



(RESEARCH ARTICLE)



Antibacterial activities of honey bee against gram-positive bacteria isolated from urine

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GSC Biological and Pharmaceutical Sciences, 2024, 26(03), 113–122

Publication history: Received on 01 February 2024; revised on 13 March 2024; accepted on 16 March 2024

Article DOI: <https://doi.org/10.30574/gscbps.2024.26.3.0092>

Abstract

The medicinal benefits of honey have long been recorded in ancient days as a solution for various ailments and infections. This study x-rayed the effects of honey on common gram-positive bacteria isolated from urine. The study evaluated the *in vitro* susceptibility of two gram positive bacteria strains, isolated from urine of students of Nnamdi Azikiwe University, Awka Anambra state, Nigeria. The end point was achieved via macroscopic, microscopic methods and biochemical examinations which includes: gram staining, urease, catalase, coagulase testing to a honey sample. The evaluation of the antibacterial activity was ascertained by the agar diffusion well method. The results obtained in this study proved that the honey sample exhibited potent antibacterial activity against the tested strains, the zone of inhibition was at a mean value of 20.5 mm and 29.5 mm respectively at 100% concentration of honey. *Staphylococcus epidermidis* was found to be less susceptible than *Enterococcus faecalis* with a mean inhibition zones being 29.5 mm. This contribution has provided a broad overview of the antibacterial activity of honey and shown that honey bee has great potential for therapeutic use as an alternative therapy for infections caused by the isolates.

Keywords: Honey bee; Gram-positive; Bacteria strains; Agar well method; Ailments

1. Introduction

Bacteria are ubiquitous, mostly free-living organisms often consisting of one biological cell they constitute a large domain of prokaryotic microorganisms (McCutcheon, 2021). Bacteria have a remarkable ability to adapt to different environments, which makes them ubiquitous in almost all niches. They can withstand different environmental stresses such as temperature, pH, and pressure changes (Madigan *et al.*, 2012). Bacteria can also exchange genetic material through various mechanisms such as transformation, transduction, and conjugation, which enable them to adapt to new environmental conditions quickly (Huddleston, 2014). *Though most bacteria species are harmless and often beneficial others can cause infectious diseases* (Ryan *et al.*, 2014).

Bacterial infections are variety of illnesses caused by bacteria (Curtiss *et al.*, 2017).

Bacteria can indeed be found in the urine of men, which can indicate the presence of a urinary tract infection (UTI) or Asymptomatic bacteriuria both gram-positive and gram-negative bacteria, are commonly found in urine samples but the most common bacteria found are gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (Foxman, 2014). These bacteria are known to colonize the gastrointestinal and genitourinary tracts of humans, and they are often introduced into the urinary tract through the urethra.

On the other hand, gram-positive bacteria such as *Enterococcus faecalis*, *Staphylococcus saprophyticus*, and *Streptococcus agalactiae* are less commonly associated with UTIs, usu although still cause infections, especially in immunocompromised individuals or patients ally find their way to the urinary tract through indwelling urinary

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catheters (Colgan *et al.*, 2011). These bacteria are typically found in the vaginal and rectal flora, and they can be introduced into the urinary tract during sexual activity or catheterization. (Foxman, 2014).

Gram-positive bacteria found in the urine often signals significant health concern indicating the presence of Urinary tract infection (UTIs), which often requires antibiotic treatment. The rise of antibiotic resistance has led to an increased interest in alternative treatments, such as natural products. Honey has been used as a traditional medicine for centuries and has recently gained attention for its antibacterial properties. The antimicrobial properties are from the honey's ability to inhibit bacterial growth, which have been demonstrated using many microorganisms, including *S. aureus*, *S. pyogenes*, *P. aeruginosa* and *E. coli* (Saeed *et al.*, 2018) the *in vitro* antimicrobial activity of honey was reported by Radwan *et al.* who observed that honey stopped the growth of *Salmonella* and *Escherichia coli*.

Many studies have analyzed the composition of honey and have studied the physical and chemical properties that may give rise to its ability to work against various microorganisms, various parameters have been identified which includes: its low water content (low water activity), the sugar content, high viscosity, and acidity (Saeed *et al.*, 2021). In addition, various compounds are associated with honey and provide its antibacterial potential, including phytochemicals, peptides, nonperoxidase glycopeptides, and proteins that are notable features of honey and are associated with its antimicrobial effects (Saeed *et al.*, 2021). Recent research has also identified bee defensin-1, a bee-derived protein, as a potential antimicrobial component in honey. (Nolan *et al.*, 2019). All of these elements are present at different concentrations depending on the source of nectar, bee type, and storage. These components work synergistically, allowing honey to be potent against a variety of microorganisms including multidrug resistant bacteria. (Saeed *et al.*, 2021). However, each elements produces different effect on the bacteria, e.g. its sugar content, exerts osmotic pressure on bacterial cells, causing flow of cell content out of the cell due to osmosis, hydrogen peroxide acts as oxidizing agent, its acidity is unfavorable to growth of certain acidiphilic organisms, etc.

2. Materials and methods

2.1. General materials

Petri dishes, Sterile wire loop, Sterile normal saline solution, McFarland 0.5 standard solution, Sterile swab, Sterile cork borer (6mm diameter), Micropipette, Honey, Antibiotic discs, Sterile distilled water, alpha naphthol, Methyl Red reagent, Lugol's iodine, safranin, acetone, 0.5% solution of crystal violet, Tryptone broth, Kovac's reagent, 3% hydrogen peroxide solution, Clean glass slides, wire loop, urine specimen containers, syringes, EMB agar (Eosin Methylene Blue agar), CLED agar (cystine lactose electrolyte-deficient agar), Blood agar, Nutrient agar, Mueller Hinton agar, MR-VP broth, peptone water, test tubes, micropipette, pipette,

2.2. Collection of specimen

Urine specimens were collected at random from students of Nnamdi Azikiwe University, Awka, Anambra state, Nigeria. A total of 18 urine samples were collected, consisting of 10 samples from male students and 8 samples from female students.

2.3. Preparation of honey

Pure honey was obtained from a local supplier and sterilized using an autoclave. The pH of the samples was checked, and they were stored at 2-8°C until they were used. After filtration, different concentrations of honey samples were prepared from the 100% pure concentrated sample. To obtain a 75% honey solution (v/v), 0.75 ml of honey was diluted in 0.25 ml of sterilized distilled water. Further serial dilutions of 0.5 ml of each and 0.25 ml of honey and 0.75 ml of sterile distilled water were added to obtain 50% and 25% honey solutions (v/v), respectively.

2.4. Isolation and identification of organisms

2.4.1. Isolation and characterization

Each of the fresh urine samples was inoculated onto Nutrient agar, Cysteine Lactose Electrolyte Deficient agar (CLED), Eosin Methylene Blue agar (EMB), and Blood agar media and incubated at 37 °C for 18–24 hours. All the plates were incubated aerobically and were initially examined for growth after 24 hours; each visible colony was visually inspected and counted manually to determine the amount of colony-forming units (CFU) present in each plate.

Discrete colonies on various plates were subcultured onto nutrient agar and incubated for 24 hours. The various isolates underwent identification testing. Identification of the isolates was performed from pure colonies using classical

biochemical tests (Gram Staining, Urease, Indole, catalase, coagulase, methyl red, and citrate test) according to the standard guidelines.

2.4.2. Gram staining

This reaction was done to identify organisms that were Gram-positive (+ve) and Gram-negative (-ve).

Procedure – A smear of the isolate was made on a clean, grease-free slide and allowed to air dry. The slide was heat-fixed before being flooded with a 0.5% solution of crystal violet for 30 seconds. The stain was then washed off with water before the slide was flooded with iodine solution (mordant) and allowed to sit for 10 seconds, after which it was washed off. Next, the slide was counterstained with safranin for 30 seconds, rinsed with water, and allowed to air dry. Finally, the stained slide was viewed under the microscope using immersion oil under a x100 objective lens.

2.4.3. Catalase Test

Procedure – A loopful of hydrogen peroxide was dropped onto a clean, grease-free slide. The isolate was then mixed with the hydrogen peroxide on the slide. The mixture was observed for the immediate production of gas bubbles, which indicated a positive reaction, while no gas bubbles indicated a negative reaction.

2.4.4. Indole Test

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to convert tryptophan into indole.

Procedure: The test organism (isolate) was inoculated into a test tube containing 3 ml of sterile tryptone water. The test tube was incubated at 37°C for 24 hours. After incubation, 0.5 ml of Kovac's reagent was added to the tube, and the mixture was gently shaken. The absence of a red ring-like color on the surface of the layer within

2.4.5. Urease Test

The urease test was performed to determine the ability of the organisms to split urea by producing the enzyme urease. This test was primarily used to differentiate between members of the genera *Proteus*, *Providencia*, and *Morganella*, which are urease-positive, and other *Enterobacteriaceae*, which are urease-negative.

Procedure: The isolate was inoculated onto a urea agar medium and incubated at 37°C for 18-24 hours. The plate was then observed for growth and a color change in the medium. If the organism was urease-positive, the urea was hydrolyzed to ammonia, resulting in a rise in pH and a color change to pink or magenta. If the organism was urease-negative, the medium remained yellow.

2.4.6. Citrate Test:

The citrate test was performed as a diagnostic test to determine whether the bacterial isolate could utilize citrate as the sole carbon source. It was primarily used to differentiate members of the *Enterobacteriaceae* family.

Procedure: The isolate was inoculated onto a Simmons citrate agar medium and incubated at 37°C for 18-24 hours. The plate was observed for the presence of growth and a color change in the medium. If the organism was citrate-positive, it utilized the citrate in the medium as the sole carbon source and produced an alkaline byproduct, resulting in a color change in the medium from green to blue. If the organism was citrate-negative, the medium remained green.

2.5. Antibacterial activity test of honey

The antibacterial activity test of honey was conducted using the agar diffusion assay method. The procedure involved the following steps:

- **Bacterial Culture:** The bacterial isolate was cultured on a nutrient agar plate and incubated at 37°C for 24 hours. A pure culture was obtained by streaking the isolate onto a fresh nutrient agar plate and further incubating it for 24 hours.
- **Bacterial Suspension:** A sterile wire loop was used to pick a few colonies from the pure culture and transfer them into a tube containing sterile normal saline solution. The tube was shaken to ensure proper dispersion of the colonies.
- **Standardization:** A turbidity standard of 0.5 McFarland was used to standardize the bacterial suspension. The suspension was compared with the standard and adjusted accordingly.

- **Preparation of Agar Plate:** The standardized bacterial suspension was streaked on a Mueller-Hinton agar plate using a sterile swab. Wells were made in the agar plate using a sterile cork-borer with a diameter of 6mm.
- **Addition of Honey:** Honey samples were added to each well using a micropipette. The plate was then incubated at 37°C for 24 hours.
- **Measurement of Zone of Inhibition:** After incubation, the diameter of the zone of inhibition around each well was measured in millimeters. The results were recorded and compared with standard values for antibiotic susceptibility testing.
- **Positive Control:** A positive control well was filled with an antibiotic disc, which served as a reference for comparison.

3. Results

Table 1 Microbial Colony Count

Specimen	Nutrient Agar	Blood Agar	E.M.B Agar	C.L.E.D Agar
M 1	53	30	NG	NG
M2	TNTC	TNTC	TNTC	NG
M3	98	TNTC	TNTC	NG
M4	250	TFTC	NG	36
M5	TNTC	NG	TNTC	NG
M6	TNTC	47	NG	NG
M7	TNTC	TNTC	TNTC	NG
M8	TNTC	NG	TNTC	NG
M9	TNTC	95	NG	NG
M10	40	TNTC	NG	NG
F1	TNTC	80	NG	NG
F2	TNTC	TNTC	115	NG
F3	40	TNTC	190	TFTC
F4	TNTC	TNTC	TNTC	NG
F5	150	TNTC	TNTC	NG
F5	56	23	NG	NG
F7	251	TNTC	249	NG
F8	180	TNTC	TNTC	NG

KEY: TNTC = Too Numerous to Count, TFTC = Too Few to Count. M= Male urine specimen, F= Female urine specimen, NG= No growth.

Table 2 Morphological Identification of the various Isolates (colony morphology)

Isolate	Shape	Surface	Colour	Elevation	Gram	Identity
BA	Circular	Smooth	White	Raised	+ cocci	<i>Staphylococcus epidermids</i>
CLED 1	Circular	Glistening	Yellowish	Raised	+ cocci	<i>Enterococcus faecalis</i>

Table 3 Biochemical Identification of Bacterial isolate

ISOLATE	CAT	COAG	IND	CITR	MET. R	UREAS	ORGANISM
CLED 1	+	-	+	+	+	+	<i>Enterococcus spp.</i>
BA	+	-	+	-	-	+	<i>Staphylococcus epidermidis</i>

KEY: CAT= Catalase test, COAG= Coagulase test, CITR= Citrate test, MET. R= Methyl Red test, UREAS= Urease test.

Table 4 Degree of susceptibility of isolates to Honey expressed in millimeters

Organism	E1	E2	X
<i>Staphylococcus epidermidis</i>	20 mm	21 mm	20.5 mm
<i>Enterococcus faecalis</i>	30 mm	29 mm	29.5 mm

KEY: E1- Test well 1, E2 - Test well 2, X-Mean value of the wells, NR- Non reactive (Resistant).

Table 4: shows the results of tested susceptibility of two bacterial isolates, *Staphylococcus epidermidis* and *Enterococcus faecalis*, to honey (which can be said to check the antibacterial activity of honey against the isolates).

For *Staphylococcus epidermidis*, the results showed that the bacterial isolate was inhibited by honey, with an average diameter of inhibition of 20.5 mm. This suggests that the isolate is moderately susceptible to the antimicrobial activity of honey.

For *Enterococcus faecalis*, the results showed that the bacterial isolate was more susceptible to honey than *Staphylococcus epidermidis*, with an average diameter of inhibition of 29.5 mm. This suggests that the isolate is highly susceptible to the antimicrobial activity of honey.

Therefore, the results proved that honey has a moderate to high level of antimicrobial activity against both *Staphylococcus epidermidis* and *Enterococcus faecalis*.

Table 5 Positive Control for the Degree of susceptibility of Isolates to antibiotics expressed in millimeters

	Abbreviations	Concentrations (mcg)	Zone of inhibition (<i>Staphylococcus epidermidis</i>)	Zone of inhibition (<i>Enterococcus faecalis</i>)
Ampiclox	APX	20	NR	NR
Amoxicillin	AMX	10	NR	NR
Nitrofurantoin	NB	10	NR	13 mm
Sulfamethoxazole	S	20	25 mm	NR
Chloramphenicol	CH	30	15 mm	25 mm
Ciprofloxacin	CPX	10	20 mm	NR
Erythromycin	E	30	15 mm	NR
Linezolid	LEN	30	20 mm	18 mm
Clindamycin	CM	10	24.5 mm	11 mm
Rifampicin	RD	30	13 mm	15 mm

KEY: NR- Non reactive (Resistant)

Table 5: illustrates the degree of susceptibility of *Staphylococcus epidermidis* and *Enterococcus faecalis* to ten different antibiotics i.e. Ampiclox (APX), Amoxicillin (AMX), Nitrofurantoin (NB), Sulfamethoxazole (S), Chloramphenicol (CH),

Ciprofloxacin (CPX), Erythromycin (E), Linezolid (LEN), Clindamycin (CM), and Rifampicin (RD), as expressed in millimeters of zone of inhibition.

The susceptibility was expressed in millimeters, with the zone of inhibition indicating the degree of bacterial growth inhibition. The following considerations can be made from the table:5

Ampiclox and Amoxicillin did not show any zone of inhibition against *Staphylococcus epidermidis* and *Enterococcus faecalis*, indicating that these two antibiotics were ineffective against these bacterial species.

Nitrofurantoin showed a zone of inhibition of 13 mm against *Enterococcus faecalis*, indicating some level of susceptibility. However, it did not show any inhibition against *Staphylococcus epidermidis*.

Sulfamethoxazole showed a zone of inhibition of 25 mm against *Staphylococcus epidermidis*, indicating a high level of susceptibility. However, it did not show any inhibition against *Enterococcus faecalis*.

Chloramphenicol showed a zone of inhibition of 15 mm and 25 mm against *Staphylococcus epidermidis* and *Enterococcus faecalis*, respectively, indicating moderate susceptibility.

Ciprofloxacin showed a zone of inhibition of 20 mm against *Staphylococcus epidermidis*, but did not show any inhibition against *Enterococcus faecalis*.

Erythromycin showed a zone of inhibition of 15 mm against *Staphylococcus epidermidis*, but did not show any inhibition against *Enterococcus faecalis*.

Linezolid showed a zone of inhibition of 20 mm and 18 mm against *Staphylococcus epidermidis* and *Enterococcus faecalis*, respectively, indicating moderate susceptibility.

Clindamycin showed a zone of inhibition of 24.5 mm and 11 mm against *Staphylococcus epidermidis* and *Enterococcus faecalis*, respectively, indicating a high level of susceptibility against *Staphylococcus epidermidis*.

Rifampicin showed a zone of inhibition of 13 mm and 15 mm against *Staphylococcus epidermidis* and *Enterococcus faecalis*, respectively, indicating moderate susceptibility.

Overall, the results indicate that different antibiotics have varying degrees of efficacy against *Staphylococcus epidermidis* and *Enterococcus faecalis*.

4. Discussion

Numerous studies carried out have shown that honey possess antibacterial against several bacterial pathogens, such as: *E. coli*, *S. Typhi*, *P. aeruginosa*, *K. pneumoniae*, *B. cereus*, and *S. epidermidis* (Mandal and Mandal 2011; Feás *et al.*, 2013; Salonen *et al.*, 2017; Matzen *et al.*, 2018; Combarros-Fuertes *et al.*, 2019).

The present research work investigated the antibacterial activity of honey on gram-positive bacteria isolated from urine, on (*Staphylococcus epidermidis* and *Enterococcus faecalis*.) The results obtained from our study demonstrate that honey exhibits a significant antibacterial effect against both of these bacteria. The bacteria isolates were susceptible to organic honey at 100% concentration.

The analysis of the diameter of inhibition zone (DIZ) values (Table 4) showed that the tested honey sample had potent antibacterial activity against both *Staphylococcus epidermidis* and *Enterococcus faecalis*, the diameters of inhibition for each of the isolate varied between 20.5 and 29.5 mm (mean inhibition value) this results are in agreement with the study of Bouacha *et al* (2016), which reported high antibacterial activity concentration of ranging from 25% - 5% of Honey against *Enterococcus faecalis* isolated from the urine of pregnant women.

A recent study by Kim *et al.* (2021) investigated the antibacterial activity of Korean honey on *Enterococcus faecalis* reported that honey exhibited a significant inhibitory effect against *Enterococcus faecalis*. The researchers suggested that the antibacterial activity of honey may be attributed to the presence of various bioactive compounds, including polyphenols and flavonoids, which have been shown to possess antibacterial properties.

In the present study Honey also exhibited a high antibacterial effect against *Staphylococcus epidermidis* with an average value of inhibition at 20.5 mm the result of this present study is in accordance with what has been reported by Balázs *et al.*, (2023) study investigated the antibacterial and antibiofilm effect of unifloral honeys against bacteria isolated from chronic wound infections. In that study, the various honeys demonstrated the highest antibacterial activity, as evidenced by the lowest minimum inhibitory concentration (MIC) values, as well as the highest rates of biofilm inhibition and membrane disruption. Among the tested bacteria, *Staphylococcus epidermidis* showed the greatest sensitivity to the honey samples. the studies suggest that honey has antibacterial activity against *Staphylococcus epidermidis*, and the antibacterial effect of honey can be influenced by the type of honey, concentration, and floral source.

Antibacterial activity of the studied commercial organic honeys against these isolates suggests the protective effect of organic honey against urinary tract infections as described by Bouacha *et al.*, (2018). Efficacy of conventionally produced honey against pathogenic bacteria has been scientifically demonstrated (reviewed by Mandal and Mandal 2011).

Honey is composed of hundreds of compounds, any one of which may act at several sites, additively or synergistically. Apparently, resistance to honey properties is therefore difficult to develop (Combarros-Fuertes *et al.*,2019). When bioactive honey compounds are used separately, at the concentrations detected in honey, they may not have an effect on bacteria (Combarros-Fuertes *et al.* 2019).

Honey also acts synergistically with several antibiotics, reducing the doses required to inhibit bacterial growth or reverting the antibiotic-resistance previously acquired (Jenkins and Cooper 2012, Campeau and Patel 2014, Hayes *et al.*, 2018).

5. Conclusion

The results of our study clearly demonstrate the significant antibacterial activity of honey against the tested bacterial strains. This finding supports the potential use of honey as an alternative therapeutic agent for bacterial infections. However, further investigations are needed to deeper into the mechanisms of action and optimize the use of honey as a therapeutic agent. Ultimately, the findings of this study provide valuable insights into the potential application of honey in the field of antimicrobial research and offer promising avenues for future studies and clinical applications

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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