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In vitro Vibriocidal Efficacy of Monofloral Honey Against Different Sero groups of *Vibrio cholerae*

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Cholera is a major public health problem in developing countries of the world. Relative resistance to antibiotics is difficult to treat cholera patients leading to spread of microbial drug resistance as a global public health challenge that results in increased illness and death rate. Newer antimicrobials or agents are urgently required to overcome this problem. The present study was conducted to investigate the antimicrobial potential of four types of honey against clinical isolates of *Vibrio cholerae* O1 and O139 sero groups Disc Diffusion method using Karanja (*Pongamia glabra)*, Mango (*Mangifera indica)*, Neem (*Azardirachta indiaca)* and Mustard (*Brassica campestris)* honey were employed for their antibacterial activity. All *Vibrio cholerae* strains were highly susceptible to almost all five concentration of Karanja honey in comparison to other three types of honey. From the antibiogram profile it was observed that the susceptibility pattern of total strains were tetracycline > gentamicin > norfloxacin > ciprofloxacin. The present findings indicated that different types of monofloral honey exhibited different antibacterial activity against *V.cholerae* strains which were almost dose dependent. These results suggest the possibility of using honey as an alternative to antibiotics to overcome the growing problem of antimicrobial resistance among *V.cholerae* strains.

Keywords: Honey; Antibacterial activity; *Vibrio cholerae.*

Vibrio cholerae, the causative agent of cholera is a gram negative bacterium responsible for severe morbidity and mortality in developing countries of the world. *Vibrio cholerae* currently has more than 200 "O" sero groups. Out of this only O1 and O139 sero groups are associated with clinical disease [1]. During nineteenth century, cholera caused large epidemics in different parts of India. Resistance to more than one antibiotic is now common among the clinical isolates of *V.cholerae*. There are reports of multi drug resistant *V.cholerae* appearing with increasing frequency [2]. World Health Organization has suggested an urgent need to find new antimicrobial or new approach to combat this serious issue. According to WHO more than 80% of the world population relies on traditional medicine for their primary health care needs [3]. There are many reports available that honey is used for treatment of some diseases.

Honey is a sweet food made by honey bees (*Apis melifera*) using nectar from flowers. It contains powerful antioxidants with antiseptic and antibacterial properties. The nutritional and medicinal qualities of honey have been documented in Vedic, Greek, Roman, Christian, Islamic and other texts [4]. Due to antibacterial effect of honey it is increasingly being used in the management of medicine, alone or in combination with other substances both orally and topically in a large number of societies from ancient time. The antibacterial property of honey was first recognized

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in 1892 by the Dutch scientist Van Ketel [5]. The use of honey as therapeutic substance has been rediscovered due to its ability to inhibit bacterial growth [6,7] have demonstrated that 40% of diluted honey and above reduced bacterial growth like *Salmonella typhi*, *Vibrio cholerae* and *Yersinia enterocolitica* where as concentrated honey exhibited greatest inhibitory effect. Aurongzeb et al, (2015) [8] have tested mono flora and multi flora honey against antibiotic-resistant strains of *Vibrio cholerae*, *Salmonella typhi*, *Shigella dysenteriae* and *Campylobacter spp.* isolated from clinical fecal samples. In the present investigation we have tested four types of monofloral natural honey against different sero groups of *Vibrio cholerae* O1 and O139.

MATERIALS AND METHODS

Honey

V. cholerae O1 and O139 strains used in this study were isolated from diarrhoea patients between 1999 to 2005 from different outbreaks in Odisha. The four types of monofloral honey were selected for antibacterial activity, against *V. cholerae*. Those are *Pongamia glabra* (Karanja honey: Ka), *Mangifera indica* (Mango honey: Ma), *Azardirachta indiaca* (Neem honey: Ne) and *Brassica campestris* (Mustard honey: Mu). These honey were used against selected 42 multi drug resistant strains of *V.cholerae* O1 (30) and O139 (12) sero groups isolated from the diarrhoea patients. This honey was produced from a local farm from Balasore district of Odisha for research purposes.

Bacteriological Analysis

Vibrio cholerae O1 and O139 strains were revived from the stock cultures on TCBS agar plates and purity was checked. The antibiotic susceptibility analysis was performed by modified Kirby Bauer Disk diffusion technique [9] along with commercially available antibiotic discs (Himedia-Mumbai). Characterization of strains as susceptible, intermediately resistant or resistant were done as based on the size of the inhibition zone according to the manufacturer's instruction which matched the interpretive criteria recommended by WHO. Antibiotics used in this study were gentamicin (G, 10µg), tetracycline (T, 30µg), chloramphenicol (C, 30µg), ampicillin (A, 10µg), nalidixic acid (Na, 30µg), furazolidone (Fr, 50µg), norfloxacin (Nx, 10µg), ciprofloxacin (Cf, 5µg), co-trimoxazole (Co, 25µg), streptomycin (S, 10µg) and Neomycin (N, 30µg).

After the lawn culture was prepared in MHA plates, the wells were cut into the media by the help of a sterile agar well punctured. 1 % agar was poured into the well as a sealing material. The varying volumes (10, 20, 40, 50, 100µl) of Mango, Mustard, Neem and Karanja honey were loaded over the wells by the help of micropipettes. After 24 hours of incubation at 37°c the plates showed a clear zone around the well produced a positive antimicrobial activity of honey [10].

Minimum Inhibitory Concentration (MIC)

Determination of (MIC) of honey was done by "tube dilution technique" [11]. This MIC value of honey was determined against each O1 and O139 sero groups of *V.cholerae* strains, which were selected from 42 *V.cholerae* isolates. Nutrient broth (NB) of 900 µl was dispensed into each of the sterilized test tube labeled 1-10, with the help of micropipette; 100µl of different concentration of honey was transferred to the tubes according to concentration and mixed properly to get a homogenous solution. Further the sample was serially diluted accordingly so as to get a one fold dilution and from the 10^{th} tube 100µl solution was discarded. Two of the test organisms each of O1and O139 sero groups were grown in TSB for 4-6 hours at 37°C. One loopful organism were inoculated to all 1-10 test tubes and incubated at 37°C for 18-24 hours. Appearance of turbidity in the tubes indicated the growth of the organism and the MIC value of the honey was determined. The least concentration of honey inhibiting the growth of the organism in the test tubes showing no turbidity. From each of the turbid tubes one loopful sample were streaked on the nutrient agar plates in order to determine the antibacterial activity of the honey. These plates were incubated at 37°C for 18-24 hours and observed for the said activity. The same procedure was repeated for all the four types of honey.

RESULTS

The in vitro vibriocidal efficacy of four types of honey exhibited different results against different sero groups of honey. The zones of

inhibition were dose dependant as tested against different sero groups of *V.cholerae* strains against different honey. At 10µl of four types of honey: Ka, Mu, Ne, Ma against *V.cholerae* O1 sero groups exhibited similar results. Whereas 20µl of four types of honey exhibited similar results (Ka and Mu showed 23.17mm and 21.2mm inhibition zone, where Ne and Ma showed around 21mm inhibition zone). Similarly 40 and 50µl of four types of honey exhibited almost similar results. But while testing against 100µl of four types of honey the pattern of inhibition zone were Ka>Ne>Mu>Ma (37mm>35mm>33mm>30mm respectively). Similarly *V.cholerae* O139 sero groups showing almost similar results. At 10µl of different honey (Ka, Mu, Ma) exhibited similar results (around 20mm inhibition zone), where Ne honey showed 19mm inhibition zone. Where as 20µl of four types of honey exhibited similar results (22.66mm – 23.33mm). Similarly 40µl and 50µl of four types of honey exhibited almost similar results. But while testing against 100µl the zone of inhibition were Ka>Ne>Mu>Ma (38.1mm> 37.2mm> 34.4mm> 33.6mm respectively), which was dose dependant against four types of honey and five types of concentration, means increasing the concentration of honey the zone of inhibition increased (Fig1, 2 and 3).

The *V.cholerae* O1 and O139 sero gruops were tested against eleven commercial antibiotics. The *V.cholerae* O1 strains were highly sensitive to gentamicin, tetracycline, chloramphenicol, norfloxacin, ciprofloxacin and neomycin, Where as these strains were resistant to ampicillin, furazolidone, co-trimoxazole and streptomycin. But *V.cholerae* O139 strains were sensitive to gentamicin, tetracycline, furazolidone, norfloxacin and ciprofloxacin, but resistant to chloramphenicol, ampicillin, nalidixic acid, co-trimoxazole and streptomycin. Interestingly the inhibition zone exhibited by the higher concentration of honey showed higher inhibition zone size while comparing to these commercial antibiotics.

MIC value of *V.cholerae* O1 sero groups showed lowest 0.00001µl/ml against Neem honey and highest concentration 0.01µl/ml against Mustard honey. Whereas *V.cholerae* O139 sero group were showing intermediate results against Mango honey and Karanja honey 0.01µl/ml and 0.00001µl/ml respectively. Interestingly the MIC values showing against Ka, Ne, Mu, Ma were 0.00001µl/ml, 0.0001µl/ml,0.001µl/ml, 0.01µl/ml respectively (Table 1).

DISCUSSION

Natural honey is an exemplary supersaturated viscous solution of sugars, minerals, vitamins and proteins abstracted by bee of *Apis* species, The *Apis mellifera* bees are widely used in apiaries for large scale natural honey production [8]. Researchers have failed to point out the active ingredient responsible for the antibacterial activities of honey. Over 100

Table 1. MIC values of different honey against *V.cholerae*

	O1 serogroups (µl/ml)	O139 serogroups (µl/ml)
Karanja honey	0.0001	0.00001
Neem honey	0.00001	0.0001
Mustard honey	0.01	0.001
Mango honey	0.001	0.01

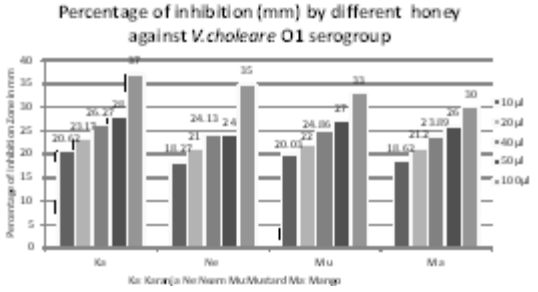

Fig. 1. Percentage of zone of inhibition at different concentrations of four types of honey against *V.cholerae* O1 sero groups

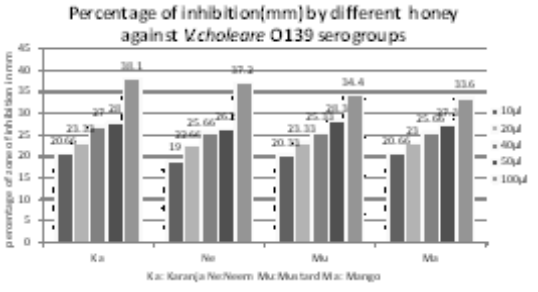

Fig. 2. Percentage of zone of inhibition at different concentrations of four types of honey against *V.choleare* O139 sero groups

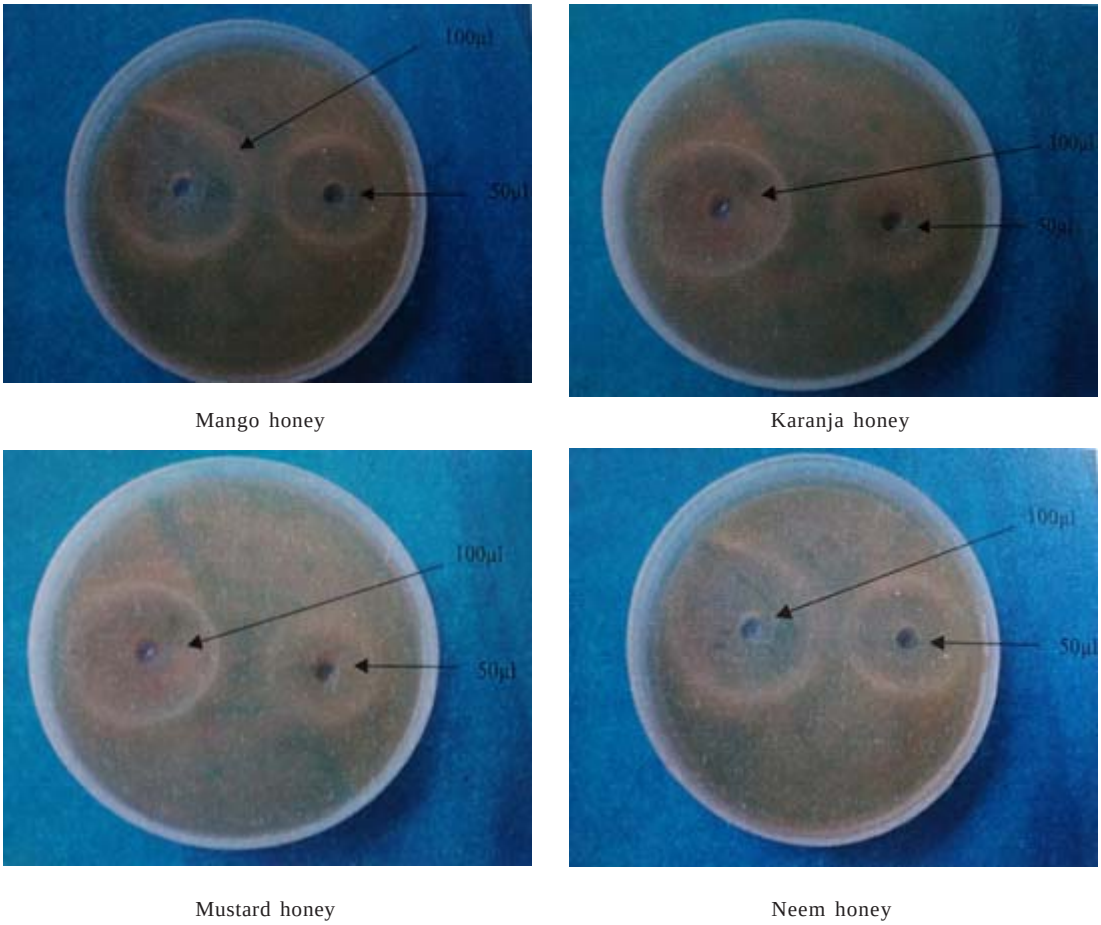

Fig. 3. Zone of inhibition (mm) with 50µl and 100µl of different honey against *V.choleare* 01 strains

substances were found to be candidates for such antibacterial activity [12]. While antibiotics destroy bacteria by attacking the cell wall, honey draws moisture out of the cell environment and dehydrates the bacteria with the aid of its hyper osmolar properties [12-14]. Other factors that contribute to antimicrobial activity of honey include the high sugar concentration, hydrogen peroxide, methylglyoxal, and the antimicrobial peptide bee defensin-1 [15]. It was found that both hydrogen peroxide (H_2O_2) and the non-peroxide components contribute to the bacteriostatic and bactericidal activity of honey. Also, H_2O_2 in honey is involved in oxidative damage causing bacterial growth inhibition and DNA degradation, but these effects are modulated by other honey components[16]. The pH of honey is commonly between 3.2 and 4.5, so due to acidic pH level prevents the growth of many bacteria [4,14].

Abd El-Moez et al (2013) [17] had explained that diluted cotton flower honey (10%) showed bacteriostatic effect against *Sh.flexneri*, *S. typhimurium*, *E. coli* and *Klebsiella* with zone of bacteriostatic effect equals 40, 35 and 30 mm, respectively, followed by *Pseudomonas aeruginosa*, *Citrobacter* and *E. fecalis* with zone of bacteriostatic 26, 20 and 19mm, respectively. In two African studies honey was used successfully to treat ulcers, wound and Fournier's gangrene (gangrene of the scrotal area). The ulcers responded to a treatment regime of washing with saline and the topical application of 15-30 ml honey daily. Pathogens isolated at the commencement of the study included *Pseudomona spyocyanea* (35 cases), *Escherichia coli* (31), *Staphylococcus aureus* (15), *Proteus mirabilis* (9), coliforms (9),

Klebsiella species (7), *Streptococcus faecalis* (3) and *Streptococcus pyogenes* (1)[18]. From another study it was found that two table spoons of honey (30 ml) given before meals three times daily was used to treat male and female patients (20 – 40 years) suffering from gastritis, duodenitis and duodenal ulcers[19]. Similarly the combination of honey-lemon juice before it was used in cough remedies was indented to kill bacteria microbial contamination and also to get the flavor and aroma were referred[20].

In this investigation the *V.choleare* different sero groups were isolated from the cholera patients. And these *V.cholerae* strains were showing multidrug resistance in different time periods reported by us. In the present study, it was found that the Karanja honey showed highest antibacterial effect against *V.cholerae* of both sero groups (O1and O139). Our results showed that the Karanja honey was more sensitive to both of the O1and O139 sero group of *V.cholerae* as compared to other three types of honey (Mango, Neem and Mustard) i.e. 37mm zone of inhibition against O1 and 38.1mm against O139 sero groups at the 100µl concentration. Again at 20µl concentration highest zone of inhibition was nearly 23.3mm against O1 and O139 sero groups of Karanja honey. Aurongzeb et al (2015) [8] have tested dilutaed honey of 15-20% (v/v) against *Salmonella typhi, Shigella sonneie, Vibrio furnissii, Yersinia pestis,* and *Escherichia coli*. The medial level potency of Pakistan honey was comparable to antibacterial activity of Australian honey [14, 21] and less than the Manuka honey from New Zealand honey [14, 22]. Obi et al (1994)[7] reported that the 40% of Nigerian local honey was showing reduced bacterial growth against *Salmonella typhi, Vibrio cholerae* and *Yersinia enterocolittica* and undiluted honey shoed greatest effect. The lower concentration like 30% and below did not inhibit pathogen growth. In this present investigation even at 10µl of concentration four types of honey inhibited 18-20mm of zone of inhibition. Similarly as increasing the dose (20µl, 40µl, 50µl, 100µl) of different honey, the zone of inhibitions were increased and the highest zone of inhibition was obtained at 100µl concentration of four types of honey as mentioned above against *V.cholerae* of O1 and O139 sero groups. However 40 µl and 50 µl of Neem honey were showing almost equal zone of inhibition against *V.choleare* sero groups which should be rechecked

It is evident from our study that there was a significant difference in MICs of the four types of honey. This might be due to the difference in the constituents. It also highlights the importance of screening of different types of monofloral honey for their antimicrobial activity against specific bacterial strain. The possible mode of action of all four types of honey against forty-two strains of *V. cholerae* might be due to inhibition through cell wall synthesis or through multidimensional activities.

Sharma et al (2007) [23] have reported that the antibiotic sensitivity pattern of *V.cholerae* between 2003 to 2005. In this study he reported 22% Tetracycline resistance as compared to 99% and 100% sensitive to both *V.cholerae* O1 and O139 sero groups respectively which were collected during 1999 to 2005. Now a days the multidrug resistant *V.cholerae* strain are being reported from the country and also from other part of the globe. It is interesting to note that the natural honey may be tested against cholera patients which need more molecular research. The future studies are warranted to find out the active ingredients of honey which should be separated and tested against different multidrug resistant *V.cholerae* strains in vitro and in vivo conditions.

The present investigation indicates that honey may be used for the treatment of cholera patients. The future study warrants to find out the in vivo experiment of honey and clinical trial of cholera patient. The molecular study will be helpful to find out the active ingredients of honey, which can act against *V.cholerae* and that may be used as an alternative for the treatment of cholera patents instead of conventional antibiotics.

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