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Investigation of The Antibacterial Efficacy of Honey Sourced from Different Markets in Abakaliki on Selected Clinical Isolates from Alex Ekwueme Federal Teaching Hospital Abakaliki, Ebonyi State. Southeastern, Nigeria.

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Abstract

The effectiveness of antibacterial agents has been essentially important in reducing the global burden of bacterial-caused infectious diseases. The efficacy of honey on pathogens of clinical importance was investingated in this study using clinical isolates of *Pseudomonas aeruginosa*, *Escherichia. coli*, *Staphylococcus aureus* and Vibrio spp. Designated differential media were used to differentiate the growth of these bacteria. Biochemical tests were also done to reconfirm the identities of these isolates. The sensitivity of the test organisms to honey was carried out using the agar well diffusion method while antibiotics efficacy was checked using the Kirby Bauer disc diffusion method. Results showed that at l00% concentration, honey has a better antibacterial effect than the standard antibiotics, with greater zones of inhibition Zone Diameter for both honey against test organisms viz: *P. aeruginosa* (10 & 16 mm), E. coli (3I & 32 mm), *S. aureus* (29- 30 mm) and Vibrio spp (l6 & 28) mm. The honey samples proved to have antibacterial effects against the isolates, which were greater than the control. Gentamicin (10ug) showed zones of inhibition ranging from 19 to 22 mm and while that of Amoxiclav (10ug) ranged from 0 to 16. Phytochemical analysis showed the presence of alkaloids, saponins, flavonoids, terpenoids, tannins, and cardic glycosides in both honey samples while phenol was ab sent. These phytochemicals may be responsible for the antibacterial activities of honey. Honey B at 25 % v/v was able to inhibit some of the isolates while honey A was able to cause inhibition of the isolates at 50% v/v concentration. This study proved that both honey samples from Abakaliki possessed antibacterial efficacy against the clinical isolates that were studied.

Keywords: Honey, Efficacy, Inhibition, Sensitivity, Pathogens, Antibacterial

1.Introduction

Antimicrobial agents are organic or inorganic substances or even substrates whose activities kill or inhibit the activities of these microbes. An example of such a substance is an antibiotic that inhibits the growth of bacterial. The effectiveness of these substances has been essentially important in reducing the global burden of bacteria-caused infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics (inorganic) is diminished. This bacterial resistance to the antimicrobial agents including the major last-resort drugs, poses a very serious threat to public health. Bacteria become resistant to antibiotics when they are exposed to them, which is a natural occurrence. This situation is amplified and accelerated by the inappropriate and overuse of antibiotics.

Currently, there are bacteria that are able to resist almost all, or even all the approved antimicrobial agents for their treatment. Consequently, some common infections have become very difficult to treat. Alexander Fleming discovered Penicillin as the first natural antibiotic. The frequencies of resistance are also increasing worldwide (Levy and Marshall, 2004; Mandal, *et al*., 2009). Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as naturally occurring substances like plants and plant-based products (organic), including honey which is the focus of this research (Mandal, *et al*., a and b 2010).

Honey composes of a complex mixture of carbohydrates (mainly fructose and glucose) and other minor substances such as organic acids, proteins, minerals, amino acids, pollen grains, vitamins, waxes, and lipids (Sanz *et al*., 2004; Saxena *et al*., 2010). In most ancient cultures, honey has been used for both nutritional and medicinal purposes. Apitherapy is an alternative medicine developed in recent years, which offers treatments using honey and other bee products against many diseases. The use of traditional medicine to treat infection has been practiced since the origin of mankind, and honey a metabolic product of Bee (*Apis mellifera*) is one of the oldest traditional medicines which is used in the treatment of several human ailments. Several researchers have reported the antibacterial activity of honey and discovered that natural unheated honey has some broad-spectrum of antibacterial activity when tested against pathogenic bacteria and food spoilage bacteria (Lusby, *et al.*, 2005; Mundo, *et al*., 2004).

Phytochemicals are found in plant-based foods and are known to have protective or disease-preventive properties acting as antioxidants, antimicrobial, anti-inflammatory, antitumor, and anticancer effects amongst others (Harborne, 1973). The medicinal properties of honey lie in some chemical substances that produce a definite physiological action on the human body. The knowledge of the chemical constituents of honey would be valuable in discovering the actual value of folklore remedies. Numerous phytochemicals, such as polyphenols, which function as antioxidants, can be found in honey. These phytochemicals have been reported to vary according to geographical and climatic conditions (Khali *et al*., 2010). Antioxidants are abundant in raw honey. Honey contains, in addition to flavonoids, other antioxidants such as catalase and ascorbic acid. Ferreire *et al.* (1992) reported that there have been 33 flavonoids identified in honey with concentrations ranging from 0.005 to 0.01%. Recently, there has been increasing interest in the determination of the antioxidant activity ofhoney. The present research work was aimed to determine the antibacterial efficacy of honey sourced from Abakaliki (a town in Ebonyi State of Nigeria) on selected clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and Vibrio spp.

2. Materials and methods

2.1. Sample Collection

Pure clinical bacteria stock cultures were collected from Alex Ekwueme Federal University Teaching Hospital, Abakaliki, Department of the Research laboratory. The isolates were subcultured into the suspected organism's differential mediums. Viz: *S. aureus* was cultured on Manitol salt agar, *P. aeruginosa* culture was sub-cultured on centrimide agar, *E. coli* on Eosin Methylene Blue agar (EMB) and Vibrio Spp was cultured on Thiosulphate Citrate Bile Salt agar (TCBS). This sub-culturing served as the first step to confirm that the right organisms were collected from the medical laboratory. The cultures were placed in the incubator for 24hrs at 37°C. After which pure cultures of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Vibrio cholera* were stored on nutrient agar slant in the Mac-Cartney bottles and kept in the refrigerator at 40℃ for future use.

2.2. Sources of pure honey

Two samples of honey were purchased from shops within the Abakaliki metropolis and labeled honey A and B. The honey samples were stored at room temperature for three weeks. The samples were taken to the microbiology Laboratory, Department of Microbiology, Alex-Ekwueme Federal University, Ndufu-Alike, Ikwo for further analysis.

2 .3.Gram staining and biochemical tests

The Gram was carried out following the procedures and steps of Faurie (2019). The staining was followed by the use of various biochemical tests to get closer to the identification of bacteria. There are numerous biochemical assays available to identify microorganisms. Depending on the bacterium, only a few of them must be done. The biochemical tests used for characterization are as mentioned. Catalase test, Coagulase test, Oxidase test, Lactose fermentation test, Indole test, Citrate test, Urease test, methyl red test and Voges-proskauer test.

2.4. Salt tolerance test (using sagar aryala method).

To demonstrate the ability of such organisms in the laboratory, broth containing 3% to 6% Nacl is used as the test medium. Organisms capable of growing in the high salinity medium will cause turbidity in the tube indicating a salt tolerance of the organism and its ability to survive and grow in a high salinity environment. I. inoculated one colony of the isolate from a 24-hours culture into 6% Nalco peptone water. I incubated the tube at 37°C for 48 hours. After which the tube was examined for turbidity after 24 hours and if negative again at 48 and 72 hours.

2.5. Antibacterial susceptibility test of pure honey

Using the Kirby-Bauer Agar well diffusion method, Muller Hinton agar (MHA) was prepared and sterilized at 121 ^{o}C, allowed to cool to around 55^{o}C, and poured into sterile plates. To prepare the inoculum 2-3 colonies of the test organism were emulsified with sterile normal saline and matching the turbidity with the standard (0.5 McFarland). The swab stick was used to streak evenly over the surface of the MHA to obtain uniform growth. Using a cork borer to make wells of uniform diameters on the MHA. Then used a dropper to collect the honey and filled the wells, then with the forceps the control antibiotics disks were placed on the media surface, and incubation of the plates was done at 37°C for 24 hours. Following overnight incubation, I measured the diameter of the zone of inhibition in millimeters (mm) around each honey well and the antibiotic discs with a ruler.

2.6. Phytochemical analysis

2.6.1. Qualitative Phytochemical Testing of the Honeys

2.6.1.1.Test for saponins (Frothing Test):

For quantitative analysis, 2ml of pure honey was dissolved in 10ml of distilled water. This was shaken vigorously for 30 seconds and allowed to stand for 30 minutes.A honeycomb color formed for not more than 30 minutes indicated the presence of saponins (Trease and Evans, 2002).

2.6.1.12.Test for flavonoids Sodium Hydroxide:

About 2ml of honey was dissolved in 10ml of water and filtered. 2 ml of the l0% aqueous sodium hydroxide was added to the honey to produce a yellow coloration. A change in color from yell.ow to colorless on the addition of diluted hydrochloric acid indicated the presence of flavonoids (Trease and Evans. 2002).

2.6.1.1Test for alkaloids (Wagner's and Mayer's Test):

About 2ml of each honey sample was stirred with 10ml of 1% HCl on a steam bath and was filtered. The reagents were added to the filtrate and were checked for the appearance of a brownish-coloured precipitate that indicated the presence of alkaloids.

2.6.1.1Test for tannins:

About 2ml of the honey samples each were stirred with 2 ml of distilled water and a few drops of 1% FeCl3 solution was added. The formation of a blue-black, green or blue-green precipitate indicated the presence of tannins (Trease and Evans, 2002).

2.6.1.1Test for terpenoids:

About Iml of the honey samples each was dissolved in ethanol. Iml of acetic anhydride was added to each followed by the addition of conc. H2S04. A change in color from pink to violet indicated the presence of terpenoids (Sofowora, 1993).

2.6.1.1Test for cardiac glycosides (Kella-Killiani Test):

About I ml of each honey sample was dissolved in a glacial acetic test tube and held at an angle of 45 0 Celsius, Iml of concentrated sulphuric acid was added down the side. A purple ring color at the interface indicated the presence of cardiac glycosides (Trease and Evans. 2002).

2.6.1.1Test for steroids:

About 2ml of the honey samples each was dissolved in 2 ml of chloroform and 2 ml concentrated Sulphuric acid. A red color produced in the lower chloroform layer indicated the presence of steroids (Trease and Evans. 2002).

2.7.Quantitative phytochemical analysis

The quantitative amounts of phytochemicals, which were found in the honey samples were determined using the standard procedure as described by (Obadoni and Ochuko, 2001; Trease and Evans, 2002; Amakura *et al.* 2009).

2.7. Minimum inhibitory concentration (mic) and minimum bacteriocial concentration of pure honey

To determine the lowest concentration of honey that prevents visible growth of the test organisms the method of Koneman's atlas of Microbiology 7th ed, and Grego *et al*., (2016) were adopted.

The minimum inhibitory concentration (MIC) for the honey was determined by the tube dilution method at concentrations of 100%, 50%, 25% (v/v). In a sterile test tube, 1ml of each concentration of honey and 1ml of Muller Hilton broth were added, sterilized and cooled to 55^{o}C). Their turbidity was observed and noted before the isolate was introduced using a sterilized wire loop and shaken for proper homogeneity of the mixture and the turbidity was noted again. The tubes were incubated for 24hrs at 37^{o}C. After the period of incubation, the test tubes were observed for change in their turbidity in other to determine the MlC of the honey at the different dilutions. The lowest concentration of the honey samples that inhibited bacterial growth was recorded as the MIC. From the tubes showing no visible sign of growth/turbidity in MIC determination, the isolates were inoculated onto nutrient agar plates by streak plate method and incubated for another at 37°C. The least concentration that did not show growth of the isolate after incubation for 24 hours was considered as Minimum Bactericidal Concentration (CLSI, 2014; Grego *et al*., 2016).

3. Results

3.1. Colony description of the isolates on the various mediums.

(*Staphylococcus aureus*) on Mannitol salt agar: yellow colonies with yellow zones, round convex with a diameter of about 1-3mm. (*Pseudomonas aeruginosa*) on Mac-Conkey Agar: flat and smooth colonies, with a diameter of about 2-3 mm. (Vibrio spp) on TCBS agar: yellow, slightly flattened colonies of about 2-4mm in diameter. (E coli) on EMB agar: raised and mucoid pinkish colonies with a diameter size of about 2-3mm. with the colonies appearing to join each other in a confluent manner.

3.2.Biochemical test results

Table1: Showing the biochemical reactions of the isolates

Isolates tests	*E. coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Vibrio* spp.
Catalase	–	+	+	-
Citrate	+	–	+	+
Coagulase	–	+	+	-
Oxidase	–	–	+	+
Indole	+	–	–	+
Urease	–	-	–	+
Salt tolerance	-	+	-	+
Lactose	+	+	-	
Methyl red	+	+	-	-
Mannitol salt	+	+	-	+

Keys. (–) stands for a negative biochemical test result**,** (+) stands for positive biochemical test results. The Keys represent the same information in Tables 1 and 6 respectively.

Table 2: Antibacterial Activity of Abakaliki Honey A and B against Standard Antibiotics on the Pathogens.

	Zones of Inhibition (mm)			
Organisms	**Undiluted Honey A**	**Undiluted Honey B**	**Gentamicin (10ug)**	**Amoxiclav (10ug)**
E. coli	31(S)	32(S)	21(S)	16(I)
Vibrio spp	16(I)	28(S)	19(S)	00(R)
Staphylococcus aureus	29(S)	30(S)	22(S)	00(R)

Table 3: Qualitative Analysis of Phytochemical of the Two Honey Samples

Phytochemical	Honey A (%)	Honey Bz(%)
Alkaloids	0.32, ±0.03	0.38, ± 0.01
Saponins	4.54 ± 0.15	3.89, ± 0.01
Flavonoids	0.38, ± 0.01	0.32, ± 0.02
Terpenoids	0.26, ±0.02	0.38, ± 0.03
Tannins	0.32, ±0.02	0.34, ± 0.04
Phenols	Absent	Absent
Cardiac glycosides	0.35, ±0.03	0.39, ± 0.07

Table 4: Minimum Inhibitory Concentration of Different Concentrations of Honey

Organisms	Dilutions of Honey A %(v/v)	Dilutions of Honey B % (v/v)
E. coli	100 no growth	100 no growth
	50 no growth[a]	50 no growth
	25 growth	25 no growth[b]
Pseudomonas aeruginosa	100 no growth	100 no growth
	50 no growth[a]	50 no growth
	25 growth	25 no growth[b]
Vibrio spp	100 no growth[a]	100 no growth
	50 growth	50 no growth[b]
	25 growth	25 growth
Staphylococcus aureus	100 no growth[a]	100 no growth
	50 growth	50 no growth[b]
	25 growth	25 growth

[a] = *Minimum inhibitory concentration of honey A against Escherichia coli, Staphylococcus aureus, Vibrio cholera, and Pseudomonas aeruginosa.*

[b] = *Minimum inhibitory concentration of honey B against Escherichia coli, Staphylococcus aureus, Vibrio cholera, and Pseudomonas aeruginosa.*

4.. Discussion

The study was carried out to assess the antibacterial activity of honey from different locations in Abakaliki Local Government Area of Ebonyí State, on some pathogenic microorganisms (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Vibrio* spp) collected from the hospital. The study confirmed the antibacterial efficacy of honey against the clinical bacterial species. Results obtained showed that honey A had a minimum inhibitory concentration of 50% v/v against *E. coli* and *P. aeruginosa* while Abakaliki honey B had a Minimum inhibitory concentration of 25% v/v against *P. aeruginosa*, and 50 % against *E .coli*, *S.aureus*, and *Vibrio* spp. These findings agree with an MIC of 25 % and 50% for Nigeria honey and Manuka honey respectively against clinically isolated ESBL producing *K. pneumoniae* and non- ESBL producing *K .pneumoniae* in the report of Tahir and Afegbua, (2020). These results also agree with the

findings of (Kwakman and Zaat, 2012) for red honey and white honey respectively against clinical isolates of *K. peumoniae* but disagree with the findings of (Usanga *et al*., 2020) whose lowest MIC was

20% v/v of natural honey from Nkalagu and Ishielu LGA. These differences in MIC could be attributed to the floral source, location, and storage of the honey which affects the phytochemical characteristics of honey.These phytochemicals are the major components for antibacterial effects of honey, according to previous reports (Mandal and Mandal, 2011), Kwakman and Zaat, (2012) Nwankwo *et al*., (2014).

In this study, honey was able to inhibit the growth of the isolated bacterial species and the inhibition was found to be positively increasing with the concentration of the honey as an increase in the concentration of the honey sample showed a corresponding increase in its inhibitory potency as referred to in table 4 respectively. This result corresponds to the findings of Usanga *et al*., (2020), who carried out a similar study on wound isolates of *E .coli*, *S. pyogenes*, and *S .aureus* using a honey sample from Nkalagu and Ishielu LGA both in Ebonyi state. This property of honey may be arrogated to the high osmotic pressure exerted by the sugar content (Molan and Cooper, 2000). This study disagrees with the findings of Usanga *et al*., (2020) and Farrag *et al*., (2018) both of whom reported *P. aeruginosa* and *S. aureus* as the isolates most susceptible to honey at the highest concentrations respectively whereas *E. coli* showed the highest susceptibility to Honey B at the highest concentration. This is likely related to the source of pathogens as *P. aeruginosa* that was reported by Usanga *et al*., (2020), was isolated from an open wound; *S. aureus* that was reported by Farrag *et al*.,(2018) was isolated from a urine sample while *E.coli* used in this study was isolated from the stool of diarrhea patient. Duration of isolate storage may also be a factor in these differences in results as some isolates may have been stored for a longer time than the other thereby causing a difference in the organism's phase of growth and interaction to antibacterial.

Phytochemical components of honey such as sugars, saponins, alkaloids, cardiac glycosides, flavonoids, and phenols possess therapeutic properties against several pathogens (Bogdanov *et al*., 2004, Phytochemical analysis results of the honey analyzed revealed the presence of flavonoids, saponins, alkaloids, cardiac glycosides. Terpenoids, tannins, in the honey samples except phenol, which tested negative for both honeys (table 6), shows the phytochemicals and the mean values obtained with honey A having higher Saponin content with ±4.54, ± 4.49, and ±3.89, ±3.86, for honey B, honey B has higher cardiac glucose of ±0.39, ±0.46, while honey A ±0.35, ±0.38 respectively, and this phytochemicals according Bogdanov, *et al*., (2004), contributed to the antibacterial effect of these honey on the selected isolates. The absence of phenols in this honey compared to the result of (Tahir and Afegbua, 2020), who reported the presence of phenol in Nigerian honey compared to Manuka honey that also had phenol present, the difference in phytochemical content could be as a result of floral source of the honey being used in both cases

Conclusion

This study has confirmed the antibacterial efficacy of honey sourced from Abakaliki against pathogenic bacteria species of medical importance as an encouraging relief to the condition of antibiotic resistance encountered by the constantly rising number of resistance patterns of the predominant isolates; *S. aureus*, *Escherichia Coli*, and *Pseudomonas aeruginosa* were found to positively respond to honey with varying concentration. Therefore, the use of honey as a non-toxic and cheap natural antibacterial agent and alternative should be globally embraced after being

subjected to proper and consistent pharmaceutical standardization and further clinical trials on pathogens from environmental sources which often constitute clinical importance

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