RESEARCH ARTICLE

Antibacterial activity of honey (*Apis mellifera*) on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* isolated from wastewater

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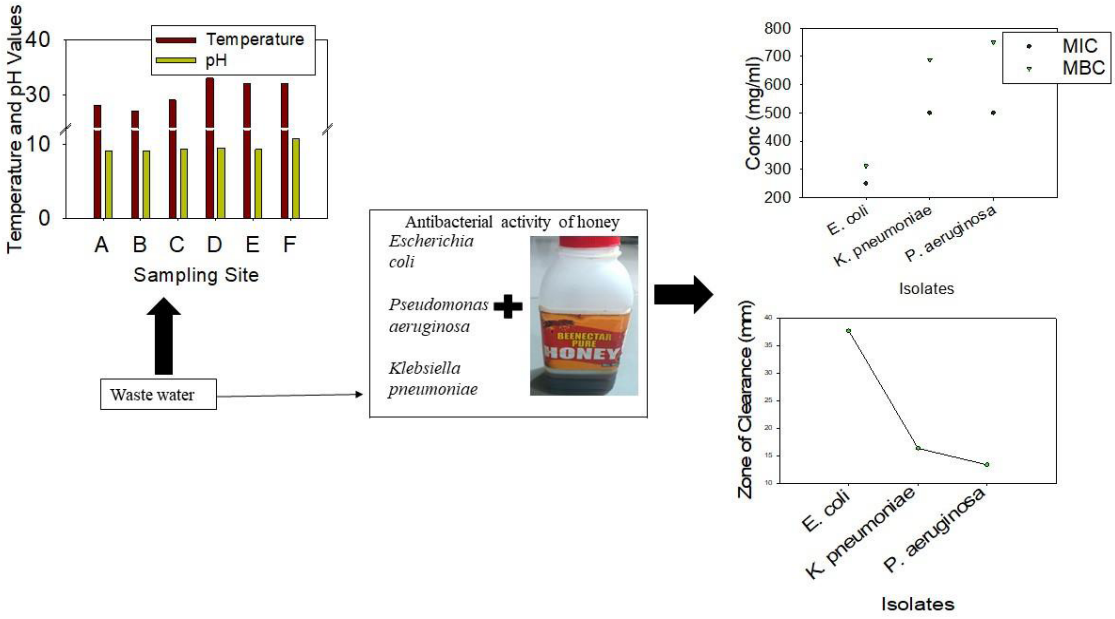

Highlights

- Wastewater samples were collected from damaged pipeline distribution system for drinking water for students within and outside the University campus.
- Three bacteria were isolated from the samples using selective media for the isolation.
- Locally produced honey sample from same source was used for antibacterial studies against the isolates.
- *Escherichia coli* and *Klebsiella pneumoniae* showed marked resistance to Amoxycillin, Chloramphenicol and Cetftriazone.
- *Escherichia coli* was most susceptible to honey which makes it a good promising agent.

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Antibacterial activity of honey (*Apis mellifera*) on *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* isolated from wastewater

T.O. Agbabiaka*, O.J. Awelogun, F.O. Otuyelu and T.O. Agbabiaka

Department of Microbiology, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Nigeria.

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Abstract: The use of honey as a remedy for microbial infections has been the reason behind recent researches on its antimicrobial activity. The research assessed the antibacterial activity of honey on *Eschericia coli, Pseudomonas aeruginosa and Klebsiella pneumoniae* isolated from environmental wastewater, using disc diffusion method at various concentrations of honey ranging from 62.5 - 1000 mg/ml while the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were determined using macro-dilution method. The zones of inhibition across the disc were measured after 24 hours of incubation. Results showed that honey has higher antibacterial activity on *E. coli* compared to other test isolates and also higher on *E. coli* than ciprofloxacin. Honey showed weaker activity on *K. pneumoniae* and *P. aeruginosa* compared to standard antibiotics. MIC was 250 mg/ml for *E. coli* while *K. pneumoniae* and *P. aeruginosa* were at 500 mg/ml. MBC for *E. coli, K. pneumoniae* and *P. aeruginosa* were observed at 312. 5 mg/ml, 687.5 mg/ml and 750 mg/ml respectively. Honey has promising antibacterial activity on infections caused by *E. coli, K. pneumoniae and P. aeruginosa* because of its antibacterial properties such as low pH, high osmolarity, and production of hydrogen peroxide.

Keywords: Disc diffusion, macrodilution, antibacterial activity, antibiotics resistance, *Escherichia coli.*

INTRODUCTION

Antimicrobial agents (antibiotics) are very essential in reducing the global burden of infectious diseases (Mandal and Mandal, 2011). With the wrong and massive use of antibiotics in underdeveloped and developing countries, resistant pathogens develop and spread. As a result, the effectiveness of antibiotics is diminished (Levy *et al.,* 2004). This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and all kinds of antibiotics including the major last-resort drugs, as the frequencies of resistance are increased worldwide (Mandal *et al.,* 2009).

Before antibiotic came into existence, it was not unusual for an experienced medical professional to even slather honey on a wound to prevent infection and hasten healing. Honey, well known as a magic drug for various diseases, contains various properties which are responsible for the antibacterial properties observed with its use. One of the modes of action of this agent includes high osmotic pressure because honey is said to draw water from other sources such as tissue or bacterial cells (Badge *et al.,* 2013).

Pseudomonas aeruginosa is one of the most common agent of infected burn injuries, community-acquired and ventilator-associated pneumonia, and is an important opportunistic pathogen in the healthcare system which cause nosocomial infection (Yetkin *et al*., 2006). *Escherichia coli*, commonly found in animal faeces, lower intestines of mammals can be classified into strains on the basis of different serotypes. A pathogenic strain *E. coli* O157:H7 is a well-studied strain of the bacterium *E. coli*, which produces Shiga-like toxins, causing severe diarrheal illnesses or disease (Atlanta, 2007). The treatment of *E. coli* infections is increasingly becoming difficult due to multi-drug resistance exhibited by the organism. Extended spectrum β-lactamase (ESBL) producing *E. coli* has spread as a major cause of hospital-acquired infections, as well as infections in outpatient settings (Oteo *et al.,* 2005). *Klebsiella pneumoniae* is common species of bacteria that cause problems in health care in recent time and can be responsible for community-acquired infections, but is most commonly observed as a major cause of hospital-acquired infections which can be fatal. *K. pneumoniae* has been observed to develop resistance to antibiotics more easily than most bacteria through the production of new enzymes to break them down (Qureshi, 2015). Resistance has been observed against beta-lactams, carbapenems, fluoroquinolones, aminoglycosides, trimethoprim, and sulfamethoxazoles. However, not all strains of *K. pneumoniae* express resistance (Kumar *et al*., 2011).

Antimicrobial resistance is most commonly associated with nosocomial infections. This is often due to the fact that hospitals are where the resistant strains tend to first develop. The development of resistance is most often due to the excessive use of antibiotics, sometimes unnecessarily and without monitoring or control (Harbath *et al*., 2015).

The study was to establish if there is any link between the odour and discomfort experienced in the use of water from the pipeline distribution system as a result of damage to some pipes along the distribution system during the rainy season and eventual erosion of soil around the distribution

**Corresponding Author's Email:* agbabiaka.to@unilorin.edu.ng
https://orcid.org/0000-0002-2908-7723

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network within the campus and outside the campus as experienced by students using such water. It also attempts to investigate if natural honey produced locally could have antibacterial effects on the isolated bacteria and establish the efficacy of honey on such bacteria.

MATERIALS AND METHODS

Sample collection

Wastewater samples were collected from different sources including "Lagos" boy's hostel (N 8.4812 E 4.6762) , "Zamfara" female's hostel (N 8.4801 E 4.6694), "Abuja" female's hostel (N 8.4839 E 4.6608), University of Ilorin, University Old Park premises, Oyin Folorunso Hospital and Maternity Tanke, Ilorin (N 8.4813 E 4.6115), and "Compound S", Tanke, Ilorin (N 8.4713 E 4.6312), Kwara State, Nigeria and were represented as A, B, C, D, E and F respectively. Samples were collected using sterile sampling bottle with fitted cap and represented as A, B, C, D and E respectively. Honey used in this study was obtained from University of Ilorin apiary.

Culture Media

Nutrient agar (NA) produced by Oxoid Ltd, UK was used for the enumeration of total bacteria in the samples, MacConkey agar (MA) by Oxoid, UK was used for the enumeration of coliform bacteria, HiCrome Klebsiella Agar (HKA) base by HiMedia Laboratories, India was used for the isolation of *Klebsiella pneumoniae*, Eosine Methylene Blue Agar (EMB) produced by Oxoid Ltd, Uk was used for the isolation of *Esherichia coli* while CM0559 Pseudomonas Agar Base (PAB) supplemented with CFC supplement was used for the isolation of *Pseudomonas aeruginosa.* Muller Hinton agar (MHA) produced by Oxoid Ltd, UK was used for antibacterial assay. Each of the medium was prepared according to manufacturer's instructions.

Determination of Physicochemical parameters of water

Temperature

A mercury-bulb thermometer calibrated in centigrade was inserted into a test tube containing some quantity of the sample and left for some time before reading its constant value. Duplicate readings were taken and the average of the temperature values of the water sample was obtained.

pH

The pH of each water sample was determined using the pH meter with glass electrode. The pH meter was first standardized using different pH values of 4, 7, and 9 in buffer solution. Fifty ml of each of the samples was introduced into test tubes. The standardized pH meter was inserted into the samples to obtain the pH. The determination was carried out in duplicates and the average values of the original water samples were obtained.

Microbiological analysis

Enumeration of microorganisms

Total bacterial counts of all samples were carried out using nutrient agar. One ml of each sample was serially diluted up to 10^{-6}. The last tube was plated for total bacterial count. Total coliform was carried out using MA, 1 ml of each sample was serially diluted up to 10^{-3}. The last tube was plated for total coliform count. *Escherichia coli* count was carried out using EMB, 1 ml of each sample was serially diluted up to 10^{-2}. The last tube was plated for *E. coli* count. *Pseudomonas* count was carried out using PAB, 1 ml of each sample was serially diluted up to 10^{-2}. The last tube was plated for *Pseudomonas* count. *Klebsiella* count was carried out using HKA, 1 ml of each sample was serially diluted up to 10^{-1}. The last tube was plated (Fawole and Oso, 2007).

Characterization and identification of bacterial isolates

Colonial features, morphological and biochemical tests were carried out to determine the species of the isolates using Bergey's manual (Breed *et al.,* 1957).

Determination of Antibacterial activity

Antibacterial activity of honey was tested using agar disc diffusion method against microorganisms (Bauer *et al*., 1966). About 100 µL of fresh culture suspension of the standardized test microorganisms adjusted to 0.5 McFarland standard (1×10^8 CFU/ml) was spread on Mueller Hinton agar plates. For screening, 5mm sterile diameter filter paper discs were impregnated with honey and plates were incubated under optimum conditions for 24 hours. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The zone of clearance was measured in millimeter and equivalent quantity of 10% DMSO was set up as a control, the plates were incubated for 24 h at 37°C. The experiment was repeated in triplicates for each isolate.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the honey sample was determined using broth dilution susceptibility test in test tubes (Akinyemi *et al.,* 2005). The honey samples were diluted to various concentration using 10% DMSO with only DMSO as the control. A stock solution 1000 µg/mL was prepared by dissolving 1000 mg extract added in 1 mL of DMSO. This was serially two-fold dilution using Mueller Hinton broth to obtain various ranges of concentrations between 62.5 - 500 µg/mL µg/mL. A volume of 100 µg/mL of standardized bacterial suspension was added to test tube containing a known quantity of the broth, and an additional tube containing broth only was used as a negative control. All the test tubes and control were incubated at 37°C for 18 – 24 hours. After the period of incubation, the tube containing the least concentration of extracts showing no visible turbidity was considered as MIC.

Minimum Bactericidal Concentration (MBC)

From the tubes showing no visible sign of growth/turbidity in MIC determination, about 0.5ml was inoculated onto sterile nutrient agar plates by streak plate method. The

lowest concentration of the agent that prevent the growth of less than 0.1% of the test organism on the recovery plate after incubation at 37°C for 24 hours was taken to be the MBC. (Akinyemi *et al.,* 2005).

RESULTS AND DISCUSSION

Determination of Physicochemical parameters

The mean temperature of samples ranged from 28 to 33.5 with sample D having the highest value while pH ranged from 9.1 to 10.8 with sample F having the highest value (Figure 1). The temperature values observed in this research is in accordance with Pavithra *et al.* (2017) who reported temperature values of different wastewater ranging from 25°C to 35°C. The temperature difference may be as a result of the collection time. The alkalinity of the samples may be as result of the activity in the waste water.

Enumeration of microorganisms

The results of microbial counts is as shown in Table 1.

Biochemical identification of bacterial isolates

Table 2 showed different biochemical tests carried out on isolates on selective and differential media. Probable organisms isolated includes *E. coli*, *K. pnuemoniae* and *P. aeruginosa.*

Antibacterial activity of Honey

In Figure 2, antibacterial activity of honey showed highest effectiveness on *Escherichia coli* with 37.67mm mean zone of inhibition while the least activity was observed on *Pseudomonas aeruginosa* with 13.33mm zone of inhibition. Alaa *et al.* (2015) reported the effect of different types of honey on *Pseudomonas aeruginosa,* it was observed that different honey showed different activities on test isolates while some showed no activity. Also, in support of this result was a study carried out by Salha *et al.* (2016) who reported highest antibacterial activity of honey on *E. coli* compared to other test isolates. The antibacterial activity of the honey has been attributed to its strong osmotic effect, moisture content and hydrogen peroxide as well as naturally low pH. This high acid values for local honey obtained in the study was also reported by Omojasola (2002).

Antimicrobial Effect of Standard Antibiotics on test isolates

Table 3 showed the result of selected antibiotics on the test isolates. Amoxicillin, Chloramphenicol and Ceftriazone showed no activity on both *E. coli* and *K. pneumoniae.* Highest activity was observed on Ciprofloxacin on all isolates, Streptomycin showed activity on *E. coli* only. In contrast to this result was a research cariied out by Osho and Bello (2010) who reported the effect of amoxicillin and chloramphenicol on selected isolates including *E. coli*, *K. pneumoniae* and *P. aeruginosa.* It was observed that both antibiotics showed high zone of clearance in the isolates. The resistivity of the isolates to these antibiotics may be as a result of mutation, overuse or underuse of antibiotics (Andersson and Hughes, 2010).

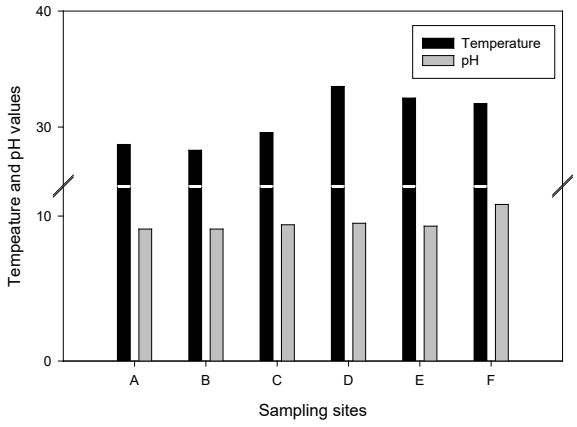

Figure 1: Temperature and pH values of wastewater samples

Table 1: Enumeration of different bacterial isolates (cfu/ml).

Samples	Total bacterial count	Total coliform count	*E. coli* count	*Pseudomonas* count	*Klebsiella* count
A	7.2×10^7	2.1×10^4	0	3.1×10^3	1.3×10^2
B	5.6×10^7	1.4×10^4	8.0×10^2	2.2×10^3	0
C	2.9×10^7	2.8×10^4	1.3×10^3	1.5×10^3	5.0×10^1
D	6.3×10^7	4.6×10^4	6.0×10^2	2.8×10^3	0
E	3.4×10^7	1.9×10^4	0	1.9×10^3	0

Table 2: Biochemical characterization of bacterial isolates

ISOLATES	Catalase	Oxidase	Coagulase	Starch	Methyl red	Voges Proskaeur	Indole	Urease	Citrate	Lactose	Sucrose	Glucose	Fructose	Probable Identity of Isolates
A	+	-	-	+	+	-	+	-	-	AG	AG	AG	AG	*Escherichia coli*
B	+	-	-	-	-	+	-	+	+	AG	AG	A	A	*Klebsiella pneumoniae*
C	+	+	-	+	+	+	-	-	+	-	-	-	-	*Pseudomonas aeruginosa*

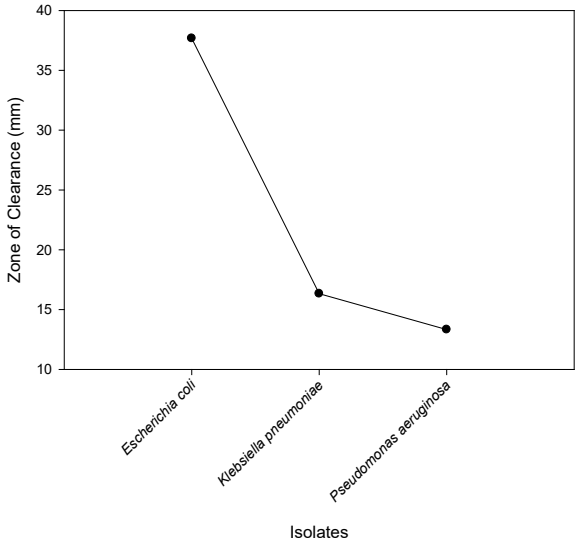

Figure 2: Antibacterial activity of Honey (*Apis mellifera*) on isolates.

Table 3: Antibiotic Susceptibility pattern of test isolates.

Concentration (mg)	Zone of inhibition (Mean ± SEM) (mm)		
	E. coli	***K. pneumoniae***	***P. aeruginosa***
Amoxicillin	0.00	0.00	11.67 ± 0.67
Ofloxacin	23.00 ± 0.57	21.67 ± 0.33	10.33 ± 0.33
Streptomycin	20.67 ± 0.33	0.00	0.00
Chloramphenicol	0.00	0.00	24.33 ± 0.33
Ceftriazone	0.00	0.00	19.00 ± 0.57
Gentamycin	13.33 ± 0.33	16.00 ± 0.57	9.67 ± 0.33
Ciprofloxacin	25.00 ± 0.57	29.33 ± 0.33	27.00 ± 0.57

Table 4: Minimum inhibitory concentration of Honey (*Apis mellifera*) on test organisms.

Test Isolates	Concentrations (mg/ml)					
	62.5	**125**	**250**	**500**	**1000**	**Control**
E. coli	G	G	NG	NG	NG	G
K. pneumoniae	G	G	G	NG	NG	G
P. aeruginosa	G	G	G	NG	NG	G

Key word: G- growth NG: no growth

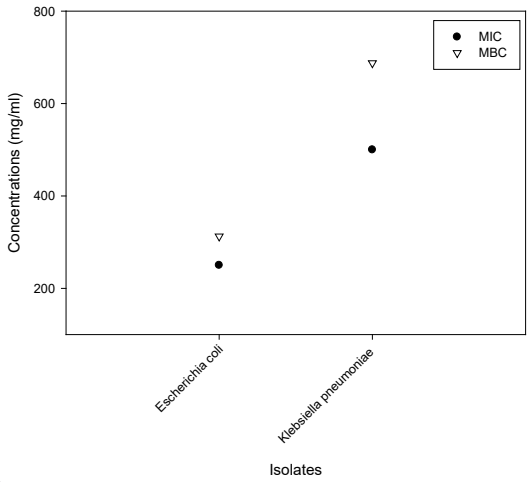

Figure 3: Minimum bactericidal concentration of Honey (*Apis mellifera*) on test organisms.

MIC and MBC of Honey (*Apis mellifera*) on test organisms

At concentrations 500 and 1000 (mg/ml), no growth was observed in all tubes as all tubes appeared clear. Only *E. coli* showed no growth at concentration 250 mg/ml as shown in Table 4. The minimum inhibitory concentration of *Apis mellifera* on *E. coli*, *K. pneumoniae* and *P. aeruginosa* were 250 mg/ml, 500 mg/ml and 500 mg/ml while the minimum bactericidal concentrations (Figure 3) were 312.5 mg/ml, 687.5 mg/ml and 750 mg/ml respectively. According to Mohapatra *et al.* (2011), it was reported that honey showed minimum inhibitory concentration at low concentrations on *E. coli* compared to other test isolates. Also supporting this result was a research carried out by Chauhan *et al.* (2010) where it was reported that *E. coli* was the most susceptible at lower concentration of honey compared to other test isolates including *P. aeruginosa.*

Comparison of the efficacy of honey to standard antibiotics

It was observed that *E. coli* was more susceptible to honey with 37.67mm mean zone of inhibition while the highest mean zone of inhibition for antibiotics was observed on ciprofloxacin. This observation agrees with Salha *et al.* (2016) as it is resistant to amoxycillin and has little susceptibility to gentamycin. Also, *P. aeruginosa* was observed to be susceptible to most antibiotics tested and little activity shown when tested against honey. *K. pneumoniae* was found to be resistant to several antibiotics, although high zone of inhibition was observed for ciprofloxacin while little activity was observed for honey. This observation agrees with Shah *et al.* (2015) where *K. pneumoniae* was susceptible to honey sample but showed resistance against almost all the antibiotics tested.

CONCLUSION

This study shows that honey has promising antibacterial activity against *Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa* which are the causative agents of commonly encountered infections including hospital- acquired infections, traveler's diarrhoea, pneumonia as well as wound infections. Therefore, there is need to characterize the active components of honey extracts and encourage investigations to the possible benefits of the use of honey among therapies in the treatment of bacterial infections.

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DECLARATION OF CONFLICT OF INTEREST

The authors have no conflict of interest.

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