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ORIGINAL ARTICLE

Evaluation of Selected Pakistani Honeys in Comparison with Manuka Honey Against *Vibrio cholerae*

Muhammad Barkaat Hussain*
Department of Microbiology, Faculty of Medicine, King Abdulaziz University, Rabigh Branch 21589, Saudi Arabia

ABSTRACT

Background: Antibacterial resistance in *Vibrio cholerae* has been reported in many parts of the world. Therefore, it is important to explore novel therapies which stand less chances of developing antimicrobial resistance. In this regard honey is getting worldwide attention because antibacterial resistance against honey is unlikely.

Objectives: To determine the antibacterial activity of locally produced Sidr (*Ziziphus jujuba*), Kalonji (*Nigella sativa*) and Eucalyptus (*Eucalyptus* spp) honey against twenty-six clinical isolates of *Vibrio cholerae* to compare the antibacterial activity of indigenous honey with medically graded Manuka honey.

Methodology: Identification of *Vibrio cholerae* was done by standard cultural, biochemical and serological methods. Susceptibility pattern of *Vibrio cholerae* was also determined. Minimum inhibitory concentrations (MIC) of locally produced Sidr, Kalonji and Eucalyptus honey, and medically graded Manuka honey was determined by agar dilution and kirby bauer test. American Type Culture Collections (ATCC) *Escherichia coli* 25922, *Staphylococcus aureus* 25923 and *Acinetobacter baumannii* 29213 were used as standard control strains.

Results: Manuka and Eucalyptus honey have comparable antibacterial activity against both sensitive and resistant clinical isolates of *Vibrio cholerae*. The lowest MICs were between 3.7 to 4% for medically graded Manuka honey, whereas Eucalyptus honey inhibited between the range 4 to 4.3%. Kalonji and Sidr honey inhibited these isolates between 6.7 to 7.0% and 6.3 to 7.0%, respectively.

Conclusion: It is concluded that Manuka and Eucalyptus honey could be evaluated in a clinical trial for the treatment of gastroenteritis caused by *Vibrio cholerae*.

Keywords
Honey, Vibrio cholerae, antibacterial activity, antibacterial resistance, eucalyptus honey

*Address of Correspondence
mbhussain1@kau.edu.sa

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INTRODUCTION

Cholera is a major life-threatening diarrheal disease confronting developing countries, particularly where proper sanitation, hygienic water and food are not available[1]. It affects approximately 2.86 million people and accounts for 95000 deaths annually in developing countries[2]. However, these figures do not reflect the true global burden of the disease because the majority of cholera cases are not reported due to various reasons[3]. According to WHO, only small fractions of cases are actually reported[4]. Medical advice for mild to moderate cases is usually not sought. Stool samples are not routinely cultured for identification of *Vibrio cholerae*[5]. Therefore, without laboratory isolation of the bacteria, the symptoms of cholera are difficult to distinguish from other causes of diarrhea. Moreover, poor epidemiological surveillance and economic disincentives for reporting also contribute to low reporting rate[2].

In Pakistan, cholera remains one of the major cause of morbidity and mortality among susceptible individuals[6].

Various reports suggest that *Vibrio cholerae* El Tor, Ogawa is endemic in Pakistan[7]. Contaminated water or food is responsible for the spread of infection. The bacterium colonizes the small intestine after passage through the gastric barrier and produces massive secretory diarrhoea and vomiting[8]. Fluid and electrolytes replacement is the primary treatment, however, in severe cholera, antibiotics are recommended to reduce the duration of illness and excretion of *Vibrio cholerae*[8]. Recently multidrug-resistant *Vibrio cholerae* has been reported in many parts of the world[9]. Moreover, prolonged antibiotic therapy inhibits the growth of beneficial flora of the gut[10]. The rise in multi-drug resistant *Vibrio cholerae* and associated side effects of antibiotics has led to the development of new therapeutic agents effective against *Vibrio cholerae*.

Honey compared to antibiotics has multiple advantages. Antibacterial resistance to honey has not been reported so far because it contains multiple bees and plants derived antibacterial substances[11]. Hydrogen peroxide is one of the important bio-active antibacterial compounds produced by oxidase enzyme (bee origin), which converts water and sugar into hydrogen peroxide and gluconic acid[12]. The enzyme remains inactive in undiluted honey and becomes maximally active when honey is diluted between 40%-60%[13]. Therefore, diluted honey may be more effective in the treatment of diarrhoea caused by *Vibrio cholerae*. Besides, there are numerous plants and bees derived antibacterial substances present in honey like flavonoids, phenolic acids, methylglyoxal, bee defensin-1, etc.[14] These substances target different sites of bacterial structure and generate synergistic effects[15]. This might be the reason that bacterial resistance to honey has never been reported. Because of these unique characteristics, honey could serve as a potential therapeutic agent for multi-drug resistant *Vibrio cholerae*. The benefits of honey are not just limited to antibacterial activity, rather it also contains beneficial lactic acid bacteria originated from honey bee stomach which includes lactobacilli and bifidobacteria[16]. The beneficial flora inhibits the attachment of pathogenic bacteria on epithelial lining of the intestinal tract and secretes antibacterial substances which have inhibitory growth effects on pathogenic bacteria[17]. Honey also contains undigested oligo polysaccharides knows as prebiotic which enhances the growth of normal flora (probiotics) of gut[18].

The level of antibacterial activity and composition of honey varies greatly and depends on the type of plant species, geographical areas, soil composition, climatic conditions and processing of honey[19]. This could also be a disadvantage of using honey. It is important to evaluate the antibacterial activity of indigenous honey because they are easily available and affordable to the local population.

In this study, the minimum inhibitory concentrations of three common locally produced honeys have been determined and comparison of their antibacterial activity with medically graded Manuka honey have been conducted.

MATERIALS AND METHODS

In this study, twenty-six *Vibrio cholerae* were isolated from stool cultures. The isolates were obtained from the National Institute of Health (NIH), Islamabad (n=15), Civil Hospital, Mirpur Khas (n=6) and Combined Military Hospital, Lahore (n=5). Identification of clinical isolates was re-confirmed by growing them on McConkey's agar, Thiosulphate citrate bile salt sucrose (TCBS), Deoxycholate citrate agar (DCA) and Blood agar. Pale coloured colonies from MacConkey agar, β-hemolytic colonies from Blood agar and yellow sucrose fermenting colonies from TCBS were selected and gram stained. Distilled water immobilization was performed and their motility was observed by hanging drop preparation. Oxidase and catalase tests were performed for identification. Biochemical identification was done by API 20NE (Biomerieux). Polyvalent and monovalent antisera (BD difco®) were used for serological confirmation. Biotype was determined on the basis of sensitivity to polymyxin B (300 IU), beta-hemolysis on sheep blood agar, Voges-Proskauer test, String test, CAMP test and agglutination of chicken red cells as Bergey's manual of determinative bacteriology.

Antimicrobial Susceptibility Test

Antibiotic-resistant profile of clinical isolates were calibrated with 0.5 Macfarland index tube was determined by Kirby-Bauer disc diffusion method on Mueller-Hinton (MH) agar. The antibiotic tested were amikacin, ampicillin,

aztreonam, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, cotrimoxazole, imipenem, levofloxacin, moxifloxacin, nalidixic acid, ofloxacin and tetracycline. ATCC 25922 *Escherichia coli* was used as a quality control strain[20].

Honey Samples

Locally produced Kalonji, Eucalyptus and Sidr honey were obtained from local apiarists. The floral identification was performed by the beekeepers based on their geographical areas, floral availability at the location of beehives (foraging radius) and season[19]. Medically graded Manuka honey (unique manuka factor-21+) derived from Manuka tree indigenous to New Zealand was included in the study for comparison.

Agar Dilution Assay

Susceptibility of *Vibrio cholerae* and ATCC reference strains to honey was evaluated by agar dilution assay as adopted by French *et al.* (2005)[21]. A stock solution of honey was prepared at 20% (v/v) and 50% (v/v) in sterile distilled water. Appropriate volumes of the honey stock solutions, double strength Mueller-Hinton (MH) agar (Thomas scientific) and sterile distilled water were mixed to obtain 1-20% incremental dilutions. These dilutions were kept at 50ºC for 20 minutes and vortex vigorously to achieve uniform homogenization. The different dilutions were dispensed into Petri dishes (Thomas scientific) in triplicate and allowed to dry for 20 minutes.

Working bacterial culture of each clinical isolates and reference strains was prepared in TSB and adjusted to 0.5 McFarland's standard (1×10^8 CFU/ml). A volume of 3µl from each adjusted culture was inoculated onto the agar plates with multi-point inoculator (Akribis Scientific Limited). The inoculated agar plates were incubated for 18 hours at 37ºC. The MIC was considered to be the lowest concentration of honey at which visible growth of bacteria was inhibited.

RESULTS

Statistical Analysis

The data were analyzed by the Statistical Package for Social Sciences (SPSS 23.0). The arithmetic mean of minimum inhibitory concentrations (MICs) and SDs of mean values of honey was calculated. The variances of mean MICs among tested honey samples were detected by applying the Kruskal-Wallis test. Bonferroni post hoc test is applied for pairwise comparison between different kinds of honey. Differences were considered significant at $p < 0.05$. All clinical isolates of *Vibrio cholerae* were identified as *Vibrio cholerae* O1 Biotype El Tor Serotype Ogawa. Table **1** & **2** show the susceptibility pattern of twenty-six clinical isolates of *Vibrio cholerae*. Twenty isolates were resistant to cotrimoxazole and nalidixic acid, two isolates were nalidixic acid-resistant and four were sensitive to all antibiotics tested.

Manuka and Eucalyptus honey have comparable antibacterial activity against both sensitive and resistant clinical isolates of *Vibrio cholerae* (Table **2**). The lowest MICs were 3.7 to 4% for medically graded Manuka honey against clinical isolates of *Vibrio cholerae*, whereas Eucalyptus honey inhibited between the range 4 to 4.3%. Kalonji and Sidr honey inhibited these isolates between 6.7 to 7.0% and 6.3 to 7.0%, respectively. There is variation between the levels of antibacterial activity of different tested honeys. Manuka honey also inhibited ATCC 25923 *Staph aureus* at lowest MIC 4%, whereas Eucalyptus honey had more inhibitory against ATCC 29213 *A. baumannii* and ATCC 25922 *E. coli* in comparison with Manuka and other honey (Table **3**). Generally, the most susceptible organisms were *Vibrio cholerae* and *Staph aureus* and the least susceptible organisms were *E. coli* (Table **3**).

There is a significant difference (Kruskal-Wallis Test, p=0.000) among the mean minimum inhibitory concentrations (MICs) (%v/v) of Kalonji, Eucalyptus, Sidr and Manuka honey against clinical isolates of *Vibrio cholerae*. There is a significant difference (p=0.00, Bonferroni post hoc test) between mean MICs of Manuka and Kalonji, Manuka and Sidr honey against *Vibrio cholerae*. Similarly, there is a significant difference (p=0.00, Bonferroni post hoc test) between MICs of Eucalyptus and Kalonji, Manuka and Sidr honey. However, there is no difference (p=0.061) between the MICs of Manuka and Eucalyptus honey (Table **4** & Figure **1**). Regarding ATCC reference strains significant difference (p=0.039) was recorded among mean MICs of Manuka, Eucalyptus, Sidr and Kalonji honey. There is a significant difference (p=0.040) between mean MICs of Eucalyptus and Sidr honey against ATCC reference

strains. There is also a significant difference (p=0.014) between the mean MIC of Eucalyptus and Kalonji honey.

against ATCC reference strains (Table **5** & Figure **2**).

Table 1. Summary of Antimicrobial Resistance Profile of *Vibrio cholerae* Isolates.

Number of Isolates (n)	Antimicrobial Resistance Pattern
4	S
20	SXT^R, NA^R
2	NA^R

SXT: cotrimaxazole, NA: nalidixic acid, S: sensitive, R: resistant.

Table 2. Susceptibility Pattern of *Vibrio cholerae* and Minimum Inhibitory Concentrations MICs (%v/v) of Honeys.

Species	Resistant Profile	MICs*			
		Kalonji	Eucalyptus	Sidr	Manuka-21
Vibrio cholerae-1	SXT^R, NA^R	6.7±0.5	4.3±0.5	6.7±0.5	Manuka-21
Vibrio cholerae-3	S	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-4	SXT^R, NA^R	6.7±0.5	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-7	SXT^R, NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-9	SXT^R, NA^R	6.7±0.5	4.0±0.0	7.0±0.0	3.7±0.5
Vibrio cholerae-10	SXT^R, NA^R	6.7±0.5	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-13	SXT^R, NA^R	7.0±0.0	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-15	SXT^R, NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-16	SXT^R, NA^R	6.7±0.5	4.3±0.5	6.3±0.5	4.0±0.0
Vibrio cholerae-17	S	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-18	SXT^R, NA^R	6.7±0.5	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-19	SXT^R, NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-20	S	6.7±0.5	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-21	NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-23	SXT^R, NA^R	6.7±0.5	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-31	NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-32	SXT^R, NA^R	6.7±0.5	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-25	SXT^R, NA^R	7.0±0.0	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-33	SXT^R, NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-27	SXT^R, NA^R	6.7±0.5	4.3±0.5	7.0±0.0	3.7±0.5
Vibrio cholerae-30	SXT^R, NA^R	7.0±0.0	4.0±0.0	7.0±0.0	4.0±0.0
Vibrio cholerae-27	S	7.0±0.0	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-26	SXT^R, NA^R	7.0±0.0	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-28	SXT^R, NA^R	6.7±0.5	4.3±0.5	7.0±0.0	4.0±0.0
Vibrio cholerae-24	SXT^R, NA^R	6.7±0.5	4.0±0.0	6.3±0.5	3.7±0.5
Vibrio cholerae-24	SXT^R, NA^R	6.7±0.5	4.0±0.0	7.0±0.0	4.0±0.0

*MIC values are the mean of triplicate determinations, and shown as Mean ± SD. S: sensitive, R: resistant, SXT: cotrimaxazole, NA: nalidixic acid

Table 3. Minimum Inhibitory Concentrations (MICs) (%v/v) of Honeys Against ATCC Reference Strains.

S.No.	Species	MICs			
		Kalonji	Eucalyptus	Sidr	Manuka
1	*Staphylococcus aureus* 25923	9.0±0.0	4.3±0.5	7.7±0.5	4.0±0.0
2	*Acinetobacter baumannii 29213*	8.0±0.0	4.0±0.0	7.0±0.0	7.0±0.0
3	*Escherichia coli 25922*	9.0±0.0	6.0±0.0	10±0.0	6.7±0.0

Table 4. Pairwise Comparison of Mean MICs of Manuka, Sidr, Kalonji and Eucalyptus by Bonferroni Post Hoc Test Against Clinical Isolates of *Vibrio cholerae.*

S.No.	Honey types	p-value
1	Manuka versus Eucalyptus	0.061
2	Manuka versus Kalonji	0.000*
3	Manuka versus Sidr	0.000*
4	Eucalyptus versus Kalonji	0.000*
5	Eucalyptus versus Sidr	0.000*
6	Kalonji versus Sidr	0.261

*denotes significant p-value

Table 5. Pairwise Comparison of Mean MICs of Manuka, Sidr, Kalonji and Eucalyptus by Bonferroni Post Hoc Test Against ATCC Reference Strains. (*Staphylococcus aureus* 25923, *Acinetpbector baumannii* 29213 and *Escherichia coli* 25922)

S. No.	Honey types	p-value
1	Eucalyptus versus Manuka	0.608
2	Eucalyptus versus Sidr	0.040*
3	Eucalyptus versus Kalonji	0.014*
4	Manuka versus Sidr	0.124
5	Manuka versus Kalonji	0.053
6	Sidr versus Kalonji	0.690

*denotes significant p-value

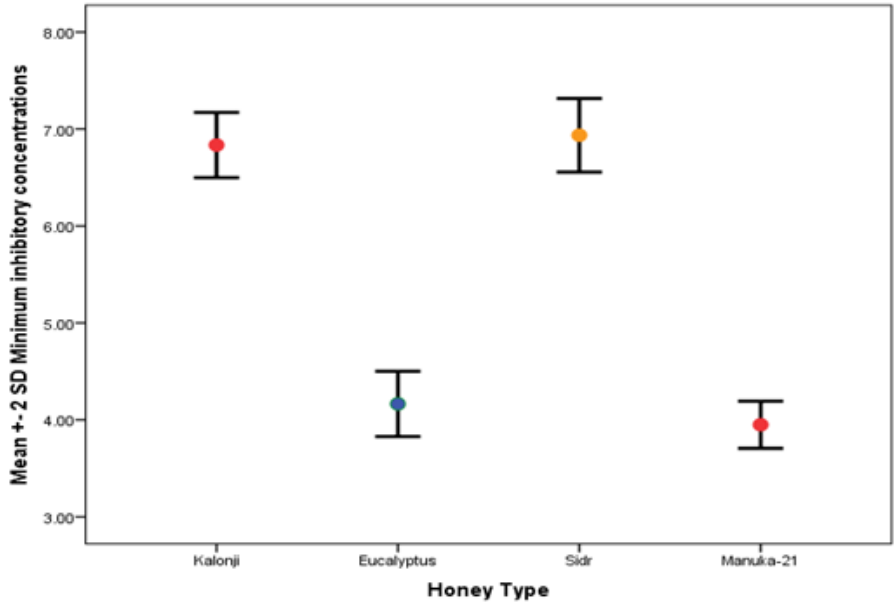

Figure 1. Minimum Inhibitory Concentrations MICs (%v/v) of Honeys Against *Vibrio cholerae*.

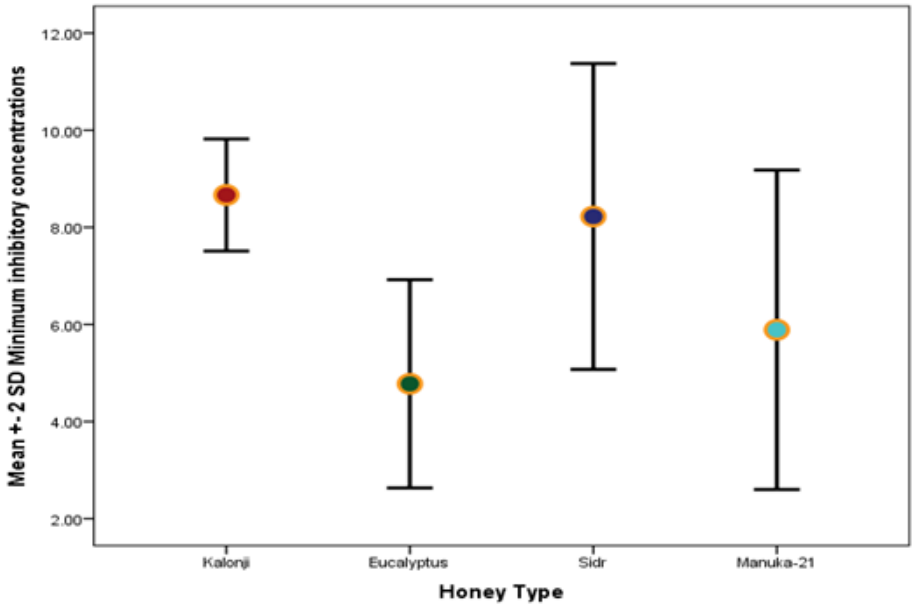

Figure 2. Minimum Inhibitory Concentrations MICs (%v/v) of Honeys Against ATCC Reference Strains. *Staphylococcus aureus* 25923, *Acinetpbector baumannii* 29213 and *Escherichia coli* 25922 (Mean Value)

DISCUSSION

Multidrug-resistant strains of *Vibrio cholerae* has been increasingly recognized around the world[22]. New resistance phenotypes of *Vibrio cholerae* have been emerged recently[23]. Both sensitive and resistant strains of *Vibrio cholerae* were inhibited by honey at the same MICs (Table **2**). This means that tested honey have different and unique mechanism of action against bacterial pathogens. Recently, studies have identified the bacterial cellular targets and underlying mechanism of action of honey[24,25]. Since, honey contains multiple antibacterial

bioactive compounds and they act synergistically on multiple targets of the bacterial cell, therefore, antibacterial resistance to honey is less likely[11].

The present study revealed that locally produced honey has significant antibacterial activity against all tested pathogens and ATCC reference strains. However, the level of antibacterial activity generated by different types of honey is quite variable (Table **2** & **3**, Figure **1** & **2**). The antibacterial activity of Eucalyptus honey is superior to that of medically graded Manuka honey (Table **2**). Eucalyptus honey is more bactericidal than Manuka honey against *A. baumannii* and *E. coli* (Table **3**). Previously, Lusby *et al*. (2005) have shown that honey other than the medically graded honey may have similar antibacterial effects[26]. Therefore, it is important to evaluate the antibacterial activity of untested and locally produced honey. It also demands standardization of honey for medical exploitataion of local honeys.

In this study, we used agar dilution assay for evaluation of the antibacterial activity of honey because it provides quantifiable and more accurate results as compared to diffusion assay[27]. Honey is uniformly distributed in agar dilution assay and is direct contact with testing organisms, whereas in agar well diffusion assay there are many factors which affect the rate of diffusion of active constituents of honey. It is likely that large size active substances present in honey may not diffuse in agar well diffusion assay[28].

In one of the clinical trials, Haffejee and Moosa (1985) have demonstrated that honey is effective in treating bacterial gastroenteritis[29]. In the present study, all clinical isolates of *Vibrio cholerae* were inhibited by Eucalyptus honey at quite low concentrations (4 to 4.3%) (v/v), which is comparable to medically graded Manuka honey (3.7 to 4% v/v). Thus, Eucalyptus honey taken orally can shorten the duration of cholera-like antibiotic and at the same time unlike antibiotic may not disrupt the growth of beneficial GIT flora. Moreover, it can enhance the growth of normal flora because honey contains prebiotics which has positive growth effect on probiotics microorganism[11].

Previously Pal *et al*. (2016) determined the antibacterial activity of four different types of honey against *Vibrio cholerae* and found that all isolates are susceptible to honey[30]. However, the authors used the disc diffusion method which is unable to provide precise and quantitative results. Secondly, no comparison was made between standardized honey. In our study, we determined the MICs of tested honey by agar dilution assay which offers precise and quantitative results and compared the results with medically graded honey.

One of the important constituents of oral rehydration solution (ORS) for the treatment of diarrhoea is glucose (2g / 100ml), based on the recommendations of WHO[31]. Honey also contains glucose and fructose. Unlike glucose, the fructose is absorbed in the intestine by diffusion instead of active transport; therefore, sodium ion is not coupled with this process[32]. As a result, water is absorbed without augmenting the absorption of sodium. It has been shown in one of the clinical trials that orally given honey supplemented with electrolytes reduces the duration of bacterial diarrhoea in comparison with ORS[33]. Another study compared the effect of honey and ORS in the treatment of diarrhoea and found that the honey treatment group had fewer bowel movements and a shorter diarrhoea period compared to the control group[34]. Recently Shariatpanahi *et al*. (2018), evaluated the efficacy of honey in diarrhoea patients in a double-blind, randomized controlled trial comprising of 32 patients and found that honey altered the gut microflora and reduced the occurrence of diarrhoea in these patients[35]. These studies highlight the importance and effectiveness of honey in the treatment of diarrhoea.

CONCLUSION

Locally produced honey exhibited variable antibacterial activity against *Vibrio cholerae* and ATCC reference strains. Eucalyptus honey showed comparable or in some cases better activity than well-known New Zealand Manuka honey. Eucalyptus honey may be used as a potential alternative therapy against diarrhoea caused by *Vibrio cholerae* in future studies.

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LIST OF ABBREVIATIONS

SD	Standard deviations
NA	Nalidixic Acid
R	Resistant
S	Sensitive
SXT	Cotrimaxazole

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