Anti-Bacterial Activity of Different Honey Samples against Bacteria Isolated from Clinical Sources

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ABSTRACT

Objectives: To evaluate the antibacterial activities of different honey samples against clinically isolated bacteria and also to compare the antibacterial activity of honey with standard antibiotics

Methods: A study was conducted to evaluate the potent attributes of eight distinct types of honey against five pathogenic bacteria which were collected from Bharatpur Hospital of Chitwan. The susceptibility of isolates to honey was evaluated using the Agar well diffusion method. In addition, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the effective honey samples were determined using tube macro-dilution method. MIC represents the lowest concentration at which visible inhibition of bacterial growth occurs, offering insights into the honey's inhibitory potential. Furthermore, MBC, determined by sub-culturing the non-turbid wells from the MIC assay, elucidates the minimal concentration required for complete bactericidal activity.

Results: Among the eight types of honey processed, Rudilo honey exhibited remarkable effectivity against *Escherichia coli* and *Staphylococcus aureus*, with zone of inhibition of 39 mm and 36 mm, respectively. Conversely, Manuka and Chiuri displayed heightened efficacy against *Klebsiella pneumoniae* with zone of inhibition 34 mm) and *Proteus vulgaris* (38 mm). Among the tested bacteria, *Pseudomonas aeruginosa* demonstrated notable resistance to all honey samples except Rudilo, Manuka, and Multiflora. Furthermore, Manuka and Rudilo exhibited the lowest MIC (6.25% v/v) against *Proteus vulgaris* and *Staphylococcus aureus*, while Rudilo displayed the lowest MBC (25% v/v) against the same pathogens. However, Chiuri presented the highest MIC and MBC against the tested bacteria. It was found that honey samples showed a greater zone of inhibition than antibiotics used against *Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae,* and *Staphylococcus aureus* but for *Pseudomonas aeruginosa* antibiotics were found to be more effective than sampled honey.

Conclusion: The study revealed that honey exhibited antibacterial properties even at its minimum inhibitory concentration (MIC), showcasing effectiveness against infections caused by *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae and Proteus vulgaris*. However, its efficacy in treating infections attributed to *Pseudomonas aeruginosa* might be limited.

Keywords: Honey, Clinical isolates, Antibacterial activity, MIC, MBC

INTRODUCTION

The emergence of multidrug resistance is one of the most significant current public health issues, posing a threat to global health (Tanwar et al. 2014 and Abdelazeem Algammal et al. 2023). According to WHO, antibiotic efficiency has declined due to microorganism resistance, particularly to synthetic antibiotics. These resistant microorganisms can withstand the attack of antibiotics, resulting in ineffective treatment, which in turn leads to the spreading of disease. Antibiotics that were once useful against bacterial illnesses are becoming ineffective in the current situation (MacGowan & Macnaughton, 2017). The main cause of this scenario is the excessive use or abuse of pharmaceuticals in order

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to cure illnesses faster rather than focusing on their efficacy, and the agriculture sectors are also blamed for this situation. Furthermore, spontaneous evolution, bacterial mutation, and the transmission of resistant genes via horizontal gene transfer are also key factors in antimicrobial resistance (Dadgostar, 2019).

Alternatives to antibiotics are required to help combat infectious diseases as it has become a global health challenge. A natural agent which has long been used for therapeutic purposes is honey as it has phytochemical (Zammit Young & Blundell, 2023), antibacterial (Mandal & Mandal, 2011), antioxidant (Ahmed et al. 2018), and anti-inflammatory (Silva et al. 2021) properties. Honey has long been known for its antimicrobial properties. Honey has been used for repairing injuries such as wounds and burns for over 8000 years (Eteraf-Oskouei& Najafi, 2013). Other applications include oral ailments, digestive issues such as diarrhoea and constipation, skin disorders, eye ailments, lung problems, and so on (Qamar & Rehman, 2020).

Different factors of honey contribute as antimicrobial characteristics, one of the major factors is hydrogen peroxide which works by damaging the cell walls, and enzymes and degrading the bacterial DNA (Brudzynski et al. 2011). However, another kind of honey, called non-peroxide honey (viz., manuka honey) produces non-peroxide components, such as methylglyoxal (MGO) which alter the adhesion and movement of bacteria by changing the structure of flagella (Girma et al. 2019). Also, the high acidity of honey (Albaridi, 2019), higher concentration of sugar and low water activities causes osmotic pressure, inhibiting the growth of microorganism (Kwakman& Zaat, 2011). Furthermore, bee defensin-1 (Almasaudi, 2020) present in honey, a peptide (Vică et al. 2021) produced by bees, is also the main ingredient responsible for the antibacterial activity of honey, except for Manuka honey (Kwakman et al. 2011) which by disrupting bacterial cell membranes.

MATERIALS AND METHODS

Study Site and Duration

The research was carried out at the Microbiology Laboratory of Balkumari College, Bharatpur, Chitwan, for three months between May and July, 2023.

Collection of sample

A total of eight honey samples (Wildlcliff, Rudilo Chiuri, Multiflora, Putka, Local, Tori) were obtained from local beekeepers and market in sterile sampling bottles whereas Manuka honey was imported from India in a well-sealed container. To ensure its sterility, the sample was streaked on a nutritional agar plate. The honey solution was handled carefully and to avoid photo degradation of its active components, honey was stored in a cool, dark place away from bright light.

Figure 1: Collection of different kinds of honey

Collection of bacterial culture

The cultured organisms under study were collected from the Bharatpur Hospital in Chitwan. The purity of the organisms was examined through subculturing in Eosin methylene blue agar, Macconkey agar and Mannitol salt agar, and further microbiological and biochemical tests, including gram staining, Indole test, Methyl Red test, Voges Proskaeur test Citrate test, Catalase test, Coagulase test, Oxidase test and TSI test were performed in order to verify the organisms. The organisms included in this study were: *Escherichia coli* and *Klebsiella pneumoniae* isolated from urine samples, *Staphylococcus aureus*, *Proteus vulgaris* and *Pseudomonas aeruginosa* isolated from pus samples.

Preparation of Honey solutions

In this study, varying concentrations (25%, 50%, 75%, and 100%) of each honey sample were meticulously prepared. 0.75 ml of honey was diluted in 0.25 ml of sterile water to make a 75% honey solution (v/v). Similarly, to make 50%, and 25%, honey solutions (v/v), 0.5ml of honey in 0.5ml of sterile water, and 0.25ml of honey in 0.75ml of sterile water were serially diluted, respectively (Mama et al. 2019).

Susceptibility testing of honey

For the susceptibility test, the Agar well diffusion method was performed (Balouiri et al. 2016). A sterile swab was dipped into the prepared standard inoculum, squeezed clear of excess fluid against the edge of the tube, and then uniformly swabbed over the Mueller Hilton Agar (MHA) by rotating the plate through a 60-degree angle and allow to dry at room temperature for 5 to 10 minutes with the lid on. With a sterile cork borer, wells were formed (22 mm apart). In the agar medium. 30 µl of honey with concentrations of 100%, 75%, 50%, and 25% was added to the wells in the plate using a micropipette. After 30 minutes of allowing honey to diffuse, the plates were incubated at 37°C for 24 hours. The average sizes of inhibitory zones were measured in millimetres and reported. Water was equally distributed in a well for positive control.

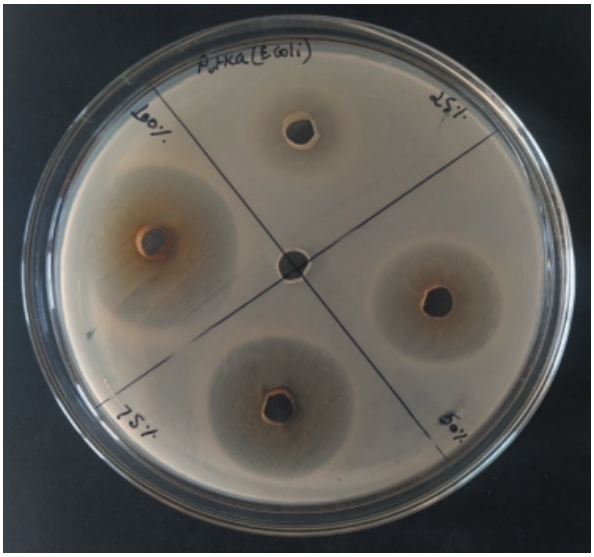

Figure 2: Susceptibility test of Putka honey against *E. coli*

Antibiotic Susceptibility Test

Kirby disk diffusion method was used for the Antibiotic Susceptibility test according to Khan et al. (2019). A sterile swab was dipped into the prepared standard inoculum, squeezed against the edge of the tube to remove the excess inoculum, and then uniformly swabbed over the Mueller Hilton Agar (MHA) and allowed to dry at room temperature for 5 to 10 minutes with the lid on. Antibiotic disk was picked and placed onto MHA gently with the help of sterile forceps. Then plates were incubated at 37°C for 18-24 hours. After the proper incubation, diameters of the zone of inhibition around the discs were measured to the nearest millimeter using a ruler and classified as sensitive, intermediate, and resistant according to the Interpretation chart (Hudzicki, 2009). 5 antibiotics were used: ciprofloxacin (5µg), gentamicin (10µg), co-trimoxazole (25µg), amoxicillin (10 µg), ceftazidime (30 µg).

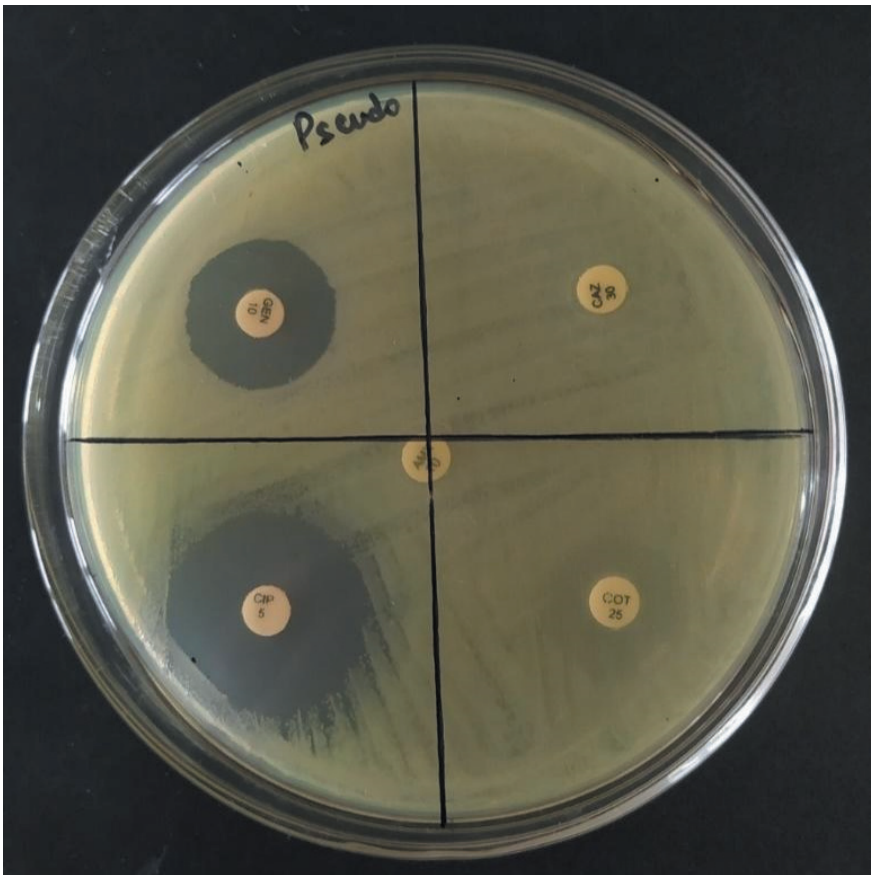

Figure 3: Antibiotic Susceptibility test against *Pseudomonas aeruginosa.*

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of antimicrobial agents was determined for each by the tube dilution method. 9 sterile test tubes were used. Out of 9, two test tubes were taken as Quality Control, one containing honey + sterile water (Honey Control) and the other containing nutrient broth + inoculum (Growth Control). 1 ml of sterile water was added to the rest of the 7 test tubes except the 1st test tube. 2 ml of undiluted honey was put into test tube 1 and 1 ml is transferred into 2nd test tube. Two-fold serial dilutions were performed by transferring 1ml of honey to tube 3 and vertexing to homogenize. After mixing, 1 ml was transferred from tube 3 and tube 4. This procedure was continued up to the 7 tubes with a dilution factor of 50% to 1.56% v/v was reached and at last 1 ml was removed from tube 8 and discarded. All seven dilutions were inoculated with the standard bacterial inoculum of the isolate of choice. Inoculated tubes were incubated overnight at 37°C. The highest dilution that inhibited growth (no turbidity) in the tube of honey tested was taken as the MIC value for that batch of honey against the bacterial species tested.

Determination of Minimum Bactericidal Concentration (MBC)

MBC was determined by sub-culturing the broths used for the determination of MIC onto sterile nutrient agar plates by the streak plate method and aerobically incubated at 37°C for 24 h. cultivated. The lowest concentration of honey that showed no growth of test microorganisms was considered MBC. Inoculated plates were scored as bactericidal for no growth of bacteria, bacteriostatic for mild to moderate growth, and no antibacterial activity for heavy growth.

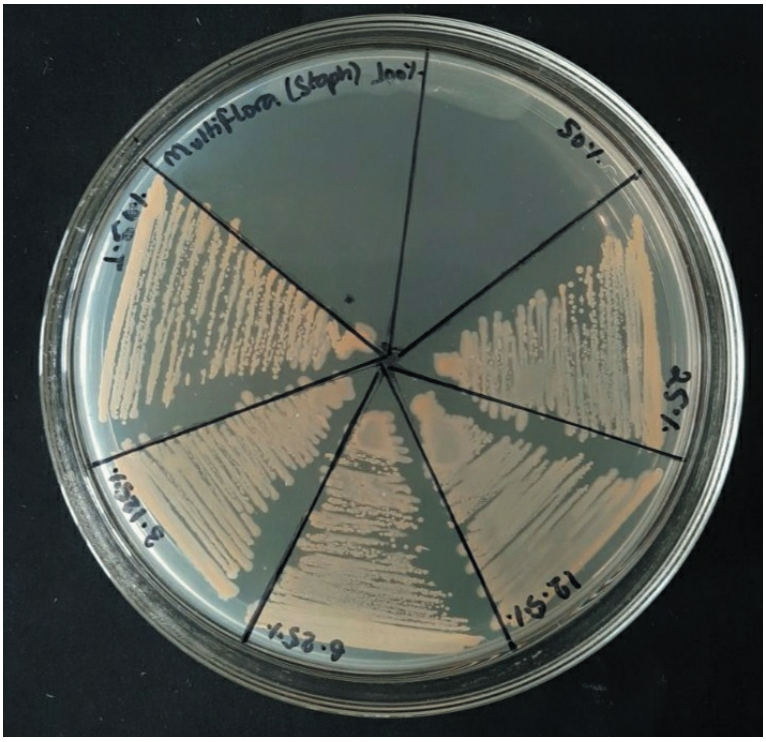

Figure 4: Minimum Bactericidal Concentration of Putka honey against *Staphylococcus aureus*

RESULTS

Antibiotic susceptibility test:

Clinical isolates were tested against selected 5 antibiotics. The susceptibility of pathogens to tested antibiotics was varied. All 5 pathogens showed high sensitivity towards Ciprofloxacin (CIP) while isolates were extremely resistant to Ceftazidime (CAZ). Two isolates were sensitive against Amoxicillin (AMX) however, Cotrimoxazole (COT), and Gentamicin (GEN) displayed effectiveness to all pathogens.

Honey Susceptibility Test:

In this investigation, antibacterial efficacy of 8 varieties of honey were examined against 5 clinical isolates. At 100% v/v concentration, all honey samples exhibited antibacterial properties; however, ZOI increased with increasing honey concentration. In general, zones of inhibition ranged from 0-39 mm where the largest zone of inhibition was shown by Rudilo honey at 100% v/v concentration against *E coli*.

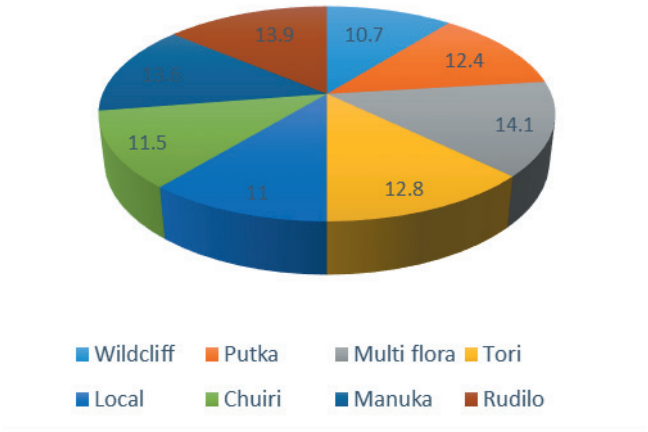

Figure 5: Average ZOI showed by Honey sample towards all isolates.

Determination of MIC

The resulting MIC value of honey samples against *S. aureus* and *P. vulgaris* varied from 6.25-50% v/v whereas MIC against *E. coli* and *K. pneumoniae* ranged from 12.5-50% v/v. However, for *P. aeruginosa*, MIC value of all honeys resulted to be 50% v/v except for Chiuri which was 100% v/v. Overall, the honeys which showed the highest MIC against the tested organisms was Manuka.

Determination of MBC:

The MBC of honey against *S. aureus* and *P. vulgaris* varied from 25-100% v/v whereas the MBC against *E. coli*, *K. pneumoniae* and *P aeruginosa* ranged from 50-100% v/v. Overall, Rudilo had the highest MBC against tested bacteria while Chiuri showed the lowest.

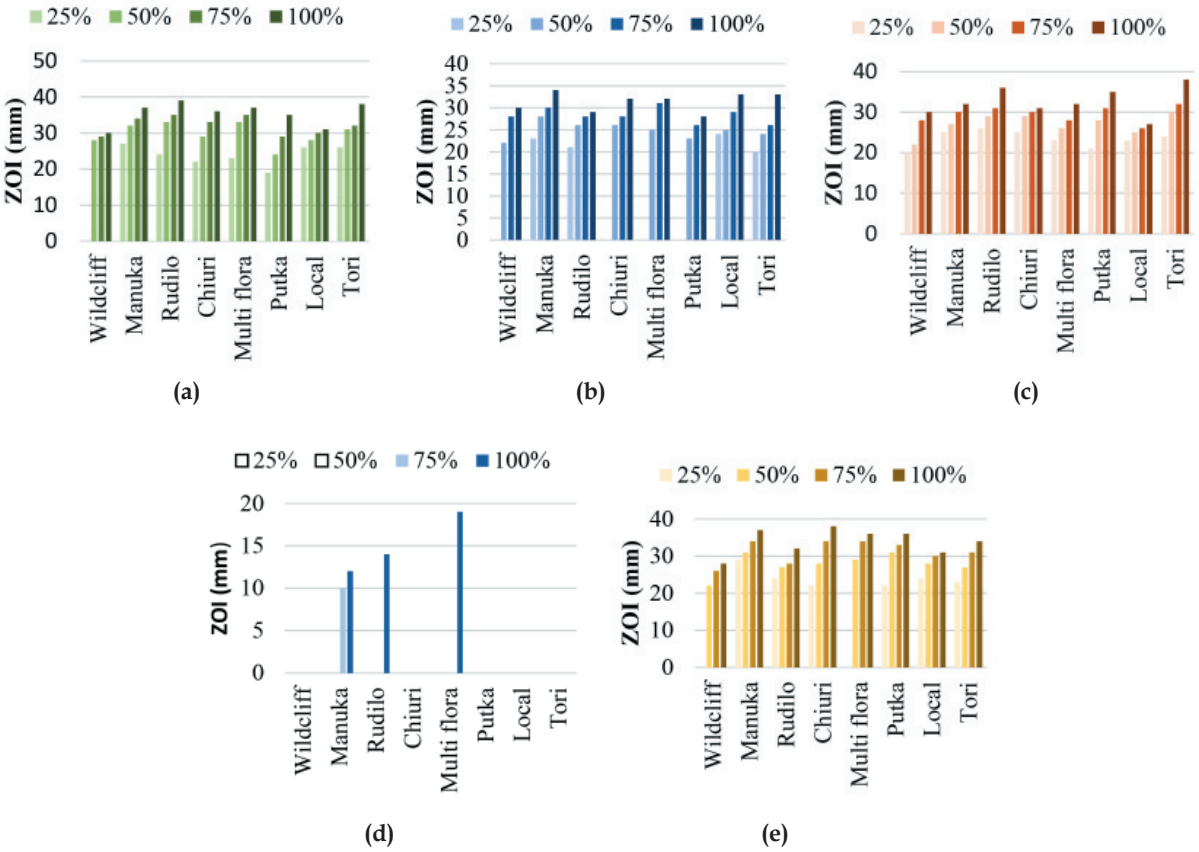

Figure 6: Honey Susceptibility Test against Different Isolates (a) *E. coli*, (4b) *Klebsiella pneumoniae*, (c) *Staphylococcus aureus*, (d) *Pseudomonas aeruginosa*, (e) *Proteus vulgaris*.

Table 1: Minimum Inhibitory Concentration (MIC) of different kinds of honey against tested bacteria

S.N.	Bacteria	Honey							
		Wildcliff	Manuka	Rudilo	Putka	Multiflora	Chiuri	Tori	Local
1	*E. coli*	25	12.5	12.5	25	12.5	50	25	50
2	*K. pneumoniae*	50	12.5	50	50	25	25	25	25
3	*S. aureus*	25	6.25	6.25	50	25	50	12.5	50
4	*P. vulgaris*	50	6.25	6.25	12.5	12.5	25	50	50
5	*P. aeruginosa*	50	50	50	50	50	100	50	50

Table 2: Minimum Bactericidal Concentration (MBC) of different kinds of honey against tested bacteria

S.N.	Bacteria	Honey							
		Wildcliff	Manuka	Rudilo	Putka	Multiflora	Chiuri	Tori	Local
1	*E. coli*	50	50	50	50	50	100	50	50
2	*K. pneumoniae*	50	50	50	50	50	100	50	50
3	*S. aureus*	50	25	25	50	50	100	50	50
4	*P. vulgaris*	50	50	25	50	50	100	50	50
5	*P. aeruginosa*	100	50	50	50	50	100	100	50

Table 3: Antibiotics Susceptibility Test against clinical isolates

S.N.	Bacteria strains	Antibiotics				
		Gentamicin	Co-Trimoxazole	Ciprofloxacin	Amoxicillin	Ceftazidime
1	*P. aeruginosa*	S	R	S	R	R
2	*S. aureus*	S	S	R	R	R
3	*E.coli*	S	S	S	R	R
4	*P. vulgaris*	S	S	S	R	R
5	*K. pneumoniae*	S	S	S	R	R

DISCUSSION

In recent years, the globe has seen a troubling rise in infectious diseases, as well as an increase in the threat of multi-drug resistance among microorganisms (Hirsch & Tam, 2014 and Keen et al. 2010). As antibiotics are losing their efficacy, scientists and healthcare workers are facing an urgent need for natural alternate therapies to tackle these growing health concerns (Ventola, 2015) and (Combarros-Fuertes et al. 2020) .

A potential replacement for traditional antibacterial medicines is honey, which exhibits broad-spectrum antimicrobial action making an inhospitable environment for bacterial growth (Simon, 2021). Honey possesses significant antibacterial, antioxidant, anti-inflammatory, and wound-healing properties. Hydrogen peroxide, pH, low water activity, high sugar concentration, bee defense-1, and methylglyoxal are some of the honey components that makes honey an antibacterial agent.

The pathogens studied in this investigation included *Staphylococcus aureus, E. coli, Proteus vulgaris, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*. The findings from this study revealed that among the five tested pathogenic strains, E. coli displayed the highest susceptibility towards honey samples; however, *Pseudomonas aeruginosa* exhibited the highest level of resistance. This evaluation was done by Agar well-diffusion method and the parameters were measured accordingly to a different zone of inhibition.

Rudilo honey was found to have a strong inhibitory action against *E. coli* with ZOI 24 mm, 33 mm, 35 mm, and 39 mm at 25%, 50%, 75% and 100% concentration, respectively whereas the honey which showed maximum inhibition against *Klebsiella pneumoniae* was Manuka with ZOI 23 mm, 28mm, 30 mm, and 34 mm at ascending concentration

In addition, *P. vulgaris* turned out to be the most sensitive to Chiuri (Butter tree honey), exhibiting the highest ZOI of 22 mm, 28 mm, 34 mm, and 38 mm at concentrations of 25%, 50%, 75%, and 100%, respectively, while Tori, (Mustard honey) was reported to be the most efficient against *S. aureus* having ZOI 24 mm, 30 mm, 32 mm and 36 mm with rise in its concentration, respectively. Moreover. Rudilo, Manuka, and Multiflora honey were the only types of honey that could prevent *P. aeruginosa* growth. Manuka honey, however, was able to successfully demonstrate ZOI at two different concentrations, namely 75% (ZOI 10 mm) and 100% (ZOI 12 mm) whereas Rudilo and Mutiflora honey only showed inhibition at 100% concentration measuring ZOI 14 mm and 19 mm, respectively.

According to the study, the zone of inhibition (ZOI) rises as honey content increases, with a concentration of 25% exhibiting the least inhibitory effect and a concentration of 100% exhibiting the strongest. Among the studied bacteria, *E. coli* was found to more susceptible whereas *P. aeruginosa* proved to be the most resistant as most of the honey was unable to exert any inhibitory action, even at its greatest concentration. Our result supports the result obtained by Wilkinson & Cavanagh (2005).

Tested honey samples were found to have both bacteriostatic and bactericidal properties as all the examined bacterial strains were susceptible to all honey samples at concentrations ranging from 6.25% to 50% (v/v). Among the honey samples examined, Rudilo and Manuka demonstrated notable effectiveness, showing low Minimum Inhibitory Concentrations (MIC) against both *S. aureus* and *P. vulgaris* which was at 6.25% v/v whereas other honey showed MIC ranging from 12.5% v/v to 50% v/v. Even for bactericidal test, Manuka and Rudilo showed the lowest MBC value which was at 25%v/v whereas the highest MBC value was shown by Chiuri honey which was at 100%. *P. aeruginosa* was found to be the least affected among the studied bacteria for MIC and MBC test.

In order to assess the bacterial sensitivity to antibiotics and make a comparative analysis with honey samples, an antibiotic susceptibility test was conducted. Among five antibiotics used, CIP-5 showed the greatest inhibition whereas all bacteria exhibited complete resistance towards CAZ. From the result obtained it was found that honey samples showed a greater ZOI than antibiotics used against *E. coli, P. vulgaris, K. pneumoniae, S. aureus*, which matches the study done by Mercan et al. (2007). However, for *P. aeruginosa,* antibiotics were found to be more effective than sampled honey.

CONCLUSIONS

This study concludes that honey has natural antibacterial qualities that can be utilized to treat bacterial infections, mild burns, and wounds. Even at its lowest concentration (MIC), honey had antibacterial properties, demonstrating its efficacy in treating infections caused *by E. coli, S. aureus, K. pneumoniae,* and *P. vulgaris*, but it may not be as effective in treating infections led by *P. aeruginosa.* Furthermore, honey could be a possible alternative to antibiotics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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