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In Vitro Antibacterial Activity of Honey Against Clinical Isolates of Multi-Drug Resistant Typhoidal Salmonellae

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Abstract.- Typhoid fever continues to be a global health problem. The emergence of resistance to conventional anti-typhoid drugs had already a major setback. The situation has further aggravated with the recent emergence of quinolone resistance especially in the developing countries. Honey is increasingly becoming a part of modern medicine and has been approved for the treatment of chronic skin infections and burns. Its role in systemic infections is, however, not known. The aim of this study was to determine, if Pakistani honey had antibacterial activity against typhoidal salmonellae, including multi-drug resistant strains. Agar dilution assay was adopted to determine the minimum inhibitory concentration. Two types of natural honeys and one simulated honey were evaluated. Black seed honey inhibited all strains at concentration median 9.0±1.0% (v/v) and shain honey at median 12.0±1.0% (v/v). Simulated honey inhibited these strains at concentration median 30±0.0% (v/v). This demonstrates that antibacterial effect of natural honey is not linked with high osmolarity alone.

Keywords: Antibacterial activity, honey, typhoidal salmonellae, typhoid fever.

INTRODUCTION

Typhoid fever remains a major cause of morbidity and mortality in the developing world. There are estimated 16 to 33 million cases globally with over 500,000 deaths in the WHO, recent report (WHO, 2008). Ninety three percent of these cases are reported in Asia (Merican, 1997; Siddiqui *et al*., 2006). The negative development showed increase of multi-drug resistance (MDR) throughout the world and these strains were responsible for Mexico catastrophe in 1970 (Bhutta, 2006; Olarte and Galindo, 1973). Ofloxacin was reported to be extremely effective against typhoid in 1986 and fluoroquinolones continued to be useful antibiotics (Hannan, 1986). However, the fluoroquinolones resistance in *S. typhi* and *S. paratyphi A* has emerged and described in various parts of Asia (Butt *et al.*, 2003). Occurrence of resistance against ceftriaxone (third generation cephalosporin) has further complicated the problem in developing countries like Pakistan (Saha *et al*., 1999). Consequently, efforts have to be made to evaluate and develop new agents to be used as anti-typhoid therapy.

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Honey has been extensively used as healing agent throughout the human history in addition to its widespread usage as popular food (White, 1966). The antimicrobial properties of honey are mainly attributed to its acidic pH, high osmolarity, release of hydrogen peroxide and plant derived non-peroxide factors (Molan, 1999; George and Cutting, 2007; Franchini *et al*., 2007). The non-peroxide substances are not specifically identified and believed to be lysozyme, phenolic acids, flavonoides and others (Snowdon and Cliver, 1996). However in case of manuka honey, recently methylglyoxal (MGO) was identified as an active ingredient, responsible for non-peroxide antibacterial activity (Marvic *et al*., 2008). Previously this compound was labeled as, Unique Manuka Factor (UMF).

In the last decade a variety of prebiotics have been identified in honey, which stimulate the growth of beneficial bacteria like bifidofacteria and lactobacilli (Shin and Ustunol, 2005). Recently, a study demonstrated that the *Lactobacillus acidophilus* secretes a molecule that control the transcription of *E. coli* genes involved in

colonization (Medellin-Penna *et al*., 2007). More recently, a study revealed that different varieties of honey possess a large amount of viable lactobacilli (6 species) and bifidobacteria (4 species) (Olofsson and Vasquez, 2008). This may explain many of the hidden therapeutics properties of honey.

A clinical trial conducted at University of Natal, South Africa indicates that honey at concentration of 5% (v/v) shortens the duration of bacterial diarrhea caused by *Salmonella, Shigella* and *E. coli* in infants and children (Haffejee and Moosa, 1985). Honey was found to be effective in the treatment of dyspepsia and of gastric and duodenal ulcers, which is mainly caused by infection with *Helicobacter pylori* (Sato and Miyata, 2000).

Besides, evidences of antibiotic activity of honey applications directly, orally taken honey may also serve as potential candidate for future prevention and treatment of typhoid fever. This study was therefore, undertaken to investigate the *in vitro* antibacterial activity of Pakistani honeys against multi-drug resistant typhoidal salmonellae.

MATERIALS AND METHODS

Bacterial strains

Twenty four clinical isolates of typhoidal salmonellae, twenty isolates of *Salmonella typhi,* two of *Salmonella paratyphi A* and two of *Salmonella paratyphi B* were evaluated. Five ATCC reference strains, *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212) and *Acinetobacter baumannii* (ATCC 29212) were included as a control. All these clinical isolates and reference strains were donated by the Department of Microbiology, Armed Force Institute of Pathology, Rawalpindi, Pakistan. All the clinical isolates were identified by standard morphological, cultural and biochemical profile (API-20E, bioMerieux, France). Serological identification was performed by using antisera (BD Difco, USA). The isolates were preserved in microbank tubes containing beads (Pro-Lab Diagnostics, UK) and 16% (v/v) glycerol in brain heart infusion (Oxoid Ltd, UK) and were stored at -70˚C.

Honey samples

Beri (*Ziziphus jujuba*) and shain *Plectranthus rugosus* wall) honey samples were obtained from Honey Bee Research Farm of National Agricultural and Research Council (NARC), Islamabad, Pakistan, and kept at room temperature in dark. Sterility of honey was checked by spreading a loopful quantity on blood agar (Oxoid Ltd, UK) medium (Mulu *et al*., 2004). Simulated honey was prepared by dissolving 40.5 g fructose, 33.5 g glucose, 7.5 g maltose and 1.5 g sucrose in 17 ml sterile distilled water according to the method described by French *et al*. (2005).

Susceptibility testing

The antimicrobial susceptibility was determined by Kirby and Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute Guidelines using commercially available antimicrobial discs (Oxoid, Basingstoke, UK) (CLSI, 2005). Following antibiotics were used: Ampicillin (10 µg), Chloramphenicol (30 µg), Sulphamethoxazole/ Trimethoprim (25 µg), Amikacin (30 µg), Nalidixic acid (30 µg), Ofloxacin (5 µg), Moxifloxacin (5 µg), Ciprofloxacin (5 µg), Cefotaxime (30 µg), Azithromycin (15 µg) and Tetracycline (30 µg). *Escherichia coli (*ATCC 25922) was used as control strain for antimicrobial susceptibility test.

Determination of minimum inhibitory concentration (MIC)

The agar dilution method was used to determine the MIC for each of the isolates. Assuming that the density of honey samples is 1.37 g ml^{-1}, honey was weighed out and dissolved in sterile deionized water to prepare a stock solution of 20% (v/v) and 50% (v/v) immediately before use (Cooper *et al*., 2002). Further 1% incremental dilutions were prepared ranging between 1-20% of honey in order to get final concentration required in a final volume of 20 ml of double strength Mueller Hinton (MH) agar (Oxoid Ltd, UK). Since honey is very viscous liquid, therefore it was kept at 50˚C before mixing to achieve uniform homogenization. Simulated honey, however was used at 5% incremental dilutions from 20-40%.

Mixing of honey solutions with autoclaved

MH agar were performed at 50˚C, vigorously vortexed and dispensed into Petri dishes (Greiner bio-one, Austria) having 90mm diameter. The poured plates were allowed to dry at 45˚C for about 10 to 15 minutes. Four to five well separated colonies from overnight blood agar were emulsified in 5 ml of sterile distilled water, adjusting to 0.5 McFarland's standard. The honey incorporated plates were inoculated with multipoint inoculator (Mast Diagnostic, UK). The plates were incubated at 37˚C for 18 hrs and observed for growth. Three control plates were also set up in parallel; one of MH agar inoculated with all strains to confirm the viability of the cultures. Second control plate contained medium only and third medium with honey to check the sterility of medium and the honey. The MIC was recorded as the lowest concentration of honey at which visible bacterial growth was completely inhibited. This experiment was performed in triplicate to ensure the reproducibility of the results.

Data analysis

For each data set three replicates were performed. The data were analyzed by using SPSS 15.0, after checking the assumption of normality. The arithmatic mean of observations and standard deviation of mean values were calculated. Median MIC + IQR (interquartile range) are given for non-normality distributed metric variables. Non-parametric Kruskal-Wallis test was applied to observe median differences, and Games-Howell multiple comparison tests was used to see which median differed.

RESULTS

Table I shows the phenotypic antimicrobial resistance profile of typhoidal salmonellae. The MIC of typhoidal salmonellae and five ATCC reference strains are shown. The median MIC of black seed honey was recorded as 9.0±1.0% (v/v) and that of shain honey as 12.0±1.0 % (v/v) against all 24 strains of typhoidal salmonellae (Table II). The control MH agar plate without honey inoculated with bacterial strains exhibited the growth of all isolates tested indicating their viability. No growth was observed in other control plates comprising only agar and agar mixed with honey after 18 hrs of incubation at 37˚C.

Significant difference was observed among the medians of MIC of black seed honey against *Salmonella typhi*, *S. paratyphi* A and *S. paratyphi* B (Kruskal-Wallis test, p=0.006) (Table III). This indicates that there is difference in susceptibility pattern of three species of salmonellae to black seed honey. However, no significant difference was appreciated regarding MIC of shain honey against *Salmonella typhi*, *S. paratyphi* A and *S. paratyphi* B (p=0.148) (Table III).

Multiple comparison (post hoc Games-Howel test) of these honey samples showed significant difference between MIC of black seed honey and shain honey (p=0.000), black seed honey and artificial honey (p=0.000), and between artificial honey and shain honey (p=0.000) against *S. typhi*. This shows that there are significant variations in the level of antibacterial activity of the honey samples evaluated in this experiment. Significant difference was also observed between the MIC of shain honey and artificial honey (p=0.024) against *S. paratyphi* A. However, there was no significant difference observed between MIC of black seed honey and shain honey (p=0.188) against *S. paratyphi* A.

DISCUSSION

To the best of our knowledge, this is the first report regarding antibacterial activity of Pakistani shain and black seed honey against typhoidal salmonellae. Moreover, according to our knowledge, no data is available regarding efficacy of honey against MDR *S. typhi, Paratyphi* A and *Paratyphi* B except one study which evaluated only one standard sensitive strain of *S. typhi* (Mulu *et al*., 2004). It has been shown in this study that black seed honey inhibited all typhoidal salmonellae strains at quite low concentration (median 9.0±1.0%) (v/v) as compared to shain honey (median 12.0±1.0 %) (v/v) (Table II). These results indicate the effectiveness of honey particularly black seed honey against typhoidal salmonellae and confirmation of variation in antibacterial activity of honey from different floral sources. The reason for such remarkable degree of variation between

different floral sources and within the same floral source is attributed to multiple factors like age,

Table I.- Phenotypic antimicrobial resistance profile and minimum inhibitory concentrations (MIC) (%v/v) of different honeys against typhoidal salmonellae and ATCC reference strains.

Strain No.	Species	Antimicrobial resistance pattern	MIC*		
			Black seed honey	Shain honey	Artificial honey
UHS-1	*S. typhi*	S	9.0 ± 0.0	11.0 ± 0.0	30 ± 0.0
UHS-2	*S. typhi*	AMP^R, SXT^R, C^R	9.3 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-3	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	11.6 ± 0.5	30 ± 0.0
UHS-4	*S. typhi*	AMP^R, SXT^R, C^R	9.3 ± 0.5	11.0 ± 0.0	30 ± 0.0
UHS-5	*S. typhi*	AMP^R, SXT^R, C^R	8.3 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-6	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	11.0 ± 0.0	30 ± 0.0
UHS-7	*S. typhi*	AMP^R, SXT^R, C^R	8.0 ± 0.0	11.0 ± 0.0	30 ± 0.0
UHS-8	*S. typhi*	AMP^R, SXT^R, C^R	9.3 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-9	*S. typhi*	AMP^R, SXT^R, C^R	8.6 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-10	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	10.0 ± 0.0	30 ± 0.0
UHS-11	*S. typhi*	AMP^R, SXT^R, C^R	8.6 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-12	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
UHS-13	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	11.6 ± 0.5	30 ± 0.0
UHS-14	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
UHS-16	*S. typhi*	AMP^R, SXT^R, C^R	8.6 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-17	*S. typhi*	AMP^R, SXT^R, C^R	9.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
UHS-18	*S. typhi*	SXT^R, C^R	9.3 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-19	*S. typhi*	SXT^R, C^R	9.0 ± 0.0	11.6 ± 0.5	30 ± 0.0
UHS-20	*S. typhi*	AMP^R, SXT^R, C^R	9.3 ± 0.5	12.0 ± 0.0	30 ± 0.0
UHS-21	*S. typhi*	SXT^R, C^R	9.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
UHS-22	*S. paratyphi A*	S	8.0 ± 0.0	10.0 ± 0.0	30 ± 0.0
UHS-23	*S. paratyphi A*	S	7.6 ± 0.5	11.0 ± 0.0	30 ± 0.0
UHS-52	*S. paratyphi B*	S	7.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
UHS-53	*S. paratyphi B*	S	7.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
ATCC 27853	*Pseudomonas aeruginosa*	NT	7.3 ± 0.5	10.0 ± 0.0	30 ± 0.0
ATCC 25923	*Staphylococcus aureus*	NT	6.0 ± 0.0	12.0 ± 0.0	35 ± 0.0
ATCC 25922	*Escherichia coli*	NT	9.0 ± 0.0	12.0 ± 0.0	30 ± 0.0
ATCC 29213	*Acinetobacter baumannii*	NT	7.0 ± 0.0	11.0 ± 0.0	30 ± 0.0
ATCC 29212	*Enterococcus faecalis*	NT	10.3 ± 0.0	14.0 ± 0.0	30 ± 0.0

*MIC values are the mean of triplicate determinations, and shown as Mean ± SD.
S, sensitive to all antibiotics tested; AMP, ampicillin, SXT, sulphamethoxazole/trimethoprim; C, chloramphenicol; R, resistant; NT, not tested.

Table II.- Median MIC (%v/v) of honeys against typhoidal salmonellae

Types of honey	No. of isolates (n)	Median MIC±IQR
Black seed honey	24	9.0 ± 1.0 %
Shain honey	24	12.0 ± 1.0 %
Simulated honey	24	30.0 ± 0.0 %

MIC, minimum inhibitory concentration; IQR, interquartile range.

storage, processing procedure, concentration of hydrogen peroxide, plant derived non peroxide factors etc (Molan, 1992). Plant derived factors which are unique to each plant species do contribute

Table III.- Comparison of MIC (% v/v) of natural honeys against typhoidal salmonellae

Types of honey	*Salmonella typhie* (Median MIC±IQR)	*S. paratyphi* A (Median MIC±IQR)	*S. paratyphi* B (Median MIC±IQR)	p-value
Black seed honey	9.0 ± 0.5	7.8 ± 0.3	7.0 ± 0.0	0.006*
Shain honey	12.0 ± 0.9	10.5 ± 1.0	12.0 ± 0.0	0.148

*Denotes significant p-value.

significantly in the variation of antibacterial activity between honeys of different plant sources (Molan and Russell, 1988; Allen *et al*., 1991). The difference within the same floral source is not yet fully elucidated; some studies indicated the effect of climate, handling, processing of honey, the propolis content, and soil composition as important contributing factors (Allen *et al*., 1991).

This underlies the importance of screening a wide range of honey samples of different floral origin from different geographical regions in order to select a honey with greater antibacterial potential. We have been collecting more honey samples for the assessment of their antibacterial activity from different geographical locations of Pakistan.

These results also revealed that black seed honey not only inhibited typhoidal salmonellae at lower concentrations as compared to shain honey but at different MIC ranges when used against different species of salmonella (Table III). *Salmonella paratyphi* B strains appeared to be more sensitive than *Salmonella typhi* to black seed honey (Table III). Shain honey inhibited typhoidal salmonellae comparatively at higher concentration and there was no significant difference when compared against different species. Both drug sensitive and MDR *Salmonella typhi* strains responded the honey at almost the same MIC (Table I). This finding is generally in accordance with Cooper *et al.* (2000) study which revealed the effectiveness of manuka honey against twenty strains of *Burkholderia cepacia* including drug resistance strains at approximately the same MIC. The number of strains and varieties of honey used in this study could be increased in future and it would be interesting if therapeutically registered honey is included for comparison.

Simulated honey inhibited typhoidal salmonellae and reference strains at quite higher concentration, negating the concept that sugar alone is responsible for antibacterial activity (Table I). Regarding ATCC reference strains, black seed honey again inhibited all strains at quite low concentration. George *et al.* (2007) recently reported the MIC of medihoney, a registered product for skin infections and burns was 4% (v/v) for *Staphylococcus aureus* (ATCC 25923), 8% (v/v) for *Pseudomonas aeruginosa* (ATCC 27853) and 8% (v/v) for *Enterococcus faecalis* (ATCC 29212). In comparison, the MIC of black seed honey against these reference strains is 6% (v/v) for *Staphylococcus aureus,* 7% (v/v) for *Pseudomonas aeruginosa* and 10% (v/v) for *Enterococcus faecalis.* These results indicate that black seed honey exhibited higher antibacterial activity than medihoney against *Pseudomonas aeruginosa* by using the same methodology. It is planned for the future investigation that active compound of black seed honey which inhibited typhoidal salmonellae can further be studied.

The ability of black seed honey to inhibit MDR typhoidal salmonellae isolated from typhoid patients at low concentration *in vitro* study necessitates the evaluation of oral/intravenous route in a suitable animal model. The application of *in vitro* results to clinical and systemic efficacy is of course an open challenge for future research. The most important issue is the reduced honey concentration in serum when diluted by the total volume of body fluid after oral intake. Nevertheless, orally taken honey promotes the growth of beneficial intestinal bacteria and recently a study has revealed that certain varieties of honey possess viable lactobacilli and bifidibacteria (Shin and Ustunol, 2005; Olofsson and Vasquez, 2008). High concentration of honey in serum of a patient with typhoid fever may be achievable by the intravenous route but possibility of some allergic reactions will have to be kept in mind. Usefulness and safety of intravenous honey has been shown in healthy sheep (Noori, 2003). Regarding varieties of honey, those honey samples that contain plant derived non-peroxide factors would be more useful in systemic illness as these factors will be not destroyed by catalase enzyme present in tissue or blood unlike hydrogen peroxide.

In the meantime, it is worthwhile that oral honey treatment may be supplemented to conventional treatment of typhoid fever. Considering the great potential of honey within a clinical environment, it is essential that research should continue beyond the topical application of honey to systemic infections particularly for multi-drug resistant microbes.

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