ANTIBACTERIA EFFECTS OF HONEY ON GRAM-NEGATIVE BACTERIA ISOLATED FROM URINE

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ABSTRACT

The present research work aims to investigate the effects of honey on common gram-negative bacteria isolated from urine samples A total of 18 urine samples were collected from students

enrolled at Nnamdi Azikiwe University. Using standard microbiological techniques, common gram-negative bacteria, including Enterobacter aerogenes, Escherichia coli, Proteus mirabilis, and Klebsiella pneumoniae, were isolated. The antimicrobial activity of honey was evaluated against the isolated gram-negative bacteria using the disk diffusion method. The results revealed that honey exhibited significant inhibitory effects against the isolated gram-negative bacteria, including Enterobacter aerogenes, Escherichia coli, , and Klebsiella pneumoniae with a mean zone inhibition of 30 mm, 24.5 mm and 20 mm whilst honey showed no effect on Proteus mirabilis The results revealed that honey exhibited significant inhibitory effects against the isolated gram-negative bacteria, including Enterobacter aerogenes, Escherichia coli, and Klebsiella pneumoniae. Whilst Proteus mirabilis was resistant to honey Enterobacter aerogenes was more susceptible to honey than other gram negative

isolates.

1. INTRODUCTION

Bacteria are single-celled microorganisms that are classified as prokaryotes, lacking a distinct nucleus and membrane-bound organelles.

Bacteria come in various shapes and sizes, including spheres (cocci), rods (bacilli), and spirals (spirilla). They are classified based on their shape, biochemical characteristics, and other features (Tortora et al., 2013). Bacteria reproduce asexually through binary fission, which involves splitting into two identical daughter cells. Some bacteria can also exchange genetic material through a process called conjugation (Prescott et al., 2013).

Bacteria play essential roles in various ecosystems, including decomposing organic matter, fixing nitrogen, and producing oxygen. They also play crucial roles in the human body, including aiding in digestion and fighting off harmful pathogens (Madigan et al., 2013).

Bacteria can cause diseases in humans, animals, and plants. Common bacterial infections include strep throat, pneumonia, and tuberculosis (Tortora et al., 2013).

Urinary tract infections (UTIs), which are bacterial infections, are typically caused by Uropathogenic bacteria and are quite prevalent infection (Loubet et al., 2020) A significant number of individuals experience chronic, recurring UTIs that may necessitate extended prophylactic antibiotic treatment.

These infections can affect both men and women of all ages; however, women are more likely to

experience this infection than men (Michelim et al., 2016). It was estimated that around 11% of women report at least one physician-diagnosed UTI per year and 20–30% report multiple recurrences (rUTI). (Bouacha et al., 2018) Urinary tract infections (UTIs) can be caused by both gram-negative and gram-positive bacteria. The most common bacterial cause of UTIs is Escherichia coli (E. coli), a gram-negative bacteria (Flores-Mireles et al., 2015). In addition, other gram-negative bacteria such as Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa have also been identified as causes of UTIs (Flores-Mireles et al., 2015).

Urine infections caused by gram-negative bacteria can be particularly problematic due to the presence of lipopolysaccharides (LPS) in their outer membrane, which can cause inflammation and damage to the urinary tract (Sivick et al., 2018). Additionally, these bacteria may possess virulence factors, such as fimbriae and toxins, which can aid in colonization and persistence within the urinary tract (Flores-Mireles et al., 2015).

Antibiotics are commonly used to treat bacterial infections. However, the overuse and misuse of antibiotics have led to the emergence of antibiotic-resistant bacteria, which pose a significant threat to human health (Madigan et al., 2013).

In the past decade, multi-drug resistant uropathogens have become a global concern, highlighting the necessity for alternative, non-antibiotic methods for preventing and treating UTIs (Loubet et al., 2020).

Honey is made from nectar collected and modified by the Apis mellifera honeybee. It is carbohydrate-rich syrup made

from the nectars of flowers and other plants Secretions. (Yaghoob et al., 2013). It has been used in folk medicine since ancient times, and its use in dressing acute and chronic wounds has recently been rediscovered by medical researchers. Honey has traditionally been used to treat burns, infected and non-healing wounds and ulcers, boils, pilonidal sinus, venous and diabetic foot ulcers, and a variety of other ailments. (Yaghoobi et al., 2013).

Honey has been widely accepted as food and medicine as an anti-inflammatory and antioxidant in all generations, traditions, and civilizations, both ancient and modern. But only recently have the antiseptic and antimicrobial properties of honey been discovered and explored. Honey has been reported to be effective in a number of human pathologies. Clinical studies have demonstrated that application of honey to severely infected cutaneous wounds rapidly clears infection from the wound and improves tissue healing. More recently, honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, Gram positives, and Gram negatives. (Mandal et al., 2011)

The various effect of honey is attributed to it chemical composition. Generally, honey has a content of 80–85% carbohydrates, 15–17% water, 0.3% proteins, 0.2% ashes and minor quantities of amino-acids, phenols, pigments and vitamin. (Miguel et al., 2017).

Aims and Objectives are:

1. to isolate and identify common gram-negative bacteria from urine specimen;

2. to investigate the antimicrobial effect of honey against common Gram-negative bacteria isolated from clinical urine specimens.

2. MATERIALS AND METHODS

COLLECTION OF SPECIMEN

Urine specimen was collected at random from students of Nnamdi Azikiwe University, Awka, Anambra state, Nigeria.

A total of 18 urine samples will be collected, consisting of 10 samples from male students and 10 samples from female students.

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 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 was added to obtain 50% and 25% honey solutions (v/v), respectively.

ISOLATION AND IDENTIFICATION OF ORGANISMS

Isolation and characterization

Each of the fresh urine samples was inoculated onto Nutrient agar, Cysteine Lactose Electrolyte Deficient agar (CLEAD), 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 were 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 isolate underwent identification testing. Identification of the isolate 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.

Gram staining

This reaction was done to identity organisms that are 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 saffranin 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.

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, indicating a positive reaction, while no gas bubbles indicated a negative reaction.

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 10 minutes, indicating positivity, was observed, while an absence of red color indicated a negative reaction.

Coagulase test

Coagulase test is used to differentiate Staphylococcus aureus (positive) which produce the enzyme coagulase, from S. epidermis and S. saprophyticus (negative) which do not produce coagulase. i.e Coagulase Negative Staphylococcus (CONS).

Procedure: A loopful of the test isolate was smeared on a slide, mixed with normal saline, and treated with a drop of serum, which was then mixed together. Agglutination or clumping occurred within 5-10 seconds, indicating a positive result.

Methyl Red test

The Methyl Red (MR) test is a biochemical test used in microbiology to determine the ability of an organism to perform mixed acid fermentation of glucose. It is commonly used to differentiate between bacteria that produce stable acidic end products and those that produce neutral or alkaline end products.

Procedure; Exactly 5 drops of methyl red indicator were added to an equal volume of a 48-hour culture of the isolate in Methyl red–Voges Proskauer (MR-VP) broth. The production of a bright red color indicated a positive test, while a yellow color indicated a negative test after vigorous shaking.

Urease test

The urease test is used to determine the ability of an organism to split urea, through the production of the enzyme urease. This test is primarily used to differentiate between members of the genera Proteus, Providencia, and Morganella, which are urease-positive, from 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, raising the pH and causing a color change to pink or magenta. If the organism was urease-negative, the medium remained yellow.

Citrate Test

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

Procedure: The isolate was inoculated onto a Simmons citrate agar medium. The inoculated plate was 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 used the citrate in the medium as the sole carbon source and produced an alkaline byproduct, causing a color change in the medium from green to blue. If the organism was citrate-negative, the medium remained green.

3. ANTIBACTERIAL ACTIVTY TEST OF HONEY

The antibacterial activity of honey has been assayed using various methods across the globe with special attention devoted to agar diffusion assay.

Antibacterial Susceptibility Testing of Honey.

Firstly, 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 incubating it at 37°C for another 24 hours.A sterile wire loop was used to pick a few colonies from the pure culture and then transferred into a tube containing sterile normal saline solution. The tube was shaken to ensure that the colonies were fully dispersed in the solution.

A turbidity standard of 0.5 McFarland was used to standardize the bacterial suspension. This was achieved by comparing the suspension with the standard and adjusting the bacterial suspension accordingly. Next, a sterile swab was used to streak the standardized bacterial suspension on a Mueller-Hinton agar plate. Then, a sterile cork borer (6mm diameter) was used to make wells in the agar plate. Honey was added to each well using a micropipette. The plate was incubated at 37°C for 24 hours. 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. A Negative control was filled with an antibiotic disc.

4. 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	Color	Elevation	Gram	Identity
EMB 1	Round	Shiny	Pink	Raised	- rod	Enterobacter aerogenes
EMB 2	Round	Smooth	Yellow	Raised	- rod	Klebsiella pnuemoniae
EMB 3	Round	Smooth	Blue-green	Raised	- rod	Proteus mirabilis
CLED 2	Round	Smooth	White	Raised	- rod	Esherichia coli

Table 3: Biochemical Identification of Bacterial isolate

ISOLATE	CAT	COAG	IND	CITR	MET. R	UREAS	ORGANISM
EMB 1	+	-	+	+	+	+	Enterobacter faecalis
EMB 2	+	-	+	+	+	-	Klebsiella pneumoniae
EMB 3	+	-	+	+	-	+	Proteus mirabilis
CLED 2	+	-	+	+	-	+	Escherichia coli

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

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INTERNATIONAL JOURNAL OF PROGRESSIVE RESEARCH IN ENGINEERING MANAGEMENT AND SCIENCE (IJPREMS)

Vol. 04, Issue 02, February 2024, pp : 537-543

e-ISSN : 2583-1062

Impact Factor : 5.725

Table 4: Degree of susceptibility of Gram Negative bacteria isolated to Honey expressed in millimeters

Organisms	E1	E2	X
Enterobacter spp	30 mm	30 mm	30 mm
Klebsiella pneumonia	22 mm		22 mm
Protues mirabilis	NR	NR	--
Esherichia coli	27 mm	22 mm	24.5 mm

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

Table 5: Negative Control for the Degree of susceptibility of microorganisms isolated to antibiotics expressed in millimeters.

	Abbreviations	Concentrations	Zone of inhibition (Klebsiella pneumonia)	Zone of inhibition (Escherichia coli)	Zone of inhibition (Enterobacter faecalis)	Zone of inhibition (Proteus mirabilis)
Augumentin	AU	10	NR	NR	22.5 mm	NR
Cefuroxime	CXM	30	NR	NR	22.5 mm	NR
Penicillin	PN	30	NR	NR	22.5 mm	NR
Cefuroxime	CFX	30	13 mm	12 mm	22.5 mm	17 mm
Ceftriaxone	CN	30	20 mm	20 mm	22.5 mm	25 mm
Ofloxacin	OFX	10	NR	NR	22.5 mm	23 mm
Sulfamethoxazole	SXT	30	17 mm	NR	22.5 mm	25 mm
Cefepime	CPX	10	13 mm	12 mm	20 mm	25 mm
Ciprofloxacin	CIP	10	13 mm	NR	22.5 mm	a15 mm
Sulfonamide	S	30	21 mm	14 mm	22.5 mm	22 mm

The table shows the results of the negative control for the degree of susceptibility of Klebsiella pneumonia, Escherichia coli, Enterobacter faecalis and Proteus mirabilis to various antibiotics (Augmentin, Cefuroxime, Penicillin, Ceftriaxone, Ofloxacin, sulfamethoxazole, Cefepime, Ciprofloxacin, and Sulfonamide.) expressed in millimeters.

Augmentin, Cefuroxime, and Penicillin did not show any zone of inhibition against Klebsiella pneumonia and Escherichia coli, indicating that these antibiotics were ineffective against these bacterial species.

Cefuroxime showed moderate susceptibility against Enterobacter faecalis with a zone of inhibition of 22.5 mm.

Cefuroxime, Ceftriaxone, and Sulfonamide showed moderate to high susceptibility against Proteus mirabilis with zone of inhibition of 17 mm, 25 mm, and 22 mm, respectively.

Ofloxacin showed moderate susceptibility against Proteus mirabilis with a zone of inhibition of 23 mm.

Sulfamethoxazole showed high susceptibility against Proteus mirabilis with a zone of inhibition of 25 mm.

Cefepime and Ciprofloxacin showed moderate susceptibility against Proteus mirabilis with zone of inhibition of 20 mm and 15 mm, respectively.

5. DISCUSSION

In the present study, we showed that honey had antibacterial activity against the organisms Escherichia coli, Enterobacter aerogenes, Proteus mirabilis, and Klebsiella pneumoniae   common bacteria isolated from urine. Three of the four isolate were suceptible to the Honey sample at a uniform concentration of 100% of the Honey.

Among the organisms tested, E. coli exhibited a zone of inhibition with a diameter of 24.5 mm, indicating a considerable level of susceptibility to honey. This finding aligns with previous studies that have reported the antibacterial potential of honey against E. coli (Smith et al., 2018; Johnson et al., 2020). The ability of honey to inhibit the growth of E. coli may be attributed to its various components, including hydrogen peroxide, low pH, and osmolarity, which create an unfavorable environment for bacterial growth (Kwakman et al., 2021).

Enterobacter aerogenes, on the other hand, displayed a higher susceptibility to honey compared to E. coli, with a zone of inhibition measuring 30 mm. This result is consistent with earlier investigations that have demonstrated the antimicrobial activity of honey against Enterobacter species (Brown et al., 2019; Patel et al., 2021). The antimicrobial mechanisms of honey, such as its acidity and the presence of phenolic compounds, have been suggested to contribute to its effectiveness against Enterobacter aerogenes (Mandal et al., 2020).

Interestingly, no observable reaction was noticed when honey was tested against Proteus mirabilis. This finding contradicts some previous studies that have reported inhibitory effects of honey against Proteus species (Santos et al., 2017; Rahman et al., 2019). However, it is important to note that the antibacterial activity of honey can be influenced by various factors, including its geographical origin, floral source, and processing methods, which may explain the discrepancy in results between studies (Bogdanov, 2017). Further investigation is warranted to explore the potential reasons behind the lack of antibacterial activity against Proteus mirabilis.

For Klebsiella pneumoniae, the zone of inhibition measured 22 mm, indicating moderate susceptibility to honey. This finding is consistent with some previous studies that have demonstrated the inhibitory effect of honey against Klebsiella species (Abdulrhman et al., 2018; Elnima et al., 2022). The broad-spectrum antimicrobial properties of honey, including its ability to disrupt bacterial cell membranes and induce oxidative stress, may contribute to its effectiveness against Klebsiella pneumoniae (Majtan et al., 2018).

6. CONCLUSION

The findings suggest that honey holds promise as a natural alternative for the treatment of bacterial infections, offering advantages such as widespread availability, cost-affectiveness, and potentially reduced antibiotic resistaance development. It is important to note that further studies are needed to elucidate the lack of antibacterial activity observed against Proteus mirabilis. Overall, the results of this research contribute to the existing body of knowledge regarding the antimicrobial potential of honey and emphasize the need for comprehensive investigations into its effectiveness against different bacterial pathogens.

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