

Effect of different doses of Manuka honey in experimentally induced mouse typhoid

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Abstract: Typhoid fever is a major cause of morbidity and mortality in the developing world. Data from World Health Organization (WHO) shows that 21 million cases of typhoid occur globally every year and over 200,000 die each year; most of them at a very young age. The situation in Pakistan is similar. Typhi and other typhoidal salmonellae have developed resistance to chloramphenicol and other first line anti-typhoid. There is a rapid increase in multi-drug resistance (MDR) throughout the world. There is an urgent need to find out alternative medicine to sort out this problem. This study was conducted to establish preventive as well as therapeutic potential of Manuka honey. A total of eighty pathogen free *BALB/C* mice between 8 weeks to 12 weeks of age, weighing 25-30 grams were taken and divided into 4 groups. Group A, B and C were infected through oral route with 10⁸ colony forming unit (CFU) of *Salmonella typhimurium* ATCC 14028 to produce typhoid like disease in mice. Group A, which comprised of 20 mice was further divided in A1 and A2 given Manuka honey at a dose of 15ml/kg and 20 ml/kg respectively. Group B, which comprised of 20 mice was further divided in B1 and B2 was given Manuka honey at dose of 20ml/kg and 25ml/kg respectively. Clinical features of mouse typhoid, like body temperature, respiratory rate, number of stools and general behavior were recorded twice daily. Blood cultures of mice in different groups were taken at different days to evaluate the establishment of infection as well as to observe the therapeutic and preventive potential of Manuka honey in mouse typhoid. Fisher's Exact, Chi-Square and t-test were used to analyze the data. Significant association was observed in the ultimate fate of mice in Group A1 and Group A2 (P<0.001), showing that from a total of 20 mice in both groups, 10 mice fall in Group A1 of which 10 (100%) developed infection as it was not prevented by honey at a dose of 15ml/kg body weight (15.00±0.00) in Group A1 and ten mice fall in Group A2 of which 10(100%) did not developed an infection as it was prevented by honey at a dose of 20ml/kg body weight (20.00±0.00) in Group A2. Significant association was observed in the ultimate fate of mice in Group B1 and Group B2 (p<0.001) showing that from a total of 20 mice in both groups, 10 mice fall in Group B1 of which 10 (100%) had an infection, which was not treated by honey at a dose of 20 ml/kg body weight. Ten mice fall in Group B2 of which 10 (100%) had an infection, which was treated by honey at a dose of 25 ml/kg body weight (25.00±0.00). Results of the present study suggest that Manuka honey (UMF25+) has a potent anti-typhoid activity *in vivo* as well. There is an intense need for a carefully designed clinical trial in which this therapeutic potential of Manuka honey should be further evaluated. There is also need for the search of local honeys comparable to Manuka honey as a therapeutic option for typhoid fever.

Keywords: Manuka honey, *BALB/C*, *Salmonella typhimurium*, multi-drug resistant.

INTRODUCTION

Typhoid fever is a major cause of morbidity and mortality in the developing world. Recent data from World Health Organization (WHO) shows that 21 million cases occur globally every year and over 200,000 die each year; most of them at a very young age (Cooke *et al.*, 2007). According to one-study 93% cases occur only in Asia (Crump *et al.*, 2004). The situation in Pakistan is no different from rest of the developing world (Siddiqui *et al.*, 2006).

Typhoidal Salmonellae; Typhi, Paratyphi A, Paratyphi B and Paratyphi C are exclusively human pathogens and are transmitted via faeco-oral route and have strong association with non-availability of clean drinking water, poor sanitation, and unhygienic living conditions (Wellcome trust Sanger Institute. 2006).

The closest animal model that can be used for any investigation of typhoid fever is that of mouse typhoid. Loeffler in 1892 described the causative agent of murine typhoid, an epidemic typhoid fever-like disease in mice. *Salmonella typhimurium*, cause of enterocolitis in human beings causes typhoid like illness in genetically susceptible mice known as mouse typhoid. It has been seen that lesions produced by *Salmonella typhimurium* in the mice are more or less the same as produced by *Salmonella typhi* in man. The route of infection and pathogenesis is also the same, thus this could be an ideal model for the study of all aspects of typhoid fever in human beings (McGovern *et al.*, 2000). Susceptible mouse lineages *BALB/C* are widely used to study the pathogenesis of serotype *typhimurium* infections (Hans-Willi Mittrücker and Stefan H.E. Kaufmann, 2000).

Typhi and other typhoidal salmonellae have developed resistance to chloramphenicol and other first line anti-typhoid (Okeke *et al.*, 2005). There is a rapid increase in

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multi-drug resistance (MDR) throughout the world and Mexico catastrophe of 1970 is still fresh in our minds (Indian Pediatr [editor]. 1999). Then MDRs of 1990 is a recent happening and if ofloxacin had not been anticipated to be extremely effective against typhoid in 1986, history of 1970 may have repeated itself (Hannan 1986). However, the fluoroquinolones resistance in *S. typhi* and *S. paratyphi A* has emerged and seen in various parts of Asia (Rahman *et al.*, 2006). The appearance of an extended-spectrum beta-lactamases (ESBL) in *S. typhi* has further complicated the situation, because these isolates are resistant to most of the extended-spectrum cephalosporins, in particular Ceftriaxone (Al-Naiemi *et al.*, 2008). As ceftriaxone, is considered the drug of choice for the treatment of infections due to MDR and fluoroquinolone-resistant *S. typhi*, therefore it will become extremely difficult to treat these cases. Consequently, health care professionals are looking forward to traditional medicines to solve this issue (Mark M. Tanaka *et al.*, 2009).

Honey has been extensively used as healing agent throughout the human history in addition to its widespread usage as popular food (Al-Jabri, 2005). 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, 2006). The non-peroxide substances are not specifically identified and believed to be lysozyme, phenolic acids, flavonoides and others (Dunford C.2000). However in case of Manuka honey, recently methylglyoxal (MGO) was identified as an active ingredient, responsible for non-peroxide antibacterial activity, previously this compound was labeled as, Unique Manuka Factor (UMF) (Kassim *et al.*, 2009).

Manuka honey has developed as a wonder drug in wound care because it offers broad-spectrum antimicrobial properties and it promotes rapid wound healing (Mavric *et al.*, 2008). Manuka honey is being extensively used for the treatment of infected wounds, ulcers, diabetic foot ulcers, burns, skin graft donor sites, surgical wounds and particularly refractory infected wounds of immuno compromised patients when conventional treatments have failed (Henriques *et al.*, 2006). Honey impregnated wound dressing are licensed for medical use in the UK, New Zealand, Australia and other countries as Manuka honey of New Zealand and Medi honey of Australia have been approved by FDA for the treatment of chronic skin conditions, infected wounds, ulcers, diabetic foot ulcers, burns, skin graft donor sites (Simon *et al.*, 2006).

Manuka honey prevents the attachment of *Salmonella enteritidis* to intestinal epithelial cells and also enhances immune system (Alnaqdy *et al.*, 2005). In a recent study, George and Cutting compared the sensitivity of 130 clinical isolates to this medical honey and demonstrated that Manuka honey is effective against a broad range of

microorganisms including multi-resistant strains (George *et al.*, 2007). In a very recent study it has been reported that Manuka honey has antimicrobial activity against clinical isolates of *Campylobacter spp* (Hern Tze Tan *et al.*, 2009). A study by Molan reported that the MIC of Manuka honey was found to be 7% (v/v) in nutrient agar against *S. typhimurium* (Molan PC. 2010). In a recent study carried by Hannan *et al* showed the MIC of Manuka honey was recorded as 7% (v/v) for both *Salmonella paratyphi A*, and *Salmonella paratyphi B* and 7% to 8% for *S. typhi* (Hannan *et al.*, 2009).

To the best of our knowledge therapeutic effectiveness of Manuka Honey has not yet been a studied yet in vivo using mouse as an animal model. So the major motive behind this study is to prove the therapeutic effectiveness of Manuka honey against typhoid using mouse as an experimental model and in future that could be possibly translated into human beings for treatment and prevention of typhoid.

Ethical permission

This research project was approved by the Ethical Committee of University of Health Sciences, Lahore, Pakistan.

MATERIALS AND METHODS

Materials

Honey

Manuka Honey UMF-25+ (Comvita, New Zealand) was used in this study

Bacterial strain

Salmonella typhimurium ATCC 14028 was used during the study.

Storage of bacterial strain

The ATCC strain was stored in Micro banks at -80°C and refreshed onto blood agar before use.

Animals

A total of eighty pathogen free *BALB/C* mice between 8 weeks to 12 weeks of age, weighing 25-30 grams were taken from Experimental Research Laboratory University of Health Sciences, Lahore and were divided in 4 groups. Animals were obtained with full health reports and were housed under specific conditions with controlled temperature of 22°C relative humidity 70+4% and 12hr light/day cycle in the animal house of University of Health Sciences, Lahore, Pakistan.

Group A (Preventive group)

Comprised of twenty mice to determine the preventive potential of Manuka honey against murine typhoid.

Group B (Experimental group)

Comprised of twenty mice to determine the therapeutic potential of Manuka honey against murine typhoid.

Group C (Positive control)

Comprised of ten mice which: were infected but not treated. This group served as a marker of infection.

Group D (Negative control)

Comprised of ten mice, which were kept separate and were given standard diet with no additive.

Sterility and storage of honey

The sample of honey was filtered with a sterile mesh and inoculated on blood agar, incubated overnight to check microbial purity and was stored at room temperature.

Methods**Preparation of Infectious Material**

Salmonella typhimurium ATCC (14028) was refreshed onto blood agar plates and incubated overnight at 37°C. Morphologically identical colonies were picked up from overnight culture and shifted to Nutrient broth supplemented with 0.3 M NaCl, diluted 1:20 in fresh medium, and incubated for 4 hours. Bacteria were washed twice in ice-cold phosphate-buffered saline (PBS) and then suspended in cold PBS to an appropriate optical density, measured spectrophotometrically at 600 nm to final concentration of 10⁸ CFU/50µl using sterile phosphate buffer saline (PBS) as a blank and a diluent and a cuvette with a 1 cm pathway (Alnaqdy et al., 2005).

Labeling of mice

All the animals were kept in separate cages according to their groups and proper labeling was done.

Experimental infection of mice

Water and food were with drawn for 4 hours prior to oral inoculation and animals were infected by oral route with the help of insulin syringe with mounted butterfly cannula at its upper end to minimize damage to oral mucosa and to make feeding easy for the animal.

Group A (Preventive group)

This group was divided into 2 subgroups each comprising 10 mice.

Group A-1

They received Manuka Honey UMF-25 + p.o. at a dose of 15 ml/kg (20.55gm/kg) body weight in two divided doses from the day one of the experiment. This group was infected with 10⁸ CFU of *Salmonella typhimurium* ATCC (14028) (50µl suspension in PBS p.o.) on the second day morning. Honey was given twice daily in divided doses for the next ten days.

Group A-2

They received Manuka Honey UMF-25 + p.o. at a dose of 20 ml/kg (27.4gm/kg) body weight in two divided doses from the day one of the experiment. This group was infected with 10⁸ CFU of *Salmonella typhimurium* ATCC

14028 (50µl suspension in PBS p.o.) on the second day morning. Honey was given twice daily in divided doses for the next ten days.

Group B (Experimental group)

They were infected with 10⁸ CFU of *Salmonella typhimurium* ATCC (14028) (50µl suspension in PBS p.o.). At this stage, this group was divided into 2 subgroups each comprising 10 mice.

Group B-1

This group received Manuka Honey UMF-25 + p.o. at a dose of 20ml/kg (27.4gm/kg) body weight in two divided doses at day five after oral infection. This continued for the next ten days, till day fourteen of the experiment.

Group B-2

This group received Manuka Honey UMF-25 + p.o. at a dose of 25ml/kg (34.25gm/kg) body weight in two divided doses at day five of the experiment. This continued for the next ten days, till day fourteen of the experiment.

Group C (Positive control)

They were infected with 10⁸ CFU of *Salmonella typhimurium* ATCC 14028 (50µl suspension in PBS p.o.). Afterwards they were given standard diet with no additives.

Group D (Negative control)

They were given 50µl of sterile PBS p.o. on day one and afterwards they were given standard diet with no additives.

All the animals were observed after 24 hours for signs and symptoms of Colitis and then were observed twice daily for next 15 days for signs and symptoms of typhoid Fever, any mortality, behavior change or other unwanted effects.

Observations

Observations included.

Recording of body temperature

Body temperature of mice was observed twice daily for fifteen days by using a thermometer and placing it in thigh fold of mice.

Recording of respiratory rate

Respiratory rate of the mice was observed by visual inspection of mice.

No. of stools

Number of stools passed by mice was recorded and visual inspection of stool was also done to rule out presence of blood or pus in the stools.

Table 1: Results of independent sample t-test on age, weight and culture day in different groups

	Comparison	Age (Mean ± S.D)	p-value	Weight (Mean ± S.D)	p-value	Culture Day (Mean ± S.D)	p-value
Group A	Group A1 n=10	9.00 ± 0.94	0.79	31.00 ± 0.94	0.40	7.30 ± 3.56	0.03*
	Group A2 n=10	10.00 ± 1.141		31.45 ± 1.38		10.00 ± 0.00	
Group B	Group B1 n=10	9.30 ± 1.64	0.35	30.70 ± 1.25	0.23	10.90 ± 2.60	0.00*
	Group B2 n=10	10.00 ± 1.63		31.40 ± 1.26		14.00 ± 0.00	
Group A and B	Group A n=20	9.50 ± 1.28	0.75	31.22 ± 1.17	0.65	8.65 ± 2.81	0.00*
	Group B n=20	9.65 ± 1.63		31.05 ± 1.28		12.45 ± 2.39	
Group B and C	Group B n=20	9.65 ± 1.63	0.19	31.05 ± 1.28	0.66	12.45 ± 2.39	0.00*
	Group C n=20	8.90 ± 0.99		30.25 ± 0.42		7.40 ± 2.55	
Group A and C	Group A n=20	9.50 ± 1.28	0.20	31.22 ± 1.17	0.18	8.65 ± 2.81	0.25
	Group C n=20	8.90 ± 0.99		30.25 ± 0.42		7.40 ± 2.55	

* Independent sample t-test (p<0.05)

Table 2: Blood culture results of Group A

	Groups		Total
Blood Culture	Group A1 n=10	Group A2 n=10	N=20
Positive	10(100%)	0 (0%)	10
Negative	0 (0%)	10(100%)	10

Table 3: Ultimate fate of Group A

	Groups		Total
Ultimate Fate	Group A1 n=10	Group A2 N=10	N=20
Infection not prevented by honey	10(100%)	0 (0%)	10
Infection prevented by honey	0 (0%)	10(100%)	10

Table 4: Blood culture results of Group B

	Groups		Total
Blood culture	Group B1 N=10	Group B2 N=10	N=20
Positive	10(100%)	0 (0%)	10
Negative	0 (0%)	10(100%)	10

Table 5: Ultimate fate of Group B

	Groups		Total
Ultimate Fate	Group B1 N=10	Group B2 N=10	N=20
Infection not treated by honey	10(100%)	0 (0%)	10
Infection treated by honey	0 (0%)	10(100%)	10

Pearson chi-square=20.000 p value<0.001

General behavior

General behavior of the mice was noticed to see whether there was some untoward response to honey given during treatment and prevention phase.

inoculation, each culture bottle was kept in an incubator at 37C⁰.A subculture was performed on very next day on blood agar plate with subsequent sub-cultures on day three and five till a positive culture was obtained.

Blood cultures

Blood cultures were taken to evaluate that infection had been established in the mice, as well as to observe that honey acted as a therapeutic agent in Group B and acted as a preventive agent in Group A. For this purpose pediatric blood culture bottles were used and about 1 ml blood was inoculated in each culture bottle. After

For all the negative cultures a subculture was done on day seven onto the Blood agar plate. For all the positive sub-cultures, biochemical identification was done by using API-20E and serological identification was done by using polyvalent salmonella antisera and monovalent O and H antisera.

Group A1

Out of ten mice, two died at day five of experiment so blood cultures were taken directly from heart. Similarly two mice died at day eight and two of them died at day nine of the experiment so same approach was followed for them. Three mice remained alive till day ten and then died, so the same approach was followed for them as well. Only one mouse remained alive till day eleven and the blood culture was taken again through direct heart puncture. All the cultures were taken under sterile conditions adopting all the precautionary measures using aseptic technique.

Group A2

In this group a slightly different approach was adopted, as all the mice remained alive till day ten of the experiment, so blood cultures of all the ten mice were taken at day ten of the experiment through direct heart puncture using aseptic technique.

Group B1

In this group one mouse died at day five of the experiment so blood culture was taken at that day through direct heart puncture. Four mice died at day ten of the experiment so blood cultures were taken on that day through direct heart puncture. Two mice died at day eleven and two of them died at day twelve of the experiment, so the blood cultures were taken on that day again through direct heart puncture. One of them died the very next day so the blood culture was taken on day thirteen through direct heart puncture.

Group B2

Slightly different approach was adopted in this group as all the mice remained alive till day fourteen of the experiment, so blood cultures of all the mice in this group were taken at day fourteen through direct heart puncture following full aseptic measures.

Group C

In this group three, out of ten mice died at day eight of the experiment so blood cultures of those three mice were taken at day eight. Six mice died the very next day, so the blood cultures of those six mice were taken at day six of the experiment. One remaining mice died at day ten of the experiment and its blood culture was taken at day ten. All the cultures were taken through direct heart puncture, using full aseptic technique.

Group D

All the ten mice in this group remained healthy and alive till day eleven of the experiment when one of them died so its blood culture was taken on day eleven. Remaining nine were normal and healthy till day fifteen when they were sacrificed and their blood cultures were taken through direct heart puncture using full aseptic measures.

Disposal of infected animals

After taking blood cultures all the animals were buried to exclude any possibility of spread of infection to other animals or to human beings.

STATISTICAL ANALYSIS

The data collected were analyzed using Statistical Package for Social Science (SPSS) software version 16.0. Fisher Exact test and Chi square test were used to analyze the results where appropriate. The p value of <0.05 is considered as statistically significant. For the comparison of the Age, weight and Day on which culture was taken in different groups independent sample t-test was applied

RESULTS

The results of comparison of the age, weight and day on which culture were taken are shown in table 1. No significant difference was observed in age and weight of group A1 and A2 ($P>0.05$) but a significant difference was there in the days on which culture were taken ($P<0.05$). In case of group B1 and B2, significant difference was found in the culture day ($P<0.05$). Similarly in the comparison of group B with A and C, significant difference was only found in days on which culture was taken ($P<0.05$). In comparison of Group A and C, there was found no significant in any variable ($P>0.05$).

The results of association of blood culture along with total number of mice in Group A are shown in table 2. Significant association was observed between blood culture results in Group A1 and A2, $p<0.001$, showing that from a total of 20 blood cultures of mice in both the groups, 10 mice fall in Group A1 of which 10 (100%) gave positive result in Group A1. Ten mice fall in Group A2 of which 10 (100%) gave negative result in Group A2.

The association of ultimate fate along with total number of mice in Group A is shown in table 3. Significant association was observed in the ultimate fate of mice in Group A1 and Group A2 ($P<0.001$), showing that from a total of 20 mice in both groups, 10 mice fall in Group A1 of which 10 (100%) developed infection as it was not prevented by honey at a dose of 15ml/kg body weight (15.00 ± 0.00) in Group A1 and ten mice fall in Group A2 of which 10(100%) did not developed an infection as it was prevented by honey at a dose of 20ml/kg body weight (20.00 ± 0.00) in Group A2.

The association of blood culture results along with total number of mice in Group B is shown in table 4. Significant association was observed between blood culture results in Group B1 and B2, $p<0.001$, showing that from a total of 20 mice in both the groups, 10 mice fall in Group B1 of which 10 (100%) gave positive result in Group B1. Ten mice fall in Group B2 of which 10 (100%) gave negative result.

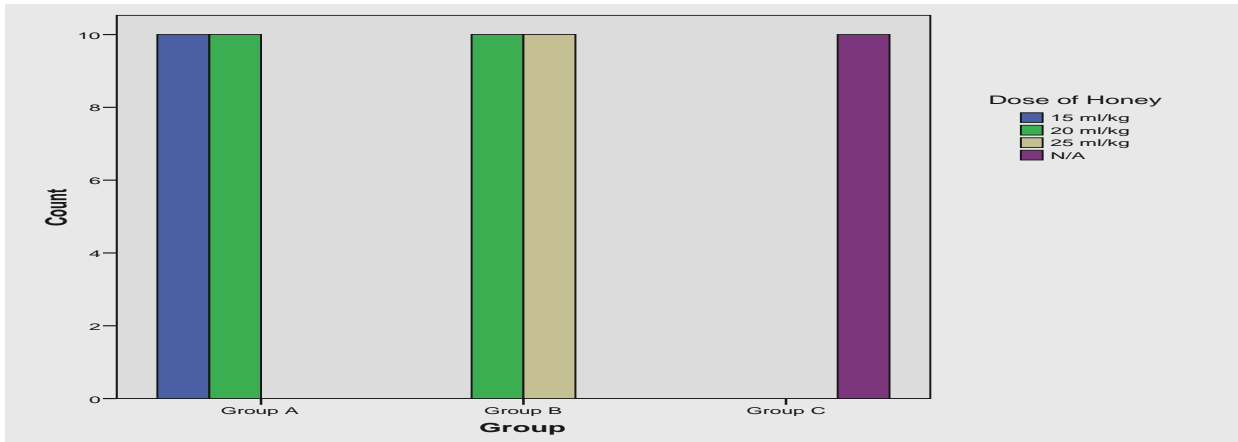


Fig. 1: Dose of honey in different groups.

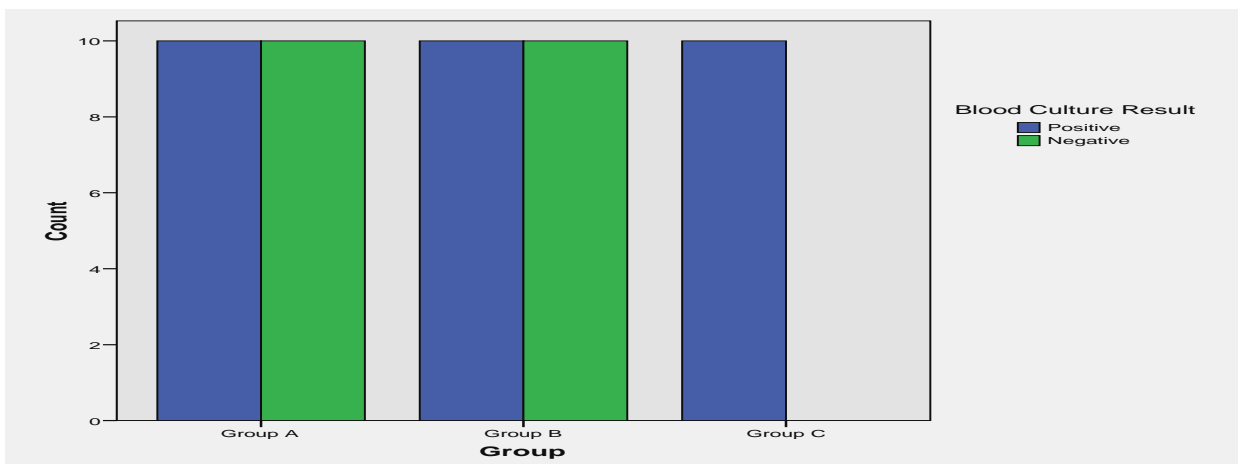


Fig. 2: Blood culture in different groups.

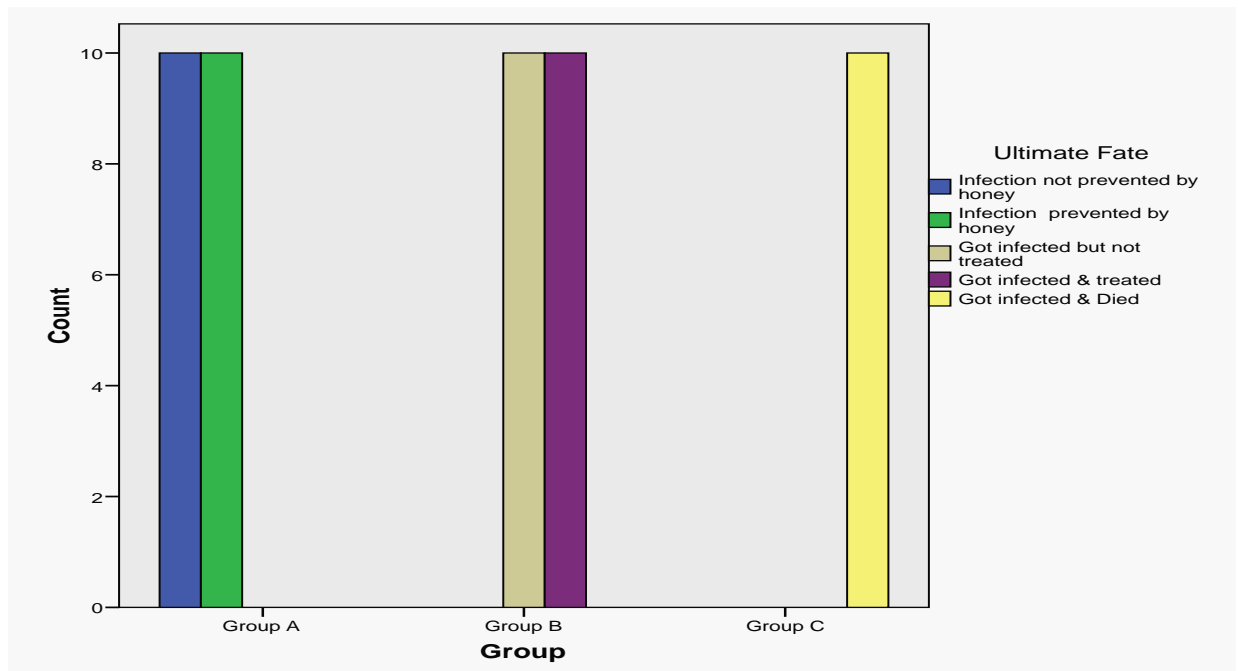


Fig. 3: Ultimate fate in different groups.

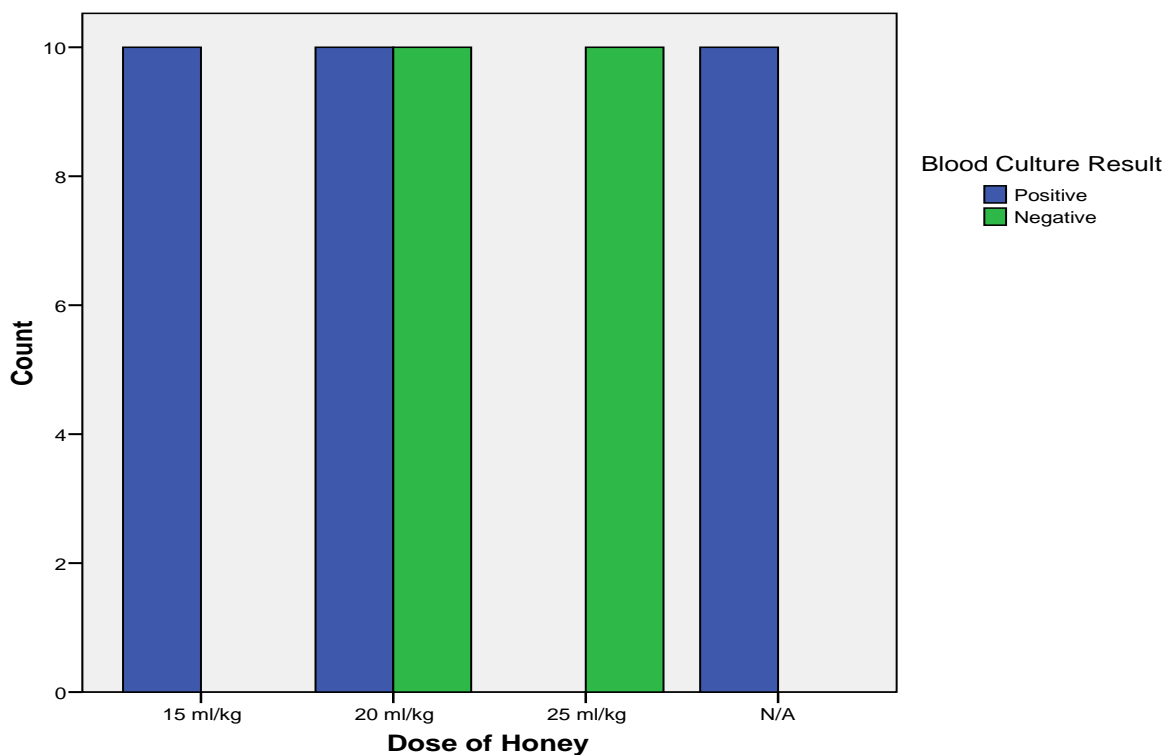


Fig. 4: Blood culture results with the dose of honey.

The results of the association of total number of mice with ultimate fate of mice in Group B are shown in table 5. Significant association was observed in the ultimate fate of mice in Group B1 and Group B2 showing that from a total of 20 mice in both groups, 10 mice fall in Group B1 of which 10 (100%) had an infection, which was not treated by honey at a dose of 20ml/kg body weight. Ten mice fall in Group B2 of which 10 (100%) had an infection, which was treated by honey at a dose of 25ml/kg body weight (25.00±0.00).

To find out the association of dose of Honey with different groups Chi-Square test was applied and there was found a strong association between the doses of honey in different groups of the study (P=0.00). Results are shown in fig. 1. The dose of honey was 15ml/kg body weight (15.00±0.00) in-group A1 and 20ml/kg body weight (20.00±0.00) in-group A2 as shown in fig. 1. In group B1 honey was used at a dose of 20ml/kg body weight (20.00±0.00) and in group B2 at a dose of 25ml/kg body weight (25.00±0.00).

Significant association was found in blood culture results in different groups (p=0.001). Results are shown in fig. 2. Blood cultures were positive in all the mice (100%) of group A1 and B1 and C were negative in all the mice (100%) of group A2 and B2.

Similarly in case of ultimate fate in different groups, again there was found a strong association in the ultimate

fate of different groups (p=0.00). Results are shown in fig. 3. In-group A1; infection was not prevented by honey (100%) at dose of 15ml/kg, while in-group A2 infection was prevented by honey (100%) at dose of 20ml/kg body weight.

Significant association was observed in the case of blood culture result and different doses of honey (P=0.00). Blood Culture results were becoming more negative as we move towards higher dose of the Honey. Results are shown in fig. 4.

DISCUSSION

The emergence of resistance to conventional anti-typhoid antibiotics, Ampicillin, Cotrimoxazole and Chloramphenicol against *Salmonella typhi* is a global health problem and is common in Asia including Pakistan. The emergence of resistance to Fluoroquinolones and third generation Cephalosporin, for example, Ceftriaxone had almost exhausted the stock of drugs available for the treatment of typhoid and there is strong realization that there should be something more than antibiotic to treat this deadly disease. This provoked us to look into vast potential of Manuka honey to act as a drug against typhoid. As one of the most important property of Manuka honey is its non-peroxide activity, which can be exploited to treat septicemias and systemic infections, like typhoid. To best of our knowledge this

would be the first report regarding anti typhoid activity of Manuka honey *in vivo* using mouse model.

Manuka honey is the most extensively researched honey in the world and famous for its non-peroxide activity. The potency of non-peroxide antibacterial activity of Manuka honey was measured and standardized by (Allen *et al.*, 1991) in accordance with phenol equivalence and was designated as Unique Manuka Factor (UMF). Manuka honey with a rating of Unique Manuka Factor 10 or more is recommended for therapeutic use and also called active Manuka honey. In 1996 an organization called Trade NZ, in conjunction with the Honey Research Unit, University of Waikato, New Zealand, establish a UMF[®] industry standard (Manuka honey, 2010).

Manuka honey in this study was used in different ways to explore its therapeutic potential against *Salmonella typhimurium* ATCC (14028) using BALB/C mice as a model which was the extension of a recent project done by Hannan *et al.*, (2008) who demonstrated that, Manuka honey showed 13.50±0.79 (mm) zone size at 25% (w/v) dilution with catalase solution against ATCC (25923) reference strain, *Staphylococcus aureus*. This finding confirms the previous reports regarding the existence of non-peroxide antibacterial factors in “active” (UMF) Manuka honey. The exact chemical nature of these non-peroxide factors are still unknown; however, recently methylglyoxal (MGO) was identified as an active ingredient, responsible for non-peroxide antibacterial activity in Manuka honey, which is commonly designated as, Unique Manuka Factor (UMF) (Efem SE *et al.*, 1992). There are very few honeys which possess non-peroxide antibacterial properties.

Manuka honey was tested to act as a preventive agent keeping in view that this honey uses multiple strategies to prevent and treat typhoid fever. On one side it directly inhibits the microorganism by high osmolarity, acidic pH, release of hydrogen peroxide and plant derived non-peroxide antibacterial substances. On the other side, it inhibits the growth of pathogenic microorganism by stimulating the growth of beneficial GIT flora, a phenomena called as bacterial interference (Shabanah OA *et al.*, 2000).

More recently a study revealed that certain varieties of honey can promote the growth of normal microbial flora like *Lactobacilli* and *Bifidobacterium* species (Kalapos MP .2008). This is not the case with antibiotics, which affect not only the pathogenic microorganisms but also inhibit the growth of normal flora. Thus, treatment with antibiotics can create an imbalance, which itself may lead to serious drug-resistant infections. Besides, honey also prevents the adherence of *Salmonella* to intestinal epithelium and also effective in disrupting the biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Henriques *et al.*, 2010). Recently identified MGO, which

is said to be the active ingredient of Manuka honey protects the intestinal mucosa from injurious stimuli and stimulates the immune system as well (Mavric *et al.*, 2008). Above all honey does not allow micro-organism to develop resistance unlike the conventional antibiotics (Kalapos, 2008).

To prove this fact that honey, especially Manuka honey which harbors potent non-peroxide activity has a potential of preventing as well as treating typhoid fever. In the first phase of the study different doses of Manuka honey were used in two groups of mice Group A1 (n=10) and Group A2 (n=10), to prove that Manuka honey can prevent the establishment of typhoid fever in genetically susceptible mouse lineage i.e., BALB/C. It was seen that Manuka honey at a dose of 20ml/kg body weight (20.00 ±0.00) which is equivalent to 27.4 gm/kg body weight prevented the establishment of disease in mice in Group A2 (n=10) as all the mice had negative blood cultures 100% (p<0.001), as compared to 15ml/kg body weight (15.00±0.00) equivalent to 20.55gm/kg (p<0.001) used in Group A1 (n=10), at which mice still developed typhoid like illness proven by the positive blood cultures 100% (p<0.001), along with symptoms of septicemia, high grade temperature, hyperventilation about 4-7 days after oral challenge.

Significant difference was observed in the dose of honey in ml/kg body weight p<0.001 showing that dose of honey in ml/kg was higher in Group A2 (20.00±0.00) as compared to Group A1 (15.00±0.00).

Significant association was observed between blood culture results in Group A1 and A2, p<0.001, showing that from a total of 20 blood cultures of mice in both the groups, 10 mice fall in Group A1 of which 10(100%) gave positive result in Group A1. Ten mice fall in Group A2 of which 10(100%) gave negative result in Group A2.

Significant association was observed in the ultimate fate of mice in Group A1 and Group A2, P<0.001, showing that from a total of 20 mice in both groups, 10 mice fall in Group A1 of which 10 (100%) developed infection as it was not prevented by honey at a dose of 15ml/kg body weight (15.000±0.000) in Group A1 and ten mice fall in Group A2 of which 10(100%) did not developed an infection as it was prevented by honey at a dose of 20ml/kg (27.4 gm/kg) body weight (20.00±0.00) in Group A2.

All above findings showed that mice in the group A1 in spite of getting honey were still having signs of infection, namely high grade fever and hyperventilation and ultimately resulting in death of mice, which led us to a thinking that those mice in Group A1 were given a suboptimal dose of honey which could not prevent the establishment of infection in Group A1.

Translation of this dose in a human being of about 60 kg weight based on BSA would make a dose of 2.22 gm/day of Manuka honey to prevent typhoid fever in human beings (Shannon Reagan-Shaw *et al.*, 2007). According to our findings this dose of Manuka Honey UMF 25+ can be safely use continuously to prevent typhoid fever in human beings.

The proposed mechanism for protection against typhoid could be that honey did not allowed bacteria to adhere to the intestinal epithelial cells as mentioned in previous reports (Okhiria *et al.*, 2004). Similarly it could be the presence of MGO that protected the intestinal epithelial cells from bacterial challenge and on the other hand MGO also stimulated the immune system of mice, thereby protecting the mice from bacterial colonization (Booth *et al.*, 2003).

Because the adhesion of bacteria to intestinal epithelial cells is crucial to the initial phase of infection blocking the adhesion in the intestine may prevent infections of bacteria such as *Salmonella*. The adherence of bacteria to cell surfaces is known to be mediated in at least three ways: by lectin-carbohydrate recognition, by protein-protein recognition, and by hydrophobic protein recognition. Oligosaccharides in human milk have been shown to prevent bacteria and toxins from attaching to intestinal cells. In a study conducted on milk a trisaccharide found in human milk, was shown to inhibit the adherence of *Streptococcus pneumonia* to buccal epithelial cells (Sugita-Konishi *et al.*, 2002).

On the basis of these findings, the oligosaccharides contained in foods are expected to provide protection against infectious gastric diseases by preventing bacteria and toxins from attaching to target cells. As it is well known fact that honey is a mixture of variety of carbohydrates and beneficial flora this could be a possibility that this carbohydrate component prevented adherence of *Salmonella typhimurium* to the intestinal epithelial cells (Sugita-Konishi *et al.*, 2002).

Another proposed mechanism could be that Manuka honey inhibited the entry of *Salmonella typhimurium* into the intestinal epithelial cells. Our finding supports the previous report by Sugita-Konishi *et al.* (2002) who reported that egg-yolk-derived sialyloligosaccharide (YDS) and its derivatives are useful for preventing *Salmonella* infection when ingested continuously. Their results suggested that YDS inhibit *Salmonella* infection not by activating macrophages but by inhibiting the entry of bacteria through the gut (Sugita-Konishi *et al.*, 2002).

Our finding also supports the previous report by Stefanova *et al.* (2007) which demonstrated that prophylactic peroral administration of coumarin or 7-hydroxycoumarin (7-OHC) enhances resistance to subsequent lethal *Salmonella enterica* Serovar

typhimurium infection in mice. This Coumarin was seen to decrease bacterial load in liver and spleen, and enhanced phagocytosis and bacterial killing by macrophages when applied in vitro and in vivo (Kassim *et al.*, 2009).

In the second phase of the study, therapeutic potential of Manuka honey against *Salmonella typhimurium* was determined. Different doses of Manuka honey were used in two groups of mice Group B1 (n=10) and Group B2 (n=10). To our surprise Manuka honey at a dose of 25 ml/kg body weight (25.00±0.00) which is equivalent to 34.25gm/kg body weight of mice did a wonder in Group B2 (n=10), (p<0.001) as blood cultures in all the ten mice were negative 100% (p<0.001), as compared to Group B1(n=10) in which Manuka honey was used in a dose of 20 ml/kg body weight(20.00±0.00), equivalent to 27.4 gm/kg body weight of mice. All mice in Group B1 (n=10) had a positive blood cultures 100% (p<0.001). Also all the ten mice died showing that they were not getting effective treatment against *Salmonella typhimurium* infection.

Significant association was observed between blood culture results in Group B1 and B2, p<0.001, showing that from a total of 20 mice in both the groups, 10 mice fall in Group B1 of which 10(100%) gave positive result in Group B1. Ten mice fall in Group B2 of which 10(100%) gave negative result.

Significant association was observed in the ultimate fate of mice in Group B1 and Group B2 showing that from a total of 20 mice in both groups, 10 mice fall in Group B1 of which 10 (100%) had an infection, which was not treated by honey at a dose of 20ml/kg body weight. Ten mice fall in Group B2 of which 10 (100%) had an infection, which was treated by honey at a dose of 25ml/kg body weight.

All above findings showed that mice in the group B1 in spite of getting honey were still having signs of infection, namely high grade fever and hyperventilation and ultimately resulting in death of mice, which led to a thinking that those mice in Group B1 (n=10) were receiving a suboptimal dose of honey which could not treat the infection in this group.

Translation of this dose that treated the mice having typhoid like illness based on BSA would make a dose of 2.77gm/day of Manuka honey in a human being of 60 kg weight (Shannon Reagan-Shaw *et al.*, 2007). According to our observation in this study using mouse as an animal model, this dose of Manuka honey UMF 25+ can be safely used in human beings for the treatment of typhoid fever.

It should be noted that the success of the Manuka honey given by oral route against *S. typhimurium* infection in

mice may be attributed to more than just antibacterial activity: it could be due to the tetracycline residues in honey and to the fact that honey stimulates the proliferation of lymphocytes and phagocytes, thus activating the immune response to infection (Tsvetanka Stefanova *et al.*, 2007).

Our results suggest that honey, would have acted as an immunomodulator against typhoid which is a condition in which patient develop septicemia along with production of mediators of inflammation. Honey behaved as immunomodulator in a study conducted by Kassim *et al.* (2009).

Our finding could also strengthen the previous report by Hannan *et al.*, (2009) which showed that Manuka honey has potent anti typhoid activity in vitro which could be translated to produce similar effects in vivo using mouse model.

This finding supports the previous report by Badawy *et al.* (2004) which showed a decrease in mortality and morbidity (93.3% morbidity and 40% mortality) of mice after treatment with Egyptian clover honey (Badawy *et al.*, 2004).

According to the results of our study, it is worthwhile that oral honey treatment be supplemented to conventional treatment of typhoid fever. The only problem that can be foreseen in this aspect is the reduced honey concentration in serum when diluted by the total volume of body fluid after oral intake. High concentration of honey in serum of a patient with typhoid fever may be achievable by the intravenous route or parenteral route (Al-Waili, 2003). But there is theoretical possibility of some allergic reactions which will have to be kept in mind. Usefulness and safety of intravenous and intrapulmonary honey has been shown in healthy sheep and further needs to be established (Al-Waili, 2003).

Manuka honey has also been used experimentally in rats to treat inflammatory bowel disease. There was significant protection with Manuka honey 5 g/kg as well as with 10g/kg body weight compared with the control. According to one study by Prakash *et al.* indicated that Manuka honey is efficacious in the TNBS-induced rat colitis model and this could be used as an experimental agent to treat this type of colitis in human beings (Prakash *et al.*, 2008).

Honey was also used by oral as well as intravenous route to treat severe pneumonia in a patient of malignant bone tumor (Mamdouh A, 2008).

Results in the present study suggest that Manuka honey (UMF25+) have potent antityphoid activity in mice and that could be used as a therapeutic agent against typhoid in human. There is an intense need for a carefully

designed clinical trial in which this therapeutic potential of Manuka honey should be further evaluated in human beings. There is also need for the search of local honeys comparable to Manuka honey as a therapeutic option for typhoid fever.

CONCLUSION

Results in the present study suggest that Manuka honey (UMF25+) have potent antityphoid activity in vivo and that could be used as a therapeutic agent against typhoid in human as well.

ACKNOWLEDGEMENTS

We would like to acknowledge the efforts of Dr. Mohammad Barakat for his valuable guidance in this project.

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