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Research Article

In vitro Inhibitory Action of Honey Against Extended Spectrum β-Lactamase Producing *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Antibiotic resistant Gram-negative bacteria, in particular, extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* are frequently involved in various human infections. Despite traditional applications of honey, a comprehensive evaluation of antimicrobial activity against ESBL-producing bacteria is lacking. This study aims to evaluate the antibacterial activity of honey against ESBL-producing and non-producing bacteria isolated from clinical samples (urine and sputum) by tube dilution method and the time kill viability assay. The minimum inhibitory concentration (MIC) of honey was found to be 25% (v/v) against most of *E. coli* (13 out of 18 isolates were inhibited by both indigenous and Dabur honey) and 50% (v/v) against most of *K. pneumoniae* (10 and 11 out of 14 isolates inhibited by indigenous and Dabur honey respectively). The MIC of honey for ESBL-producing and non-producing bacteria was found to be almost similar ($p > 0.05$). The complete reduction of bacterial population (8 $\log_{10}$ CFU/ml) was observed after 24 hours of exposure to honey at 50% (v/v) concentration. The honey samples showed bacteriostatic and bactericidal activity against tested isolates of ESBL-producing and non-producing *E. coli* and *K. pneumoniae*. These findings suggested indigenous honey serve as a promising complementary therapeutic agent in managing resistant bacterial infections.

Keywords: ESBL; *E. coli*; Honey; *K. pneumoniae*; Time kill assay

Introduction

Antibiotic resistant bacteria pose a serious threat to public health. The frequencies of bacterial resistance, to all kinds of antibiotics including the last resort drugs, are increasing worldwide. Among them, extended spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* are major bacterial pathogens being isolated and reported from urine globally (WHO, 2017). The high occurrence of ESBL producing bacteria has not only been reported in clinical settings but also from environmental samples (Bhatt et al., 2007; Talukdar et al., 2013; Zhang et al., 2016;

Khanal et al., 2025). Considering the ever-growing pool of antimicrobial resistance among bacterial pathogens, there is an urgent need for antimicrobial agents which can be used as an alternate therapeutic option against ESBL infection.

Various herbal and animal extracts have shown antimicrobial characteristics against different pathogens (Bajracharya et al., 2008; Stephens et al., 2010; Khan et al., 2013). Moreover, some plant extracts exhibit antibacterial effect against multidrug-resistant human pathogens (Shalayel et al., 2017). At present, considerable focus is being directed toward the use of honey as an antimicrobial agent. This growing interest concerns the adverse effects of antibiotics in humans, as well as the declining effectiveness of conventional antibiotics against the resistant pathogens. Many researchers have reported the antibacterial activity of honey against several pathogenic bacteria those are aerobes, anaerobes, Gram positives, Gram negatives and multidrug-resistant (Kwakman et al., 2008; Al-Waili, 2013; Wasihun & Kasa, 2016). The high reducing sugar, osmotic nature, low pH (Kwakman & Zaat, 2012), ability to produce hydrogen peroxide (H_2O_2), bee defensin-1 (Kwakman et al., 2011), phenolic compounds, methylglyoxal (MGO) (Stephens et al., 2010), glycoprotein (Brudzynski & Sjaarda, 2015), flavonoids (Al-Waili, 2013) and lysozyme (Molan, 1992) are the key factors attributing an inhibitory effect on bacteria. The antimicrobial potency of indigenous honey and Dabur honey against ESBL producing bacteria has not been assessed in Nepal. Investigating this could provide valuable insight into the potential of locally available natural products as alternative and adjunct therapeutic option against multidrug-resistant pathogens. Moreover, such evidence may support the development of cost-effective, sustainable strategies to combat antimicrobial resistance in resource-limited healthcare settings. Thus, this study aimed to investigate the possible application of honey as an effective and safe alternative for the treatment of multiple antibiotic-resistant infections, in particular those caused by ESBL producing *E. coli* and *K. pneumoniae*.

Materials and Methods

Test bacterial pathogens

Two bacterial pathogens, *E. coli* and *K. pneumoniae,* were isolated from non-invasive urine (n = 838) and sputum (n = 34) samples from the patients who were requested for microbiological investigation (culture) during the study period from October 2017 to March 2018. Collected specimens were inoculated in parallel on Blood agar and MacConkey agar and incubated at 37 ºC for 24 hours. The isolated bacterial colonies were sub-cultured on nutrient agar and identified based on their colony morphology, Gram's staining and biochemical characteristics as described by Cheesbrough (2006). The study was conducted after ethical approval from the Institutional Review Committee of Human Organ Transplant Center, Bhaktapur, Nepal (Approval Ref. No. 074/75 dated 2017.09.22).

Antibiotic susceptibility test

Antibiotic susceptibility test of *E. coli* and *K. pneumoniae* was done on Mueller-Hinton agar (MHA) by the modified Kirby-Bauer disk diffusion method. The commercially available antibiotics, meropenem (10 µg), imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), piperacillin/tazobactam (100/10 µg), amoxicillin (20 µg), ofloxacin (5 µg) and ciprofloxacin (5 µg) were selected and test results were interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2016). Control strain of *E. coli* ATCC 25922 was used for quality assurance and comparison. Bacteria showing resistance to at least one of these antibiotics were referred as multidrug resistant bacteria.

Phenotypic test for ESBL production

ESBL producing isolates were screened based on the size of inhibition zone for ceftazidime (< 22 mm) (CLSI, 2016). A phenotypic confirmatory test was carried out by combining disc diffusion method following the standard protocol (CLSI, 2016). The ESBL producing isolates were confirmed by an increase in the diameter of the inhibition zone by ≥ 5 mm due to the combined disc with ceftazidime-clavulanate (30/10 µg) than ceftazidime alone.

Honey sample

Indigenous honey was collected from Natural History Museum, Tribhuvan University whereas Dabur honey was purchased from the local market. The diluted honey samples (50%) were sterilized by using membrane filter of pore size 0.45 µm. Sterility of honey samples was checked by streaking on nutrient agar plate and incubated at 37 ºC for 24 hours and assurance of sterility was confirmed only after 7 days. Sterile honey samples were stored at room temperature until use.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the honey samples was determined using the broth tube

dilution method (Wasihun & Kasa, 2016). Briefly, one milliliter of honey was serially two-fold diluted with nutrient broth so as to maintain v/v concentrations of 50.0%, 25.0%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781% and 0.391% honey in respective tube. Except for the negative control tube, each tube was inoculated with 0.1 ml of the test bacterial culture of standard inoculum that matched 0.5 McFarland turbidity. The presence and absence of growth (assessed by turbidity evaluation with reference to positive control and negative control) was noted following the 24-hour aerobic incubation at 37 °C. The MIC is the lowest concentration inhibit bacterial growth as detected of visible turbidity. The least concentration that showed no visible growth of organisms inoculated by streaking on nutrient agar at 37 °C for 24 hours considered as the minimum bactericidal concentration (MBC) of the honey (Wasihun & Kasa, 2016).

Time kill viability assay

Time kill assay was done for selected ESBL- producer and non-producer isolates of *E. coli* and *K. pneumoniae* as described by Jayaraman et al. (2010) by using MBC concentration (50% v/v) of the honey samples. To a 1 ml volume of 50% diluted honey sample, 100 µl of 10^8 CFU/ml of bacterial isolate was added. A bacterial suspension of 10^8 CFU/ml was used as a control. Bacterial suspension incubated (37 °C) in the presence of honey sample and without honey (control) were sampled out (100 µl) at 0, 2, 4, 6, 8, 10, 12, 18, 24 and 48 hours, and microbial load was determined. The killing rate was determined by plotting log of viable colony counts ($\log_{10}$ CFU/ml) against time (hour). The time kill curve was drawn using Origin-Pro 2019 software.

Data analysis

All the experiments were done triplicate for reproducibility. For the statistical analysis of the results, SPSS version 21 was used. Fisher's exact test was applied to determine the association between different variables and a $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Out of the total 872 clinical samples (urine = 838 and sputum = 34) collected during the study, 70 samples showed growth of either *E. coli* (n = 43) or *K. pneumoniae* (n = 27). The majority of the isolates were resistant to penicillin and fluoroquinolones but susceptible to carbapenems (Figure 1). Among the total isolates, 77.1% (*E. coli*, n = 33 and *K. pneumoniae*, n = 21) were found to be multidrug resistant (Table 1). On primary screening for ESBL, a total of 39 isolates (*E. coli*, n = 23 and *K. pneumoniae*, n = 16) were resistant to ceftazidime (Figure 1) and therefore suspected as ESBL-producers. Among suspected ones, 43.6% isolates (*E. coli*, n = 10 and *K. pneumoniae*, n = 7) were confirmed as ESBL-producers. All ESBL-producing isolates (n = 17) and randomly selected fifteen ESBL non-producing isolates (*E. coli*, n = 8, and *K. pneumoniae*, n = 7) were used as test organisms for evaluation of the antibacterial activity of honey.

The antibacterial activity of honey samples was assessed by determining minimum inhibitory concentration (MIC) (v/v) value. The results revealed that all the test organisms were inhibited either at 50% or 25% (v/v) concentrations of the tested honey samples. The MIC of indigenous honey (Table 2) and Dabur honey (Table 3) was found to be 25% to 50% for the majority of *E. coli* and *K. pneumoniae* isolates, respectively. The indigenous honey showed bacteriostatic activity at a concentration of 25% for most of the *E. coli* isolates (77.8%) and that of 50% for *K. pneumoniae* isolates (78.6%). Similar results were also revealed by the Dabur honey.

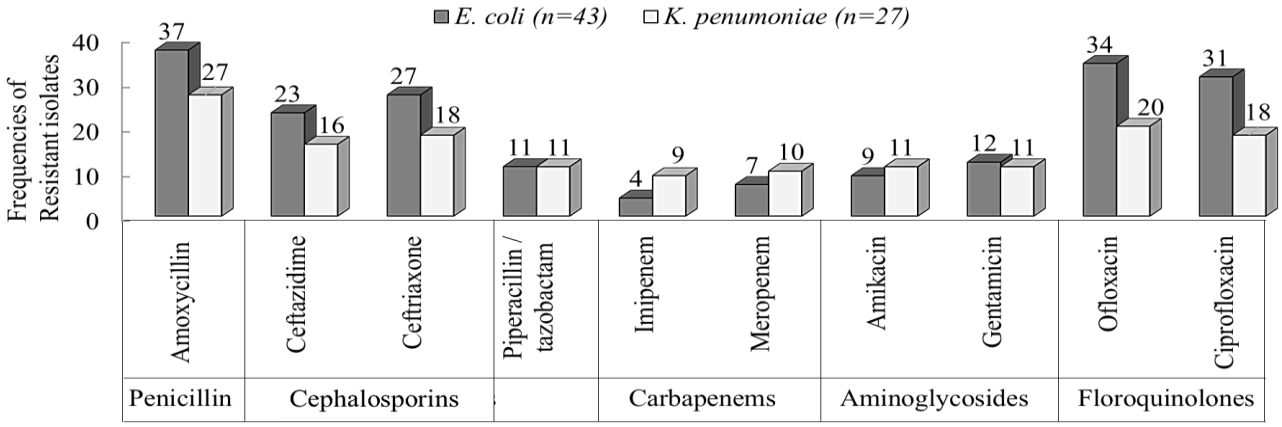

Figure 1: Frequencies of resistant isolates of *E. coli* and *K. pneumoniae* belonging to different classes.

Table 1: Frequencies of multidrug-resistant (MDR) isolates.

	E. coli	*K. pneumoniae*	Total isolate
MDR	33 (76.7%)	21 (77.8%)	54 (77.1%)
Non-MDR	10 (23.3%)	6 (22.2%)	16 (22.9%)
Total isolate	43	27	70

Table 2: Minimum inhibitory concentration (MIC) of indigenous honey against bacterial isolates.

	Bacterial isolate	MIC value of indigenous honey		*p*-value
		25% (v/v)	50% (v/v)	
ESBL producer	*E. coli*	6 (60%)	4 (40%)	0.502
	K. pneumoniae	3 (42.9%)	4 (57.1%)	
ESBL non-producer	*E. coli*	7 (87.5%)	1 (12.5%)	
	K. pneumoniae	1 (14.3%)	6 (85.7%)	

Table 3: Minimum inhibitory concentration (MIC) of Dabur honey against bacterial isolates.

	Bacterial isolate	MIC value of Dabur honey		*p*-value
		25% (v/v)	50% (v/v)	
ESBL producer	*E. coli*	6 (60%)	4 (40%)	0.476
	K. pneumoniae	2 (28.6%)	5 (71.4%)	
ESBL non-producer	*E. coli*	7 (87.5%)	1 (12.5%)	
	K. pneumoniae	1 (14.3%)	6 (85.7%)	

On comparing the bacteriostatic activity of both types of honey on ESBL-producer and non-producer isolates, almost identical activities were observed. Most of the ESBL-producers (52.9%) and non-producer (60%) isolates were inhibited by indigenous honey at 50% and 25% concentration respectively (Table 2). Likewise, Dabur honey at 50% concentration showed inhibitory activity against 64.7% isolates of ESBL-producer and 46.7% non-ESBL isolates (Table 3). The maximum proportion of ESBL isolates and non-ESBL isolates were inhibited at a concentration of 50% and 25% by the two types of honey, respectively. However, the association was statistically insignificant ($p>0.05$). The MBC of both types of honey on tested bacterial isolates was found to be 50% v/v.

The time kill analysis of indigenous and Dabur honey at 50% (v/v) concentration on randomly selected ESBL-producer and non-producer *E. coli* and *K. pneumoniae* during 48 hours is shown in Figure 2 and 3 as time kill curve. The curves were well fitted in the sigmoid curve model. As shown in the curves, both types of honey samples inhibited the growth of all test organisms within 24 hours exposure. Both types of honey showed little effect (<1 $\log_{10}$ CFU/ml reduction) until 12 hours but then after bacterial population decreased rapidly. There was a decrease up to 4 $\log_{10}$ CFU/ml (half of initial population) following 18 hours exposure to the honey samples and complete inhibitions (reduction of total 8 $\log_{10}$ CFU/ml) of bacteria were observed after 24 hours exposure to the honey samples. However, both *E. coli* and *K. pneumoniae* (ESBL and non-ESBL producing) exhibited typical sigmoidal growth curves in absence of honey, with rapid exponential growth during the first 8-12 hours followed by a stationary phase around 9.0-9.1 log CFU/ml (Figure 4).

The primary research question of this study was to understand susceptibility of ESBL-producing bacteria towards the Nepalese honey. The indigenous and Dabur honey samples showed the inhibitory effects either at 25% or 50% v/v concentrations against all the tested isolates of *E. coli* and *K. pneumoniae*. Similar results were also shown by other researchers from different countries (Agbaje et al 2006; Gomashe et al 2014; Ahmed et al 2014). In the present study, the minimum inhibitory concentration for both types of honey against the tested bacteria was observed to be 25-50% v/v. Shah and Williason (2015) found 30-40% v/v concentration of honey were sufficient to inhibit the growth of multidrug resistant bacteria. Likewise, Al Waili et al (2013) investigated antibacterial potential of different types of honey and reported 40% and 40-50% concentrations as MIC for *K. pneumoniae* and *E. coli* respectively. In contrast to this, Tan et al (2009) found that comparatively low concentrations of Tualang and Manuka honey (MIC, 8.75-25%) were inhibitory against wound and enteric microorganisms. This difference in the antibacterial activity of honey over place might be due to the difference in the species of bees and plant sources used for nectar and the difference in the test methods used and test organisms. It may be due to composition of bioactive compounds in honey.

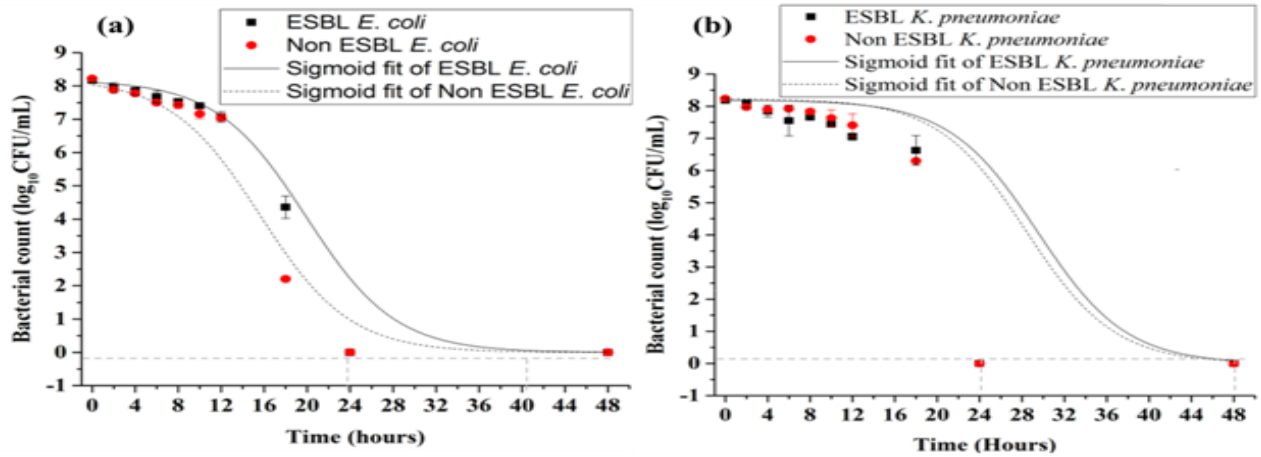

Figure 2: Time kill curve of *E. coli* (a) and *K. pneumoniae* (b) with indigenous honey. Error bars indicate standard deviation from mean. The Sigmoid curve was fitted using OriginPro2019 software. The curve revealed a pattern of slower inhibition rate until approximately 16 hours and then a sharp fall in viable cell numbers. Irrespective of test bacteria, no viable cells were obtained at 24 hours and onwards.

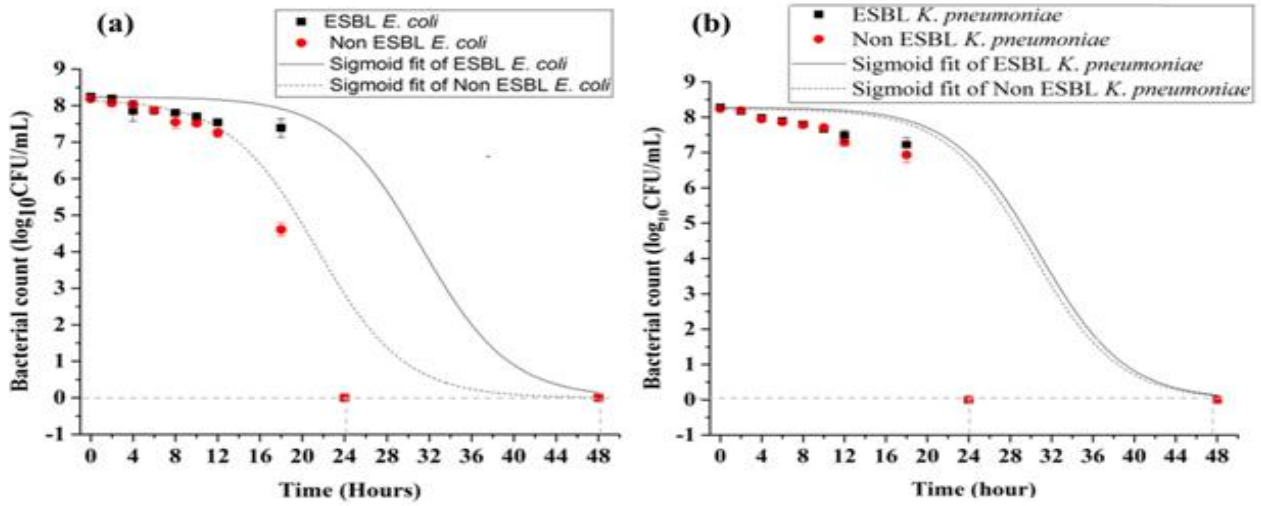

Figure 3: Time kill curve of *E. coli* (a) and *K. pneumoniae* (b) with indigenous honey. Error bars indicate standard deviation from mean. The Sigmoid curve was fitted using OriginPro2019 software. The curve revealed a pattern of slower inhibition rate until approximately 16 hours and then a sharp fall in viable cell numbers. Irrespective of test bacteria, no viable cells were obtained at 24 hours and onwards.

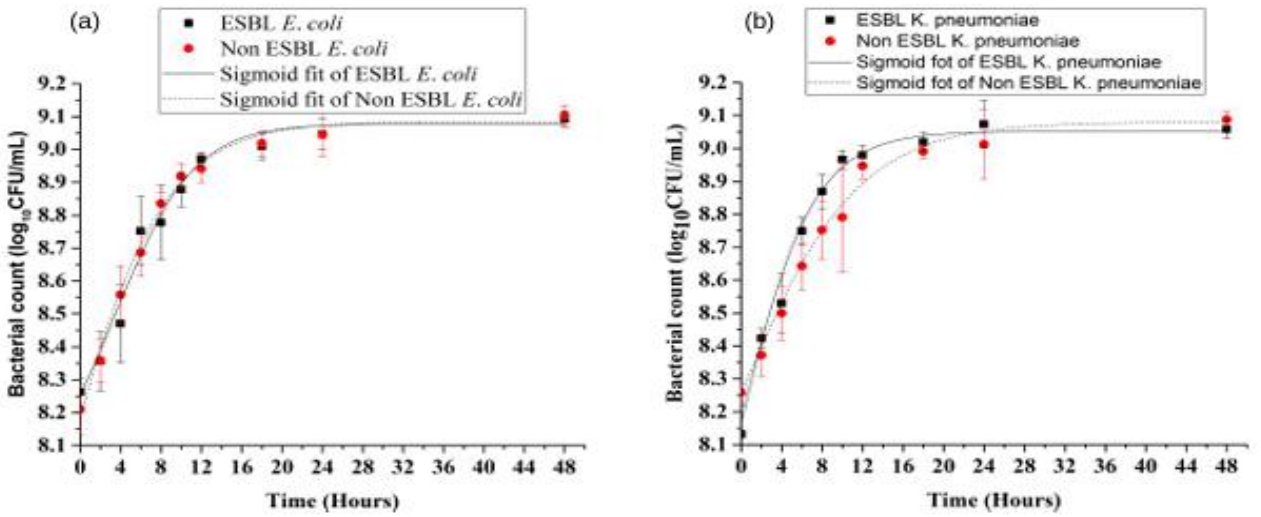

Figure 4: Time kill curve of *E. coli* (a) and *K. pneumoniae* (b) without honey. Error bars represent standard deviation from mean. The well fitted sigmoid growth curve demonstrated exponential growth of bacteria up to the 12 hours of incubation followed by stationary growth after 20 hours.

Antibacterial properties of honey have been well documented along with several compound that contributed to its activity such as H_2O_2, bee defensin-1 (Kwakman et al., 2011), phenolic acids and methylglyoxal (Stephens et al., 2010), glycopeptides (Brudzynski and Sjaarda 2015), flavonoids (Al-Waili, 2013), and lysozyme (Molan, 1992). Antibacterial activity of the test honey samples may also be influenced by osmotic inhibition as the controls like sugar or artificial honey was not tested in this study.

The emergence of ESBL producing bacteria may present an increasing risk of transmission of resistant strains in humans and animals. It is a worrying global public health issue as infections caused by such enzyme-producing organisms are associated with a higher morbidity and mortality and greater economic burden. The problem is clearly severe in developing countries where studies on this subject, drug availability, and its appropriate use are limited and resistance rate is high (Pitout et al., 2005; Ayukekbong et al., 2017; Khanal et al., 2024). In this study, considering insignificant statistical (p>0.05) association between the bacteriostatic activity of indigenous and Dabur honey against ESBL-producing and non-producing bacteria, we found that the antibacterial activity of honey was not affected by ESBL enzymes produced by bacteria. This was in agreement with the study by Kwakman et al. (2008) and Boorn et al. (2010). Similar to bacteriostatic activity, the minimum bactericidal concentration of honey was also found to be 50% v/v for all the tested isolates. This finding was consistent with reports from other researchers in various regions (Al-Waili, 2004; Kwakman et al., 2008; Ahmed et al., 2014; Wasihun & Kasa, 2016). Therefore, it is understood that the same concentration of honey can inhibit both the ESBL-producing and non-producing strains. The study also revealed that around 50% dilution of the honey samples had more antibacterial activity. The honey accumulates maximum H_2O_2 at the concentration of 30-50%, however this declines rapidly at <30% concentration due to the relatively low affinity of glucose oxidase of honey for its substrate, glucose (Schepartz & Subers, 1964). It has been shown that the antimicrobial activity of honey may range from concentrations < 3% to >50% (Wilkinson & Cavanagh, 2005; Al-Waili, 2013; Agbaje et al., 2006).

In order to explore the approximate time taken for inhibition of a given population, we assessed the time kill effect of honey at 50% (v/v) concentration on ESB-producing and non-producing *E. coli* and *K. pneumoniae* for 48 hours. In this study, the initial population of test bacteria was taken 8 $\log_{10}$ CFU/ml which are generally an infective dose to cause the infections (Schmid-Hempel & Frank, 2007). We found that complete inhibition of bacterial population (8 $\log_{10}$ CFU/ml) could be achieved in 24 hours exposure to the honey samples. The result was in accordance with the findings of previous researchers (Al-Maaini, 2011; Kwakman et al., 2008). The potential bioactivity of honey used in this study was equivalent to 30% (w/v) of Manuka honey, and 10-40% (v/v) of Revamil honey (a medical grade honey) (Kwakman et al., 2008; Kwakman et al., 2010; Kwakman et al., 2011). Nishio et al. (2016) found a significant decrease in *S. aureus*, up to 4 $\log_{10}$ CFU/ml, following 4 hours of treatment with the honey. Hashim (2014) found reduction of *E. coli* NCTC 10418 by 2 log and 3 log CFU/ml in 6 hours and 9 hours respectively at 50% (w/v) concentration of Sudanese honey and in 5 hours and 7 hours respectively at 25% (w/v) concentration of Manuka honey. All these results indicated that Gram negative bacteria require more exposure time for the same result. Though the targeted organisms were Gram negative in this study, 2 $\log_{10}$ CFU/ml diminution was observed only after 12 hours exposure to honey.

There were little differences in the killing effect of indigenous and Dabur honey against tested bacterial isolates. The comparatively higher killing rate was observed by indigenous honey (1.5-6 $\log_{10}$ CFU/ml) than Dabur honey (1-3.5 $\log_{10}$ CFU/ml) within 18 hours. Also, high and rapid killing effect of both type of honey was observed in case of *E. coli* isolate. In general, *K. pneumoniae* commonly displayed higher rates of resistance than *E. coli* (Hyle et al., 2005; Mohsen et al., 2016). However, both honeys showed complete inhibitory effect in 24 hours for all isolates indicating requirement of equivalent time of exposure for bactericidal activity. The physicochemical properties of indigenous honey (Shrestha, 2000) and Dabur honey (Shenoy et al., 2012) have been determined and the moisture content, reducing sugar, sucrose, hydroxymethyl furfuraldehyde (HMF) and pH of both honeys were within the range. The low pH, high sugar content, low moisture, elevated HMF or accumulation of H_2O_2 might be responsible for reduction of bacterial survival. Regarding the botanical origin, a variety of natural floras around the museum are speculated to be of polyfloral origin (Shrestha, 2006) and Dabur honey also have the floral source from Himalayas, Nilgiris, and Sunderbans of India (Shenoy et al., 2012).

Although, limitation in terms of geographic variation and sample size, this study clearly showed the antibacterial activity of honey against clinical isolates of *E. coli* and *K. pneumoniae* within 24 hours exposure. More research is needed to establish the potential antimicrobial activity of indigenous honey. As a limitation, the mode of action of honey against different bacterial species was not investigated. Screening of bioactive compounds and understanding their molecular mechanism would be important scientific evidence for alternative therapeutic application of the indigenous honey.

Conclusion

Indigenous honey and Dabur honey have both bacteriostatic and bactericidal activity against ESBL-producers and non-producers when tested *in-vitro*. The MICs of honey against ESBL producers and non-producers are almost similar (25% and 50%) however; it is different according to species of bacteria. The MBC of honey against all the bacterial isolates is identical (50%). Both types of honey reduced the microbial load by 8 $\log_{10}$ CFU/ml in 24 hours of exposure. However, pharmacological standardization and clinical evaluation on the effect of honey are essential before using honey as a preventive and curative measure to common diseases related to the tested bacterial species.

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