Effectiveness of Commonly Used Antibiotics in Combination with Honey Against Bacterial Infection

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ABSTRACT

Objectives: The study was carried out to compare the inhibitory effects between commonly used antibiotics and bee honey samples, so as to correlate the inhibitory effects between bee honey alone and in combination with antibiotics.

Methods: This study was carried out between December 2012 to September 2013. A total of one hundred and twenty-two clinical microbiological specimens and five different floral sourced honey samples were collected between December 2012 to September 2013. Twenty-three multi-drug resistant organisms were selected. Then, AST for commonly used antibiotics, honey alone and combination of honey-antibiotics discs was done. The difference in ZOI of antibiotic contrasting with the antibiotics containing honey were statistically analysed to define the synergism.

Results: The inhibition due to honey is variable among bacteria types ($F=39.17$, $p<0.05$). From means plot, *Staphylococcus* and *Acinetobacter* were recognized as highly susceptible bacteria for honey ($\bar{x}$ = 21.1 ± 6.2 mm and $\bar{x}$ = 18.3 ± 3.3 mm respectively) but *Acinetobacter* species could not show synergism to honey-antibiotic combination. The tested organisms from *Enterobacteriaceae* family showed effective susceptibility to Chloramphenicol-honey mixture. Imipenem-honey combination and Gentamicin-honey combination showed significant effects against *Pseudomonas aeruginosa*.

Conclusion: Thus, honey can be used in various bacteria-directed infections and found to be effective in various infections. Incorporation of honey in antibiotics like Chloramphenicol, Imipenem, and Gentamicin works better in healing various infection.

Key words: Honey, Antibiotics, Synergism, Antibiotic-honey combination

INTRODUCTION

Honey has four modes for the antibacterial effects (Molan 1992). They are osmophilic effect (Molan 1992), acidic pH (Nassar et al. 2012), hydrogen peroxide production due to glucose oxidase of bee gut (Irish et al. 2011, Bizerra et al. 2012), and antioxidants such as catalases, polyphenols, Maillard reaction products and ascorbic acid (Bizerra et al. 2012) and other components in nectar produced by the plants (Molan 1992).

It is bacteriostatic and bactericidal for gram positive and gram-negative bacteria (Pimentel et al. 2013). It also possesses antifungal character (Lane et al. 2019) and can be used against antibiotic resistant bacteria (Sharp 2009) like MRSA (Müller et al. 2013) and VRE (Boukraâ and Sulaiman 2009).

The protein now identified as universal stress protein A (UspA) is involved in the stress stamina response and its down-regulation could help to explain the inhibition of MRSA of Manuka honey. The level of expression was found to be changed at least two-folds following treatment with the honey (Jenkins et al. 2011).

However, a review article and meta-analysis suggests that the previous works are not sufficient to prove the

Date of Submission: September 22, 2020
Date of Acceptance: November 5, 2020
Published Online: December, 2020
DOI: https://doi.org/10.3126/tujm.v7i0.33797

antimicrobial effects of honey (Wijesinghe et al. 2009). In Nepal, the similar studies were performed where honey samples used were not measured properly during the tests. Thus, this study was carried out to determine the present condition of bacteria for the susceptibility towards natural compound honey and to define the statistically significant synergism with various antibiotics.

MATERIALS AND METHODS

This study was performed between December 2012 to September 2013. One hundred and twenty-two microbiological specimens included pus specimen, wound swabs, blood, urine, body fluids were obtained for routine culture in microbiology laboratory.

Sample collection:

1. Study population: All age groups and both sexes visiting KNFH were included from whom the samples were obtained for routine culture and AST. Patients or their relatives refusing to give informed consents were excluded from the study. The samples from the patients who were already in medication were also excluded from the data.

2. Collection and transportation of clinical specimen:

   (a) Pus samples: A sterile cotton swab moistened with physiological saline was used to collect two pus swabs from each patient, one for Gram staining and other for culture. The both swabs were transported aseptically to laboratory for further processing.

   (b) Blood samples: 1 volume of blood sample collected from the patient was mixed with 5 volume of Brain Heart Infusion (BHI) and transported to laboratory for further processing.

   (c) Urine samples: About 10 to 20 ml of mid-stream urine sample was collected in a sterile container and transported to laboratory for further processing.

3. Processing of samples: Pus samples were observed for their consistency and blood contents. The physical appearance of urine samples was recorded for future reference.

   The pus samples, blood samples, and the urine samples were streaked on Nutrient Agar (NA), Blood Agar (BA) then MacConkey Agar (MA). The plates were incubated at 37 ± 0.2 °C for 24 to 48 hours. The colour, appearance, pH and turbidity of urine were evaluated during macroscopic examination of the urine sample (Vandepitte et al. 2003 Collee et al. 2001). The urine samples were cultured onto the BA and MA plates by the semi-quadrant streak technique using a standard calibrated loop having internal diameter of 4 mm. The protocol was followed as recommended by WHO (Vandepitte et al. 2003). The plates were then aerobically incubated at 37 ± 0.2 °C for 24 to 48 hours before reporting negative. Semi-quantitative counting method was performed to calculate the number of cfu mL^{-1} of urine and the bacterial count was reported as:

Significant bacteriuria:

- Urine containing more than 10^5 (100000) bacteria per ml that is 10^5/ml urine-is an indication of UTI.
- Women with symptomatic UTI ≥ 10^5 cfu mL^{-1} :
- Men ≥ 10^3 cfu mL^{-1} (if 80% of the growth is due to single organism in both cases) (Scottish Intercollegiate Guidelines Network, 2012).

If the culture indicates presence of two uropathogens both showing significant growth, definitive identification and antimicrobial susceptibility testing of both were performed whereas in case of ≥ 3 pathogens, it was reported as multiple bacterial morphotypes and asked for appropriate recollection with timely delivery to laboratory (Vandepitte et al. 2003).

The culture plates were examined for the visual growth of the organisms. The colour and the morphology of the colonies were observed carefully. Then biochemical tests were performed for the isolated colonies for their identification.

Antibiotic susceptibility test (AST) by Kirby Bauer disc diffusion method was performed to select out the drug resistant organisms and multiple drug resistant organisms were selected for the study. The organism which showed resistance to at least three or more antibiotics of different classes were considered as MDR (Sahm et al. 2000). For the identification of the characters, the ZOI chart provided by CLSI guidelines (2011) was followed. Five honey samples of various origins and of two varieties of bees (*Apis mellifera* and *Apis dorsata*) were used for the tests.

Honey discs were prepared by using dry sterile filter

papers of same thickness (1 mm) and same size (6 mm diameter) as the antibiotic discs on which about 50 microlitres of honey samples were dispensed individually. Same volume was poured on antibiotic discs to meet the concentration same as honey discs used. To ensure the transfer of same volume of honey, calibration of the micropipette was done at regular interval of time. The following steps were followed for the calibration. 1 ml of deionized water was carefully pipetted into the plate in a sensitive balance. Its weight was measured. After continuous repetition for 3 times, the average weight was calculated. Then the following formula was used to calculate the accuracy of the pipette.

$$accuracy\ (\%) = \frac{Pipette\ volume - average\ value}{Pipette\ volume} \times 100\ (\%)$$

All the bacterial isolates were tested for antibiotic susceptibility test by Kirby Bauer disc diffusion method with Mueller–Hinton Agar using the guidelines and interpretive criteria of the CLSI guidelines (CLSI 2011). The antibiotic discs used were Amoxycillin-Clavulanic acid (20/10µg), Ceftazidime (30µg), Ceftriaxone (30µg), Chloramphenicol (30g), Ciprofloxacin (5 µg), Cotrimoxazole (1.25/23.75µg), Gentamicin (30µg), Imipenem (10µg), and Oxacillin (1µg). The diameter of Zone of Inhibition (ZOI) was recorded for each disc. During this procedure, the measured diameter for honey discs was deducted from the diameter of the sterile discs.

The differences in Zone of inhibition (ZOI) of antibiotic contrasting with the antibiotic containing honey were statistically analysed to define the synergism. Data entry was performed using Microsoft® Excel® and data analysis was performed using Statistical Package for Social Sciences (SPSS® 16.0 for Windows®) software. The one-tailed t-statistics with α *value* 1% was implied for the statistical confirmation.

The following formula was used to calculate the test statistics t:

$$t = \frac{\bar{x}_{diff}}{\sqrt{n}}$$

$\bar{x}_{diff}$ = where

s = sample mean of the differences

s = sample standard deviation of the differences

n = sample size (i.e. number of pairs)

Null hypothesis setup: There is no difference in the use of honey and antibiotic or antibiotic alone ($\mu_1 - \mu_2 \geq 0$).

Alternate hypothesis setup: There is enhanced effect of honey and antibiotic than antibiotic alone ($\mu_1 - \mu_2 < 0$).

Quality control

Laboratory equipment like incubators, hot air oven, autoclave, refrigerator etc. were regularly monitored for their performance and immediately corrected if any deviation occurred. The temperature of the incubators and refrigerator were monitored every day. Reagents and biochemical media were checked for manufacture and expiry date and proper storage. After preparation, each media and reagent were labelled with preparation date, expiry date and stored in proper conditions. Sterility testing and performance testing were carried out using standard control strains.

1. Quality control during isolation and identification

Culture media that passed quality control for performance and sterility were used. During the identification, pure and isolated colony of the organism was used. After inoculation into different biochemical media, the inoculum was verified for pure culture and no contamination occurred during inoculation.

2. Quality control during antimicrobial susceptibility testing

Muller Hinton agar and antibiotic disc were checked for each lot number, manufacture date and expiry date and stored properly. Before use, each antibiotics disc and Muller Hinton Agar was monitored for their performance quality with *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. For antimicrobial susceptibility testing, standard inoculum (matched with 0.5 McFarland solutions) was used.

RESULTS

Twenty three culture positive samples were collected from Korea Nepal Friendship Hospital (KNFH). Among them, six (26.09%) were *S. aureus*, one was coagulase negative staphylococcus (CONS), eight (34.78%) were *E. coli*, four (17.39%) were *Klebsiella* species and again four (17.39%) were *Salmonella* species. After screening by AST, 10 samples (43.48%) were finalized as multi-drug resistant. In addition, 4 metallo-β-lactamase producers (2 *Acinetobacter* species and 2 *Pseudomonas aeruginosa*) (that were helped by Mr. Saroj Chandra Lohani, a researcher of Birendra Sainik Hospital, Chhauni) were also used for the study.

Among the 10 isolates collected from KNFH, 4 were MDR *S. aureus* (17.39%), 2 each were MDR *E. coli* (8.70%), MDR *Klebsiella* species (8.70%) and MDR *Salmonella* species (8.70%).

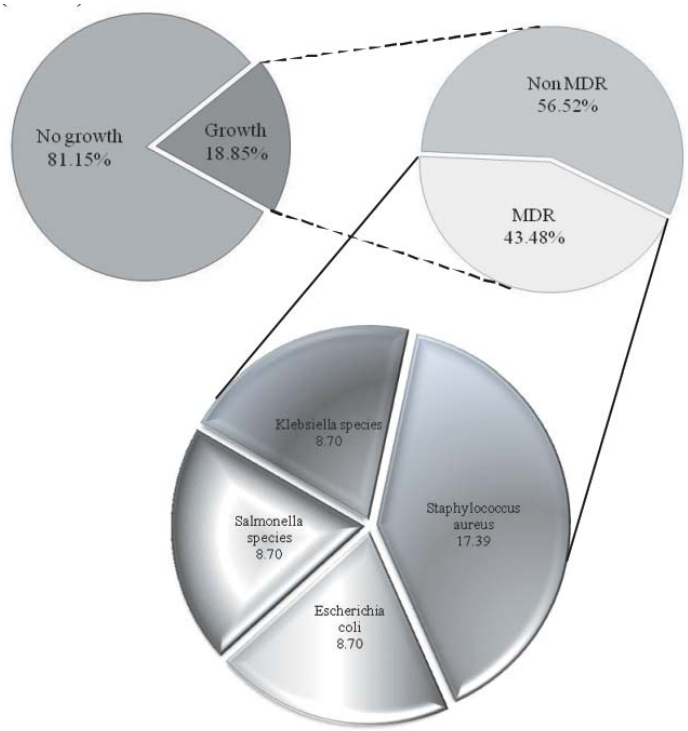

Figure 1: Distribution of organisms in the samples with MDR isolates

Five honey samples were also collected from The Beekeeping Shop, Lalitpur. Four of the samples were from single floral source, which nectar is collected by different colonies of *Apis mellifera.* One sample (Wild) was from mixed flower source, collected by *Apis dorsata.*

Table 1: Physical property of honey samples

Honey type	Floral source	Bee variety	Colour*	Specific gravity at RT†
Buckwheat	*Fagopyrum esculentum* (Buckwheat)	*Apis mellifera*	038R, 018G, 017B Or Hex#261211	1.4
Chiuri	*Diploknema butyracea* (Nepali butter tree)	*Apis mellifera*	079R, 071G, 032B Or Hex#4F4720	1.7
Mustard	*Brassica campestris* (Mustard)	*Apis mellifera*	245R, 235G, 097B Or Hex#F5EB61	1.5
Rudilo	*Pogostemon benghalensis* (Rudilo)	*Apis mellifera*	131R, 100G, 036B Or Hex#836424	1.45
Wild	Wild or mixed floral source	*Apis dorsata*	102R, 026G, 000B Or Hex#661A00	1.32

*Red Green Blue format in upper line, and hexadecimal code in lower line, data taken in average
†Room temperature

Antibiotic susceptibility pattern of different isolates

1. *Staphylococcus aureus*

Oxacillin resistant (MRSA) *Staphylococcus aureus* were selected for this study. They were also Gentamicin resistant. These organisms showed variable inhibition zone when honey was incorporated with these antibiotics. In average, the susceptibility of the organism was increased by 3 mm for Chloramphenicol and Cotrimoxazole, 2 mm for Ciprofloxacin and Gentamicin. The increase by 8 mm in average was seen when honey sample was added in Oxacillin for the susceptibility test (Table 2).

Table 2: Zone of inhibition of Antibiotic and Antibiotic incorporated with honey for *Staphylococcus aureus*

	Mean	S.E.#	SD§	Minimum	Maximum
Chloramphenicol	26.5	0.8	3.59	21	30
Chloramphenicol with honey	29.65	1.41	6.33	17	40
Ciprofloxacin	23.75	0.85	3.8	19	29
Ciprofloxacin with honey	25.9	0.95	4.27	19	38
Cotrimoxazole	21.25	0.44	1.97	18	23
Cotrimoxazole with honey	24.35	0.7	3.12	19	30
Gentamicin	20	0.93	4.17	15	26
Gentamicin with honey	24.15	1.21	5.43	14	34
Oxacillin	12.5	0.95	4.26	7	17
Oxacillin with honey	20.5	1.84	8.22	0	38

#Standard Error of Mean §Standard Deviation

2. *Escherichia coli*

At least one among Amoxycillin-Clavulanic acid, Ceftazidime, Ceftriaxone, Ciprofloxacin, and Cotrimoxazole resistant *E. coli* was taken to detect the synergism. Figure 2 shows that there is slight change in susceptibility pattern when honey was incorporated with the antibiotics. However, there was no statistical difference in T-score (Table 3) except gentamicin ($p>0.01$) and the correlation was strongly positive for the same antibiotic ($p<<0.01$). Other antibiotics could not show statistical significance in T-score and correlation ($p>0.01$) (Table 3).

Table 3: T-statistics and correlation of antibiotics with antibiotics and honey for *E. coli*

Variables	T score	p-value	R (correlation)	p-value
amoxyclav and amoxyclav with honey	1.217	0.255	-0.358	0.31
ceftazidime and ceftazidime with honey	3.337	0.009	0.25	0.486
ceftriaxone and ceftriaxone with honey	1.637	0.136	0.448	0.194
chloramphenicol and chloramphenicol with honey	-0.238	0.817	0.560	0.092
cotrimoxazole and cotrimoxazole with honey	2.297	0.047	0.287	0.421
gentamicin and gentamicin with honey	3.000	0.015	1.000	<0.001

3. *Klebsiella* species

For the study, *Klebsiella* species used were resistant to Amoxycillin-Clavulanic acid, Ceftazidime, Ceftriaxone, Ciprofloxacin, Cotrimoxazole and/or Gentamicin, but all were susceptible to Chloramphenicol. On the test, there was sudden increase in susceptibility with the combination of honey and antibiotics (Figure 2). Ceftazidime, ceftriaxone, and chloramphenicol had shown significant synergism ($p<<0.01$) and among them, chloramphenicol showed strong positive relation ($R = 82.7\%$) with honey but a slight negative relation ($R = -6\%$) between ceftriaxone and honey (Table 4).

Table 4 T-statistics and correlation of antibiotics with antibiotics and honey for *Klebsiella* species

Variables	T score	p-value	R (correlation)	p-value
amoxyclav and amoxyclav with honey	2.984	0.015	0.00	1.000
ceftazidime and ceftazidime with honey	5.155	0.001	0.342	0.333
ceftriaxone and ceftriaxone with honey	4.287	0.002	-0.06	0.869
chloramphenicol and chloramphenicol with honey	10.301	<0.001	0.827	0.003
cotrimoxazole and cotrimoxazole with honey	0.404	0.696	-0.720	0.019

4. *Salmonella* species

Ceftazidime, Cotrimoxazole and/or Gentamicin resistant *Salmonella* species were used for this study. Except Ciprofloxacin ($R= -89.4\%$, $p<<0.01$), all other antibiotics showed enhanced inhibition zone when honey was incorporated, but could not show the significant statistical difference ($p>0.01$) (Table 5).

Table 5: T-statistics and correlation of antibiotics with antibiotics and honey for *Salmonella* species

Variables	t score	p-value	R (correlation)	p-value
amoxyclav and amoxyclav with honey	3.972	0.003	0.715	0.02
ceftazidime and ceftazidime with honey	1.374	0.203	-0.186	0.606
ceftriaxone and ceftriaxone with honey	1.134	0.286	0.502	0.139
chloramphenicol and chloramphenicol with honey	2.160	0.059	-0.492	0.148
ciprofloxacin and ciprofloxacin with honey	-0.519	0.616	-0.894	0.000
cotrimoxazole and cotrimoxazole with honey	2.067	0.069	-0.279	0.436
gentamicin and gentamicin with honey	3.000	0.015	1.000	<0.001

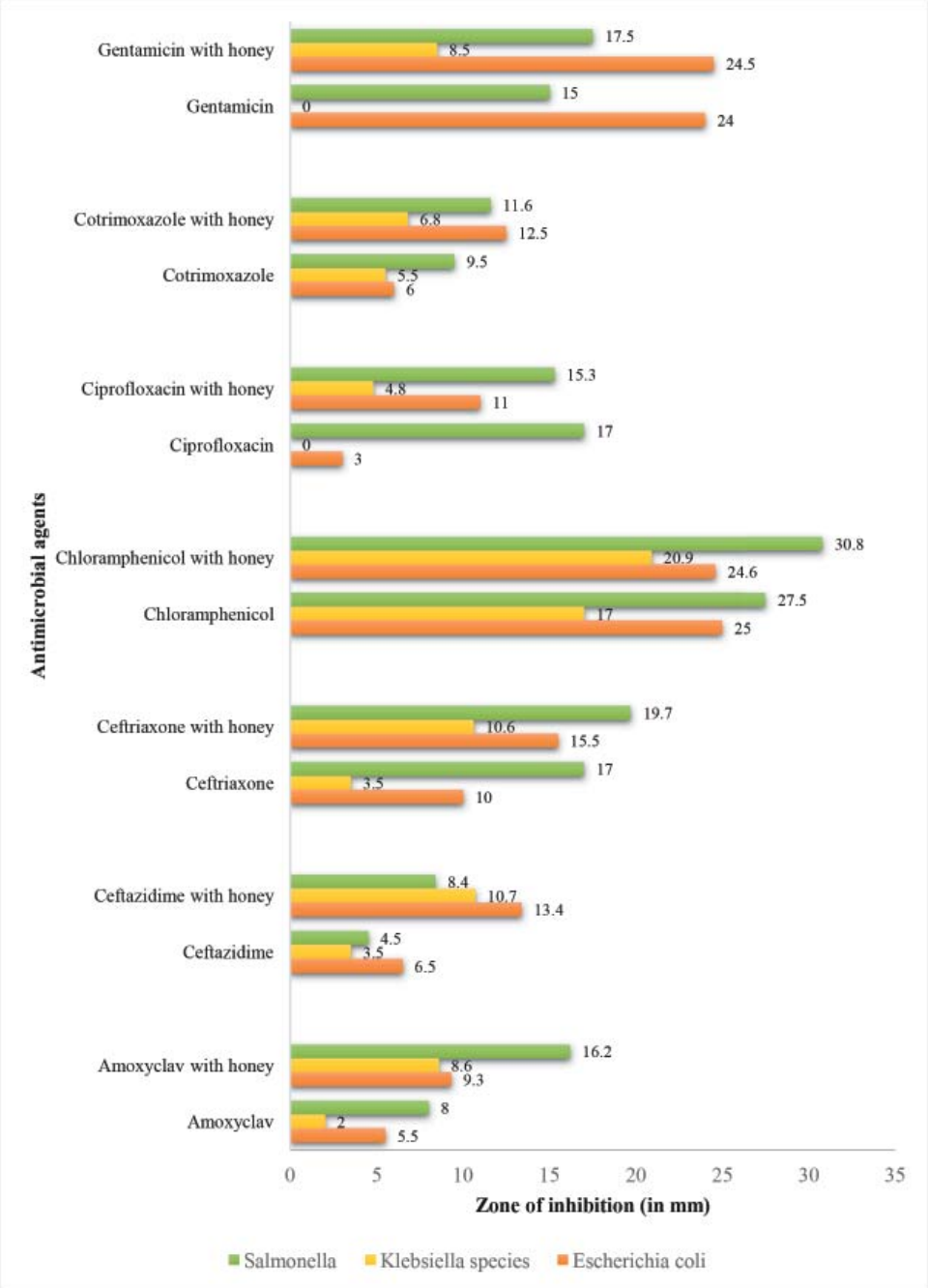

Figure 2: Comparative chart of Zone of inhibition for *Enterobacteriaceae* between antibiotics and antibiotics with honey

5. *Pseudomonas aeruginosa*

When metallo β–lactamase producing (MBL) *Ps. aeruginosa* were used, there was a drastic and significant increase in zone of inhibition ($p<<0.01$) (Figure 3). But the relation was not much stronger and lies between 8% to 41.9% (Table 6).

Table 6 T-statistics and correlation of antibiotics with antibiotics and honey for *Pseudomonas aeruginosa*

Variables	t score	p-value	R (correlation)	p-value
ciprofloxacin and ciprofloxacin with honey	8.444	<0.001	0.419	0.228
gentamicin and gentamicin with honey	16.058	<0.001	0.082	0.821
imipenem and imipenem with honey	8.508	<0.001	0.103	0.776

6. *Acinetobacter* species

MBL *Acinetobacter* species showed decreased susceptibility with Ciprofloxacin and Imipenem when honey was added (Figure 3). It was found that there was 28.9% relation of honey with Ceftazidime and –4.9% with Gentamicin (Table 7).

Table 7: T-statistics and correlation of antibiotics with antibiotics and honey for *Acinetobacter* species

Variables	t score	p-value	R (correlation)	p-value
ceftazidime and ceftazidime with honey	3.955	0.003	0.289	0.417
ceftriaxone and ceftriaxone with honey	0.631	0.544	0.071	0.846
ciprofloxacin and ciprofloxacin with honey	-0.303	0.768	0.296	0.406
cotrimoxazole and cotrimoxazole with honey	2.666	0.026	0.095	0.795
gentamicin and gentamicin with honey	0.923	0.380	-0.049	0.894
imipenem and imipenem with honey	-0.419	0.685	0.921	<0.001

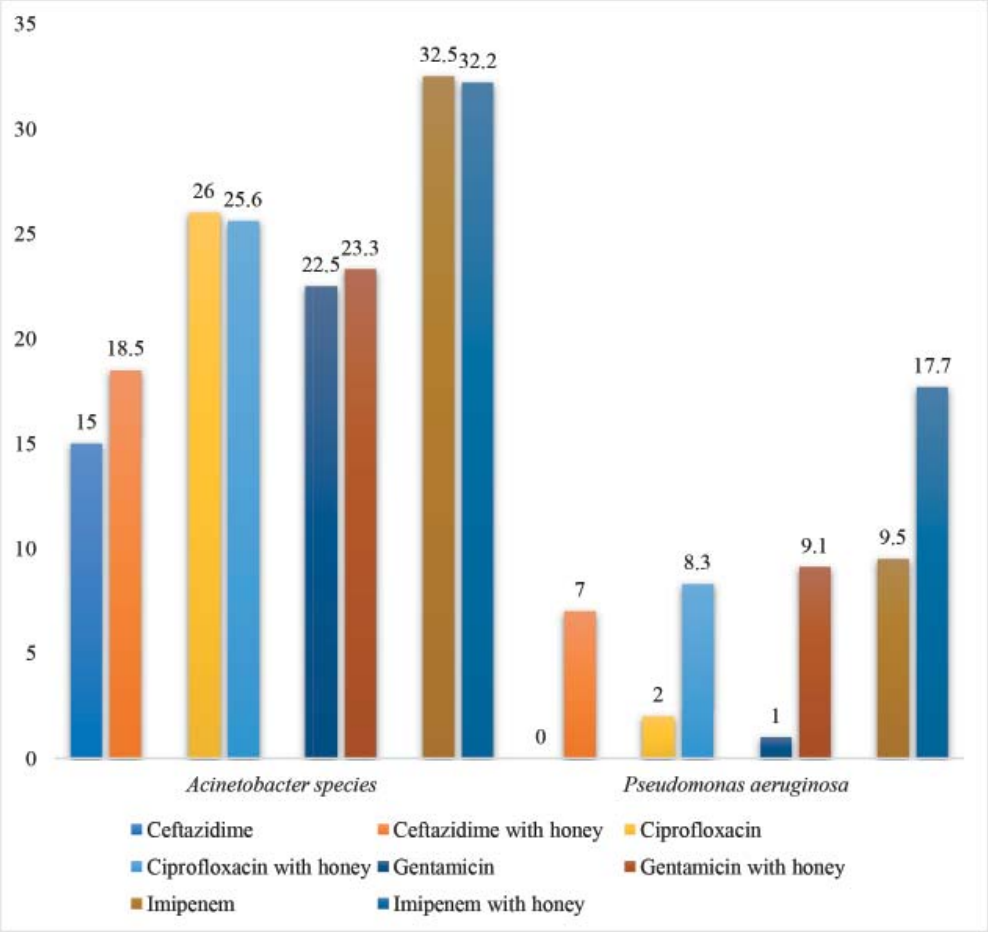

Figure 3: Zone of Inhibition of Antibiotic and Antibiotic incorporated with honey for *Acinetobacter* species and *Pseudomonas aeruginosa*

Susceptibility test to honey compared via ANOVA

In this study among the 6 bacterial samples used, honey was found to be most effective against *S. aureus* ($\bar{x}$ = 21.2 ± 4.44 mm) and the effect was decreased gradually to *Acinetobacter* species ($\bar{x}$ = 18.2 ± 2.49 mm), *Pseudomonas* species ($\bar{x}$ = 7.2 ± 1.10 mm), *Klebsiella* species ($\bar{x}$ = 6 ± 2.45 mm), *E. coli* ($\bar{x}$ = 5.4 ± 3.58 mm) and *Salmonella* species showed lowest inhibition zone ($\bar{x}$ = 5.2 ± 1.30 mm) for the honey.

When the relation between the groups was compared, null hypothesis that average ZOI differences in different groups are equal is rejected. It means the inhibition is variable among the bacteria (F=39.17, p<0.05). From means plot, it was found that *S. aureus* and *Acinetobacter* species were highly susceptible whereas *Ps. aeruginosa* and organisms of *Enterobacteriaceae* family are less susceptible to honey (Figure 4).

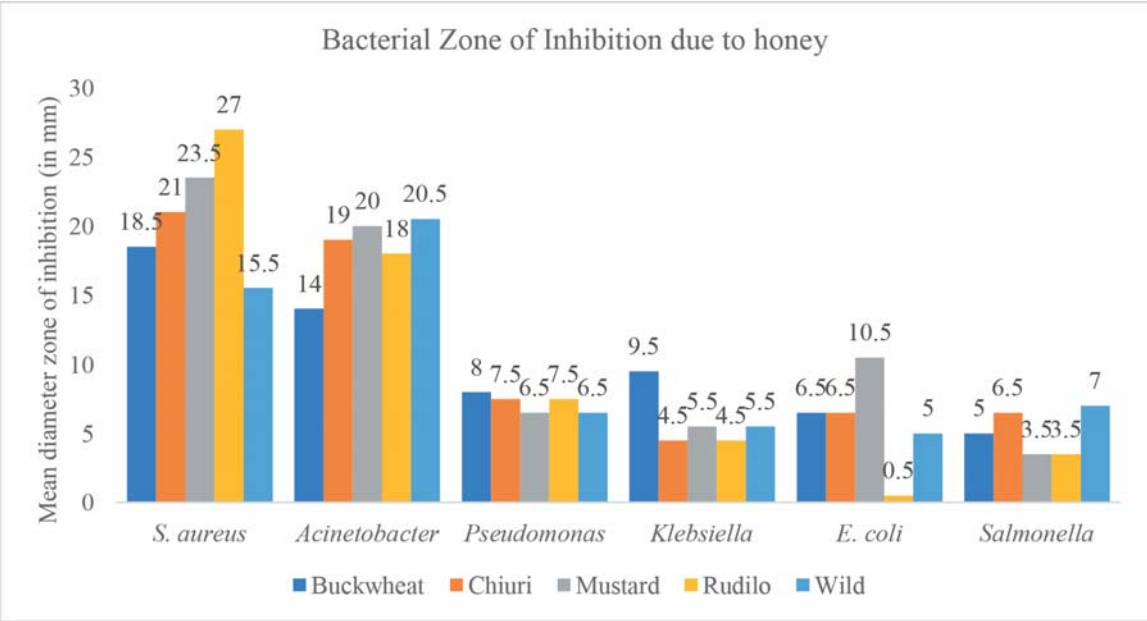

Figure 4: Clustered bar chart of Zone of Inhibition of different bacteria by different honey samples

DISCUSSION

The antibacterial and antifungal effect of honey has been repeatedly noticed from ancient time. The traditional method of treatment using honey is a remarked gateway for the study of microbiology. The advancement of tools and techniques and modernization of the lifestyle has reduced the use of honey. Antibiotics are now gaining their path as the first selection for the treatment of bacterial infection. This caused uncontrollable increase in drug resistant organisms. Pokhrel (2004) found that 47.57% pathogens in sputum and 60.40% pathogens in urine were MDR. Bomjan (2005) found that 60% urinary and sputum isolates were MDR pathogens. This study shows 43.48% multi-drug resistant organisms were isolated from various samples. These data show that the distribution of MDR pathogens is different on different geographical areas within Nepal. Indiscriminate & inadequate use of antibiotics causes losing their potency against various types of organisms (Bajaj et al. 2018). Due to the different properties of honey, it is used traditionally for treatment of different infections of wound, burns and blood infections (Sharp 2009). Thus the synergism of antibiotics and honey is an interest of study.

In this study, 5 honey types derived from different floral sources were screened for their antibacterial activity. Initial screening with the Kirby Bauer disc diffusion method demonstrated that all tested honey types exhibited more or less susceptibility to all the clinical bacterial isolates used. *S. aureus* showed greater sensitivity and the members of Enterobacteriaceae were less sensitive to honey. This finding is in accordance with the findings of previous studies (Abd-El et al. 2007, Moussa et al. 2012, Ahmed et al. 2013). These results are very much important for clinical implementation such as wound and burn management (Sufya et al. 2014).

Antibacterial activity of honey can be described due

to different factors. Due to low water concentration of honey and its hygroscopic nature, its osmophilic activity extracts water from bacteria and makes the organism inactive. The acidic pH inactivates the organism enzymes for metabolism and thus the organisms are inhibited. In addition, glucose oxidase obtained from bee gut that is regurgitated by bee during honey making process, degrades glucose to produce hydrogen peroxide. The H_2O_2 oxidises the organism enzyme and is inactivated and leads to its death. Different phytochemicals like methyl glyoxal and methyl syringate from the floral source is taken by bee during collecting nectar. These metabolites coordinates with the acidic pH to act for the bacterial inhibition. Recently, other components were also identified in honey that help it as antibacterial effect. Catalase, Maillard reaction products, polyphenols, ascorbic acid, bee defensin-1 protein are some of them (Bizerra et al. 2012).

In different studies, significant synergism was found between honey and antibiotics when tested in vitro. Honcrivine (honey plus acriflavine 0.1%) is efficient for debridement of wound without any inflammatory or allergic responses (Efem 2009) which is due to activation of protease due to H_2O_2 from honey dressing (Lane et al. 2019). In a study for 16 clinical pathogens including *S. aureus*, *Salmonella* species, *Streptococcus pyogenes*, *B. cereus* and *B. subtilis*, were up to 60% more resistant than equal reference strains, and concluded for variability in the antibacterial effect of honey (Voidarou et al. 2011). A research in Pakistan has concluded that methyl glyoxal can be a good inhibitory agent against MDR and non-MDR *Salmonella* and other Gram-negative organisms (Afzal et al. 2018).

This study found increased zone of inhibition when honey is added to antibiotics. Though the reason behind the synergism is not clear, a study described the synergism of methylglyoxal (found in natural honey) with Piperacillin, a β-lactam antibiotic. But a study in New Zealand concluded methylglyoxal is not the sole factor for Manuka honey to act synergistically with rifampicin against MRSA (Jenkins and Cooper 2012). From these studies it can be concluded that various factors are responsible for the synergistic or antagonistic action of antibiotics with honey.

In this study, honey showed synergetic effect with Gentamicin against *E coli* and *Salmonella* species, with Chloramphenicol against *Klebsiella* species (R=82.7%) and with Ceftazidime against *Acinetobacter* species. The results are incompatible with the results of a study performed by Karayil et al. in 1998, where antibiotics Gentamicin, Amikacin and Ceftazidime were synergetic with honey against *Pseudomonas* species and not with *Klebsiella* species. This leads to the mystery if the organisms in different geography can lead to different synergism or changing their genetic behaviour with time lapse.

CONCLUSION

Honey is found to be very effective against *Staphylococcus* infection whereas it is least effective against *E. coli*. The synergetic effect of honey with different antibiotics are found to be effective against *S. aureus, Pseudomonas aeruginosa* and the members of *Enterobacteriaceae* family involved in this study but found to be less or no effective against *Acinetobacter* species.

ACKNOWLEDGEMENTS

We are most grateful to the Executive Officers of Korea Nepal Friendship Hospital for providing the clinical facilities and support. We are thankful too Mr. Saroj Chandra Lohani for providing the metallo-*β*-lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter* species for this study. We also express admiration towards the director of the Beekeeping Shop and Research Centre for providing the valuable bee honey samples and the priceless information about them. Our hearty gratitude to the faculties and laboratory staffs of Tri-Chandra Multiple Campus for their support and help to complete this study.

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