical location harvest seasons were shown in Table 1. In Saudi Arabia, there are over 300 bee-associated floral species that include shrubs, herbs, vines, and trees. Nevertheless, Sidr (*Ziziphus spina-christi*), Sumra (*Acacia tortilis*), Talh (*Acacia origena*), Lavendula, and Dahiana (*Acacia asak*) are the most common bee flora in the Al-Baha, Taif, and Aseer regions[42]. *Ziziphus* and *Acasia* are both heat and drought-tolerant species and are located mainly in Saudi Arabia's tropical and subtropical regions[43]. Sidr honey is dark brown in color and is the most popular and expensive because of its unique fragrance and taste[44]. However, honey derived from Acacia species is widely consumed due to the medicinal and nutritional properties. Talh honey color is pale yellow, and the Sumra honey color is dark brown[45]. In Saudi Arabia, honey is not only used as popular food rather consumed as a therapeutic agent for the treatment of several diseases including diarrhea because its healing properties are mentioned in Muslim's sacred book, the Holy Quran, and also in Prophetic Medicine [46].

3.2. Identification of bacterial isolates

The identification of bacterial isolates by the 16s RNA gene from macro-genre (Korea) and analyzed with NCBI GenBank database (htt://www.ncbi.nlm.nih.gov) indicated that out of twenty bacterial isolates, twelve were *Escherichia coli* and four were *Salmonella* and *Shigella* each (Table 2).

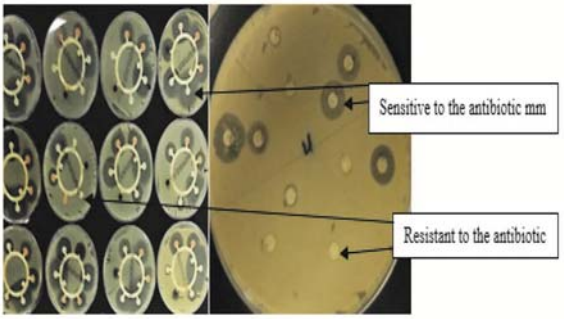

Figure 1. Phenotypic antimicrobial susceptibility profile of tested bacteria (mm)

3.3. Resistance profile of bacterial isolates

The resistance profile of bacterial isolates has been shown in Table 3. Regarding *E. coli*, 91.6% of all isolates tested were resistant to one or more of the antimicrobials tested. 75% of *E. coli* were resistant to Cephalothin (CPF), while 66.6% of this bacteria were resistant to Ticarcillin (TC), and with moderate susceptibility of 41.6% were resistant to both Piperacillin (PRL) Ciprofloxacin (CIP), 33.3% showed resistance to both Cefepime(CPM)

Table 3. Phenotypic antimicrobial susceptibility profile of tested organisms

No	Bacteria	AK	GM	TM	CPM	CTX	CEF	FOX	TC	PRL	IMI	NOR	CIP
1	*Escherichia coli-1*	25±0.57	22±0.5	10±0.5	28±0.5	15±0.4	9±0.5	9±0.5	20±0.5	23±0.6	30±0.55	20±0.54	28±0.6
2	*Escherichia coli-2*	20±0.5	22±0.5	R	13±0.5	9±0.6	R	9±0.55	14±0.6	18±0.5	38±0.5	R	R
3	*Escherichia coli-3*	18±0.5	20±0.6	9±0.6	20±0.6	R	R	R	16±0.6	17±0.6	27±0.6	R	R
4	*Escherichia coli-4*	20±0.57	21±0.6	15±0.6	R	R	R	13±0.5	R	R	30±0.5	R	R
5	*Escherichia coli-5*	18±0.6	19±0.6	13±0.6	25±0.6	13±0.6	R	20±0.5	R	12±0.6	30±0.5	16±0.5	14±0.5
6	*Escherichia coli-6*	21±0.6	22±0.5	12±0.5	25±0.5	18±0.5	R	9±0.6	R	20±0.5	28±0.5	18±0.6	19±0.5
7	*Escherichia coli-7*	21±0.6	23±0.6	12±0.6	28±0.5	17±0.5	R	19±0.5	20±0.5	25±0.5	28±0.6	18±0.6	13±0.5
8	*Escherichia coli-8*	16±0.6	R	13±0.6	R	R	12±0.6	12±0.6	R	R	29±0.6	17±0.6	R
9	*Escherichia coli-9*	18±0.5	20±0.5	13±0.5	R	R±0.5	10±0.6	13±0.5	R	R	30±0.5	10±0.6	R±0.5
10	*Escherichia coli-10*	15±0.5	R	R	R	R	R	14±0.6	R	R	27±0.6	12±0.6	R
11	*Escherichia coli-11*	20±0.5	24±0.5	12±0.5	R	16±0.5	R	17±0.5	R	R	30±0.6	17±0.6	13±0.6
12	*Escherichia coli-12*	22±0.6	16±0.5	17±0.5	25±0.5	12±0.6	R	16±0.6	R	13±0.5	28±0.5	R	12±0.6
13	*Shigella sonnei-1*	20±0.6	20±0.6	R	R	R	R	11±0.5	R	R	30±0.6	R	R
14	*Shigella sonnei-2*	22±0.5	22±0.5	14±0.6	26±0.5	16±0.6	9±0.6	14±0.5	R	18±0.5	28±0.6	16±0.6	R
15	*Shigella sonnei-3*	20±0.5	21±0.5	12±0.5	13±0.5	13±0.5	13±0.5	15±0.5	R	11±0.5	28±0.5	R±0.0	17±0.6
16	*Shigella sonnei-4*	20 ±0.5	23 ± 0.5	R	22±0.5	R	R	10±0.5	12±0.5	22±0.5	28±0.5	10±0.5	R
17	*Salmonella enterica-1*	21±0.6	19±0.6	16±0.5	18±0.6	15±0.5	R	15±0.5	R	12±0.5	29±0.5	17±0.5	R
18	*Salmonella enterica-2*	22±0.6	21±0.6	17±0.5	25±0.6	18±0.5	R	13±0.5	18±0.5	22±0.5	28±0.5	12±0.6	13±0.5
19	*Salmonella sp. -3*	22±0.5	21±.057	R	30±0.5	18±0.5	R	R	20±0.5	25±0.5	26±0.5	25±0.57	R
20	*Salmonella Typhimurium-4*	22±0.6	18±0.6	22±0.5	22±0.6	12±0.5	R	R	R	14±0.5	27±0.5	20±0.6	R

Zone of inhibition (mm) (average ± standard deviation, n = 3), Antibiotics, Amikacin (**AK**) (30µg), Gentamicin (**GM**) (10µg), Cefepime (**CPM**)

and Cefotaxime(CTX). *E. coli* tested strains were the least resistant (16.6%) to Tobramycin (TM) and Gentamicin (GM). On the other hand, all tested *E. coli* strains were susceptible to Amikacin (AK) and Imipenem (IMI) (Figure 1).

Regarding *Shigella sonnei*, 66.6% of all isolates tested were resistant to one or more of the antimicrobials tested. 75% of *Shigella sonnei* were resistant to Ticarcillin (TC) and Ciprofloxacin (CIP), tested strains were least resistant to Cefepime (CPM), Piperacillin (PRL) and Norfloxacin (NOR), (25 %), and with moderate susceptibility of 50% to both Tobramycin (TM), Cefotaxime (CTX) and Cephalothin (CPF). On the other hand, all tested *Shigella sonnei* strains were susceptible to Amikacin (AK) and Imipenem (IMI), and Gentamicin (GM). Regarding *Salmonella* spp. hundred percent resistance was noted against Cephalothin (CPF), and with moderate susceptibility of 50% against both Ticarcillin (TC) and Ciprofloxacin (CIP), while 25% of them were resistance to Tobramycin (TM).

These results are in good agreement with previous studies that have found that *E.coli* (ATTC) was sensitivity to all of the antibiotics [47-49]. Also, this study showed that all bacteria tested sensitive to Amikacin (AK), and Imipenem (IMI). With varying rates between the highest value of (38±0.5 mm) and the lowest value (20±0.5 mm) of IMI and AK antibiotics, respectively (Table 3). While, in this study that bacterial multidrug-resistant and resistant to Tobramycin (TM), Cephalothin (CPF), Cefoxitin (FOX), and Ciprofloxacin (CIP) Norfloxacin (NOR). In addition, several studies also indicated resistance to antibiotics such as Cephalothin and Tobramycin [50, 51]. This raises fears of overuse, abuse, and/or misuse to use antibiotics without regulation and/or supervision.

Escherichia coli, Shigella sonnei, and *Salmonella typhimurium* used in this study were multidrug-resistant and resistant to Tobramycin, Cephalothin, Cefoxitin, and Ciprofloxacin. MDR *S. sonnei* strains are quite common and have been reported in many parts of the world [10, 52]. Another worrisome development is the emergence of carbapenemase-producing and pan-resistant Enterobacteriaceae in many parts of the world, including Saudi Arabia [53, 54]. Under these alarming situations, honey offers the best possible alternative because it is broad-spectrum and inhibits the growth of pathogenic bacteria which may be sensitive MDR resistant or pan-resistant at almost the same concentrations[55]. Bacterial resistance to honey has not been documented anywhere in the world [56].

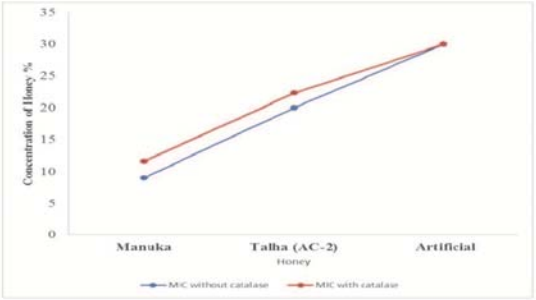

Figure 2. Minimum inhibitory concentration (MIC) (%v/v) of Manuka honey, Talh honey, and Artificial honey against bacteria with and without catalase.

Table 4. Inhibition zone (mm) of Saudi honey samples at 50%, 25% (w/v) dilution in sterile distilled water and 50%, 25% (w/v) dilution in catalase solution by agar well diffusion method against *Shigella sonnei*, *Salmonella typhimurium* and *Escherichia coli.*

Honey samples	Inhibition zone (mm)											
	50% in sterile distilled water			25% in sterile distilled water			50% in catalase			25% in catalase		
	S. sonnei	*S. typhimurium*	*E.coli*	*S. sonnei*	*S. typhimurium*	*E.coli*	*S. sonnei*	*S. typhimurium*	*E.coli*	*S. sonnei*	*S. typhimurium*	*E.coli*
Manuka	14 ± 0.5	13 ± 0.5	11 ± 0.5	10 ± 0.5	10 ± 0.5	9 ± 0.5	11 ± 0.5	12 ± 0.5	10± 0.5	9 ± 0.28	9 ± 0.5	0
SI -1	10 ± 0.57	10 ± 0.5	0	0	0	0	0	0	0	0	0	0
AC - 1	9 ± 0.2	11 ± 0.5	10 ± 0.5	0	0	0	0	0	0	0	0	0
SA - 1	10 ± 0.5	12 ± 0.5	9 ± 0.5	0	0	0	0	0	0	0	0	0
BA - 1	0	0	0	0	0	0	0	0	0	0	0	0
SI - 2	0	0	0	0	0	0	0	0	0	0	0	0
ACT - 1	0	0	0	0	0	0	0	0	0	0	0	0
AC - 2	17 ± 0.5	16 ± 0.5	18 ± 0.5	13 ± 0.5	14 ± 0.5	16 ± 0.5	0	0	0	0	0	0

*The values represent mean ± standard deviation of three replicates, SI; Sidr, AC; Acacia (Talh), SA; Saffy,BA; Baahah, ACT; Acacia tortilis. *S. sonnei; Shigella sonnei.* ; *Salmonella typhimurium. E.coli; Escherichia coli.*

3.4. Screening antibacterial activity of honey by agar well diffusion assay

Results obtained through screening assay (agar well diffusion assay) have shown that there is a lot of variation in the size of the zone of inhibition of tested honey against *Escherichia coli, Salmonella typhimurium,* and *Shigella sonnei* . A statistically significant difference (Kruskal-Wallis test, p=.002) was also noted among the mean inhibition zone of tested honey against bacteria (Table 4). This means that there is a significant difference in the level of antibacterial activity of indigenous honey. Multi-flowers (BA-1), collected from the Al-Bahha region, Sidr (SI-2) and Samara (ACT-1) from the Southern region of Saudi Arabia did not produce any inhibition zone in all tested dilutions, indicating an absence of antibacterial activity in these honey samples. Talh (AC-2) honey collected from Abha produced the highest inhibition zone as compared to other local honey as well as from Manuka honey (Table 4). Most of the tested honey exhibited variable activity against *Shigella sonnei,*

Escherichia coli, and *Salmonella typhimurium*. One way-ANOVA revealed that significant variations in the inhibition zone against all bacteria. This means that there is a significant difference in the level of antibacterial activity of indigenous honey against tested pathogens. The range of inhibition was between 13.5 ± 0.5 - 18± 0.57 mm for the concentration of 50-25% against three tested bacteria compared to Manuka 9 ± 0.57 – 14 ± 0.5 mm at these concentrations (Table 4). The Kruskal -Wallis Test showed that there is a significant difference in the level of antibacterial activity of Talh and Manuka honey (*p-value =.0001*).

The results indicated that Talh (AC-2) honey has a higher total or hydrogen peroxide related antibacterial activity than Manuka honey. In contrast, none of the indigenous honey samples showed antibacterial activity in the catalase solution. This shows that the absence of plant-derived antibacterial substances in local honey. However, four indigenous honey showed a zone of inhibition at 50% (w/v) dilutions and only one showed a zone of inhibition at 25% (w/v) dilutions. This shows that antibacterial activity in this honey is mainly derived from the hydrogen peroxide levels and high sugar content. Manuka honey exhibited an inhibition zone against bacteria at all tested dilutions both in water and catalase solution. This means that Manuka honey is a possessor of both hydrogen peroxide and plant-derived antibacterial substances. Methylglyoxal has been identified as an active antibacterial compound in Manuka honey, which is derived from plant sources [57].

The variation of antimicrobial activity among the different types of honey is related to their botanical sources, type of honey bee, soil composition, climatic conditions, harvesting, processing time, and storage conditions [58].

The absence of antibacterial activity of honey may result from poor processing or storage conditions. Heating of honey can destroy the hydrogen peroxide related to its antibacterial activity because the oxidase enzyme is protein and, thus, is heat-sensitive [59]. Sidr, Talh, Sifi and Talh 2 kinds of honey exhibited higher antibacterial activity than Baha, Sidr 2, and Acacia tortilis honey. Previous studies also showed the effectiveness of Saudi honey against the tested pathogens [60-63], Antibacterial activity of the different types of honey may be due to the presence of some of the bio-active compounds, hydrogen peroxide and others [64, 65]. Several studies revealed that honey collected from Saudi and other regions like New Zealand, Egyptian and Yemeni Honey has a better antibacterial efficacy [24, 49, 50, 64, 66].

3.5. Minimum inhibitory concentrations of Saudi honey

Talh honey (AC-2), which showed a larger zone of inhibition against the tested bacteria in agar well diffusion assay as compared to other honeys including Manuka honey was further evaluated for its antibacterial activity by microdilution assay, which is a more sensitive method (Fig 3). Minimum inhibitory concentrations of Talh, Manuka, and Artificial honey were evaluated using twenty bacteria (*Shigella spp, Escherichia coli,* and *Salmonella spp*) by this assay (Table 5). Manuka honey inhibited the growth of all bacteria at a mean of 8±0.3% (v/v), whereas Talh and Artificial honey at 16±0.8% and 30±0.0% respectively, without catalase (Fig 4). These values represent the overall or combined antibacterial activity of tested honey against *Shigella sonnei, Escherichia coli*, and *Salmonella typhimurium* of all factors present in honey samples. The addition of catalase in honey samples reduced the level of antibacterial activity in both Talh and Manuka honey. These results found that the concentration was enhanced in the presence of catalase to (11±0.45 %) in the case of Manuka honey. Whereas the bacteria treated with Talh honey with catalase was inactivated at a concentration up to (21 ±0.95%). while Artificial honey inhibited it with and without catalase at a concentration of 30±0.0 % against all other tested bacteria (Fig 5). The antibacterial activity of artificial honey remained the same after the addition of

Table 5. Minimum inhibitory concentration (MIC) (%v/v) of Manuka honey, Talh honey, and Artificial honey against selected bacteria with and without catalase.

		MIC without catalase			MIC with catalase		
	Bacteria	Manuka	Talh	Artificial	Manuka	Talh	Artificial
1	*Escherichia coli-1*	8.6±0.4	15.6±0.57	30±0	11.6±0.4	21.6±0.5	30±0
2	*Escherichia coli-2*	8.3±0.4	14.6±1	30±0	11.3±0.4	22.3±1	30±0
3	*Escherichia coli-3*	8.3±4	14.3±1	30±0	11.3±0.4	21.3±0.5	30±0
4	*Escherichia coli-4*	8.3±0.4	15.3±1	30±0	11.3±0.4	21.6±0.5	30±0
5	*Escherichia coli-5*	8.6±0.4	15.3±0.57	30±0	11.6±0.4	23.6±0.5	30±0
6	*Escherichia coli-6*	7.3±0.4	15.3±0.57	30±0	10.6±0.94	22.3±0.5	30±0
7	*Escherichia coli-7*	8.3±0.4	15.6±0.57	30±0	10.3±0.4	22.3±0.5	30±0
8	*Escherichia coli-8*	8.6±0.4	15.3±1	30±0	11.6±0.4	22.6±0.5	30±0
9	*Escherichia coli-9*	8.6±0.4	16.3±1	30±0	11.6±0.4	22.3±0.5	30±0
10	*Escherichia coli-10*	8.3±0.4	15.3±1	30±0	11.3±0.4	21.6±0.5	30±0
11	*Escherichia coli-11*	7.3±0.4	15.3±0.57	30±0	10.6±0.94	22.3±0.5	30±0
12	*Escherichia coli-12*	8.3±0.4	15.3±0.57	30±0	11.3±0.4	21.3±0.5	30±0
13	*Shigella sonnei-1*	7.6±0.4	16.6±0.57	30±0	11.6±0.4	22.6±0.5	30±0
14	*Shigella sonnei-2*	8.6±0.4	15.6±0.57	30±0	11.3±0.4	22.6±0.5	30±0
15	*Shigella sonnei-3*	8.6±0.4	18.3±0.57	30±0	12.3±0.4	22.6±0.5	30±0
16	*Shigella sonnei-4*	8.6±0.4	17.3±0.57	30±0	12.3±0.94	22.3±0.5	30±0
17	*Salmonella enterica-1*	8.3±0.4	15.3±0.57	30±0	12.3±0.94	21.6±0.5	30±0
18	*Salmonella enterica-2*	9.3±0.9	15.3±1	30±0	11.3±0.4	21.3±0.5	30±0
19	*Salmonella sp. -3*	7.6±0.4	15.6±0.57	30±0	10.3±0.4	22.3±0.5	30±0
20	*Salmonella Typhimurium-4*	8.6±0.4	16.6±0.57	30±0	12.3±0.94	22.3±0.5	30±0

catalase. This means that hydrogen peroxide has an important role in the total antibacterial activity of both Manuka and Talh honey, whereas there is an absence of hydrogen peroxide in artificial honey. Moreover, both the Talh and Manuka honey also inhibited the bacteria tested at a lower concentration as compared to artificial honey even after the addition of catalase, meaning that both Talh and Manuka also contain non-peroxide factors besides the hydrogen peroxide. The results showed that honey's antibacterial activity is not just related to high osmolarity, but also due to the hydrogen peroxide and non-peroxide factors. These results are by previous studies[67, 68]. However, interestingly in

comparison to agar well diffusion assay where none of the indigenous honey revealed an antibacterial activity in catalase solution whereas, in this assay (microdilution), only Talh honey had non-peroxide activity.

This demonstrates that agar well diffusion assay is a less sensitive technique and could not detect non-peroxide factors present in Talh honey. This reason could be attributed to low concentrations of non-peroxide factors or the presence of larger size antibacterial substances present in Talh honey which were unable to diffuse in agar assay. Therefore, it is important to identify plant-derived antibacterial substances in Talh honey in future studies. All the three tested honey had also statistically significant variation in minimum inhibition concentration (*p-value =0.023*). Previous studies have also shown similar results and these differences are due to variation in the level of acidity, amount of sugar bio-active compounds, hydrogen peroxide, and non-peroxide factors[64, 65, 69].

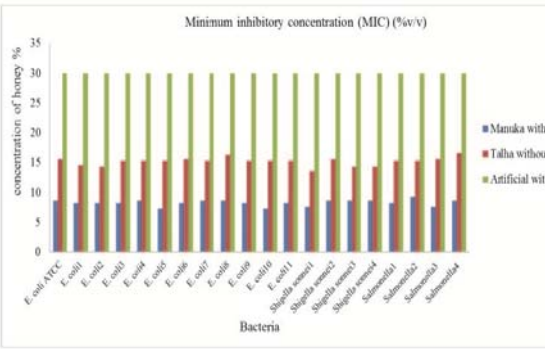

Figure 3 Minimum inhibitory concentration (MIC) (%v/v) of Manuka honey, Talh honey, and Artificial honey against selected bacteria without catalase.

3.6. The physical-chemical properties

The physicochemical properties of the three types of honey samples tested are summarized in Table 6. The mean honey densities (g/cm3) ranged from 1.44 ± 0.04 for Manuka, 1.50 ± 0.04 for Talh, and 1.47 ± 0.04 for Sidr honey, with significant differences ($p < 0.05$). Additionally, these results are in good agreement with previous studies that have found honey samples from Mambilla Plateau honey to be in the range of 1.30 -1.51g/cm3 [70]. The local Saudi honey was less acidic with a mean pH value of 4.86 ± 0.06 (Talh) and 5 ± 0.2 (Sidr) compared to 4 ± 0.04 for Manuka honey. The pH values for this study were in agreement with those values investigated for samples from Lagos, Osun, Ogun, (4.93, 5.05, 4.55) [71, 72]. Even the pH value of the sample from Unayza (4.58) Al-Qassim region of Saudi Arabia reported by Sanz et al. [73] was lower than the values obtained from this study area.

Table 6. Physicochemical parameters of honey samples (average ± standard)

Parameters	Manuka (M)	Acacia (Talh)	Ziziphus (Sidr)
Density (g/cm^3)	1.44 ± 0.04	1.50 ± 0.04	1.47± 0.04
Moisture %/100 g	17.30 ± 0.00	19± 0.00	14.6± 0.00
pH	4.40 ± 0.04	4.86 ± 0.06	5 ± 0.2
Carbohyderate Content %/100 g	82.00 ± 0.58	75.6 ± 0.5	81.14± 0.5
Total phenol (mg GAEs/100g)	59.6 ± .019	94.4± .02	25.3 ± .02

Carbohydrates are the main component of honey and most of this consists of sugars. The mean carbohydrate content of 100 g of honey with values of 82 ± 0.58% (Manuka), 75.6 ± 0.5 % (Talh), and 81.14± 0.5 % (Sidr) was not significantly different. Furthermore, the mean percentage of water contents (moisture) %/100 g of honey was 17.30 % (Manuka), 19%

(Talh), and 14.60 % (Sidr). Several studies reported that the moisture contents were 15.7, 14.8, 14.45, and 15.95% for honey obtained from Buridah, Uyanza, AlMalida, and Al-Midhnab in Saudi Arabia respectively [73].

Honey is a natural substance appreciated for its therapeutic abilities since ancient times. The phenolic content in honey plays a key role in human health, thanks to the high antioxidant and anti-inflammatory properties that they exert. The results for the total content of phenolics of the honey in the three types (Manuka, Talh, and Sidr) are presented in Table 6. A major variation in terms of total phenolic content was observed among the honey samples (59.6, 94.4, and 25.3 mg GAE/100g honey) Manuka, Talh, and Sidr, respectively. Talh had a high level of total phenolics in comparison with Sidr honey. The results of this study revealed that the tested Talh honey sample contained the higher phenolic contents compared to the Malaysian Tualang and Gelam honey (87.7±4 mg GAE/100g and 48.4±4 mg GAE/100g, respectively) [74, 75]. Although the Folin

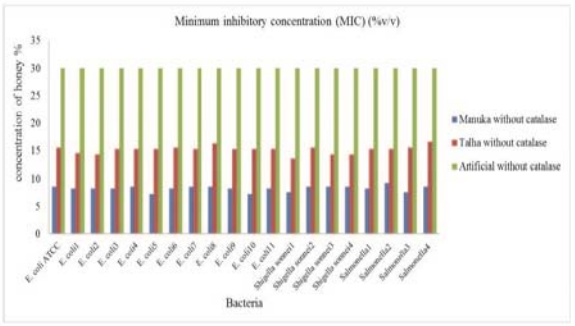

Figure 4 Minimum inhibitory concentration (MIC) (%v/v) of Manuka honey, Talh honey, and Artificial honey against selected bacteria with catalase.

Ciocalteu assay method is widely used to evaluate total phenolic compounds in plant extracts and honey samples, compounds were overestimated by the phosphotungstic acid and phosphomolybdic acid mixture which interact with other non-phenolic reduction compounds such as ascorbic acid, some sugars, and amino acids known to interfere with the test results [74, 76, 77]. Nonetheless, the method remains useful and is primarily used to evaluate the relative content of total polyphenolic compounds in honey sample varieties from various floral origins.

4. Conclusion

The resistance of bacteria to antibiotics has caused global concern. So, it is necessary to look for an alternative antibiotic. The presence of such a large amount of antibacterial properties in the honey product makes it a promising candidate for the treatment of diarrheal diseases. Both kinds of honey have effectively inhibited the growth of bacteria in standard media without catalase as well as in catalase solution. The results indicate that the oral administration of honey for the treatment of infectious diarrhea caused by Multidrug-resistant (MDR) bacteria could be an effective and safe alternative. However, Manuka honey is more effective against the tested bacteria as compared to Talh honey. Further studies are required to evaluate the effectiveness of honey *in vivo* models of infectious diarrhea. Also, it requires more in-depth studies to determine the active ingredients in the Saudi honey responsible for this antibacterial activity.

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حساسية بكتيريا الإسهال المعوية الممرضة والمقاومة للعديد من الأدوية للعسل السعودي

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مستخلص. لا يزال الإسهال المعوي هو السبب الرئيسي الثاني لوفاة الأطفال. تم في البداية فحص النشاط المضاد للبكتيريا للعسل السعودي وعسل مانوكا باستخدام طريقه الانتشار وطريقه تقدير ادنى تركيز مثبط باستخدام بيئه المرق المخففه في وجود وغياب انزيم الكاتاليز. علاوة على ذلك، تم تقييم الخواص الفيزيائية والكيميائية لجميع أنواع العسل المختبرة وتضمنت: الكثافة، ورقم الأس الهيدروجيني ، والكربوهيدرات، ومحتوى الرطوبة، وإجمالي الفينولات.

عسل المانوكا المخفف في الماء المقطر في غياب الكاتاليز ثبط نمو البكتريا المختبره عند تركيز 0.5 ± 8.3 v/v بينما تحت نفس الظروف وجدنا عسل الطلح السعودي ثبط نمو البكتريا عند تركيز 0.57 ±15.3 v/v وفي وجود محلول الكاتاليز تم منع نمو البكتريا تماما بواسطه عسل المانوكا عند تخفيف 0.4 ± 11.3 v/v بينما منع عسل الطلح نمو البكتريا عند تخفيف 0.5 ± 22.3 v/v عند نفس الظروف .وعلى الجانب الاخر، منع محلول العسل الاصطناعي نمو البكتريا عند تخفيف 0.0%±30. أظهرت النتائج أن أنواع مختلفة من العسل السعودي لها نشاط تثبيطي ضد البكتيريا الممرضه المسببة للإسهال. هناك حاجة إلى مزيد من الدراسات لتوضيح آلية العمل الأساسية وتحديد المكونات النشطة في العسل السعودي، والتي تعتبر مسؤولة عن النشاط المضاد للبكتيريا.

الكلمات الدالة: نشاط مضاد للبكتريا، عسل سعودي، إسهال، طلح، مانوكا