Phytochemical Screening and Antibacterial Efficacy of Apiary Honey on some bacteria isolated from Diabetic Foot Ulcer

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Abstract: Diabetic wounds unlike typical wounds are slower to heal, making treatment with conventional topical medications an uphill process. Among several different alternative therapies, honey is an effective choice because it provides comparatively rapid wound healing. The apiary honey samples used in this study were purchased from Umudike research Institute Umuahia, in Abia State, Southeastern Nigeria. Phytochemical screening, antibacterial susceptibility test, minimum inhibitory concentration and minimum bactericidal concentration were performed using standard methods. Phytochemical analysis revealed the presence of reducing sugar, saponins, glycosides, alkaloids and flavonoids and absence of Terpenoids, Phlobatanins, phenols and tannins. Antibacterial activity of apiary honey on some medically important bacteria including *Staphylococcus aureus, Escherichia coli, Klebsiella* spp, *Bacillus subtilis, Proteus vulgaris* and *Pseudomonas aeruginosa* shows that apiary honey exhibit strong antibacterial activity producing zones of inhibition against the tested bacteria. Also, honey sample used in this study showed antibacterial activity than the commercially available antibiotics, both of which were of the same concentration of 2.5ml. The results from this study shows that apiary honey, apart from its roles as food and supplements, could be used as an antibacterial agent, as they may be an excellent alternative to curtail the further spreading of drug resistant bacteria in Nigeria.
Keywords: Antibacterial susceptibility, Diabetic wounds, Apiary Honey, Phytochemical screening.

INTRODUCTION

Honey is a thick, sweet liquid made by bees from the nectar of flowers. It contains water, glucose, fructose proteins, vitamins and minerals (Al-Waili, 2004). It is also defined as the natural sweet substance produced by honeybees from the nectar of blossoms or from the secretions of living parts of plants or excretions of plants sucking insects on the living parts of plants, which honeybees collect, transform and combine with specific substances of their own, store and leave in the honeycomb to ripe and mature (Codex, 2001).

There are basically two types of honey, apiary and forest honeys. Honey produced by the honeybees, *Apis ceranaindica* and *Apis mellifera*, in apiaries and collected by modern extraction method are called apiary honey. They are transparent and free from foreign materials. In contrast, those produced by rock bee, *Apis dorsata*, or by wild nest of *Apis ceranaindica* in forests and collected by the crude method of squeezing the comb are known as forest honeys (Subrahamanyam, 2007).

They are turbid owing to the abundance of pollen, wax, brood (bee larvae), parts of bees and plant materials. It is therefore necessary to filter the honey to separate the suspended particles (Subrahamanyam, 2007). Honey primarily contains sugar and water. Sugar accounts for 95-99% of honey dry matter, majority of these are simple sugars, fructose (38.2%) and glucose (31.3%), which represents 85-95% of total sugars. These are "simple" sugars, 6-carbon sugars that are readily absorbed by the body (Peter *et al.,* 2007). Other sugars include disaccharide such as maltose, sucrose and iso-maltose, few oligosaccharides are also present. Water is the second most important component of honey. Its content is critical, since it affects the storage of honey (Peter *et al.,* 2007). The final water content depends on numerous environmental factors during production such as weather and humidity inside the hives, but also on nectar conditions and treatment of honey during extraction and storage (Peter *et al.,* 2007).

The colour of honey can vary from nearly colourless to dark brown and its consistency can be fluid, viscous or partly to entirely crystallized.

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The nectar source visited by the honey bees leads to variation in colours, flavors and aroma (Codex Standard for Honey, 1996). Honey is characteristically acidic with pH of between 3.2 and 4.5, which is low enough to be inhibitory to many animal pathogens (Allen *et al.,* 1991). The multi facet properties of honey anchored in the scientific world is regarded as a sweetener, functional food, antioxidant, antimicrobial, antiseptic, pre-biotic probiotic, immunomodulatory, anti-inflammatory, anti-tumor and anti-cancer effect amongst others (Conway *et al.,* 2010; Fauzi, *et al.,* 2011; Giorgi *et al.,* 2011; Jenkins *et al.,* 2011). Honey is gaining acceptance as an agent for the treatment of ulcers, bed sores and other skin infections resulting from burns and wounds (Mohapatra *et al.,* 2011; Cooper *et al.,* 2012). Moreover, it can be used on skin grafts and infected skin graft donor sites successfully (Misirliogu and Eroglu, 2003). The healing properties of honey can be ascribed to the fact that it offers antimicrobial activity, maintain a moist wound environment that promotes healing and has a high viscosity which helps to provide a protective barrier to prevent infection (Lusby *et al.,* 2005).

There is increased development of resistance to every antibiotic introduced in clinical practice (Payne *et al.,* 2007). Wound infections caused by drug-resistant organisms are common and lead to increased costs, morbidity and mortality. There is an urgent need for the discovery of new antibiotics with novel modes of action. Honey has been utilized as a wound care product and its usage as a wound healing agent is reported in the treatment of venous leg ulcers (Gethin, and Cowman, 2008; Jull *et al.,* 2008), burns (Subramanyam, 1993), chronic leg ulcers (Oluwatosin *et al*., 2000), pressure ulcers (Weheida *et al.,* 1991) and exit sites of central venous catheters. There has been growing interest by Nigerian health care professionals in wound-care products based on use of honey in treating diabetic foot ulcer patients (Molan and Betts, 2004). In Nigeria, local honeys have been used in homes to treat diabetic foot ulcers (DFU) which have failed to heal by conventional therapeutic methods. This suggests that honeys may confer antimicrobial activity against the species infecting DFU and this inference was tested in this study. Infected foot ulcers are a common cause of morbidity in diabetic patients leading to complications like gangrene and amputations. In Nigeria, one in every five people has diabetes and the incidence of foot problems and amputations remains high (Anyanwu, 2011). Full thickness penetration of the dermis of the foot of diabetic people allows colonization of microbial species and initiates a complex series of reactions which leads to transient wound contamination or clinical infection. The initial microbial burden is low in DFU; however lack of proper care promotes microbial density and diversity. Most of the DFU are polymicrobial in nature (Brodsky and Schneider, 1991; Ramani, *et al.,* 1991).

The present study was therefore carried out to screen the Phytochemical components and antibacterial efficacy of apiary honey on some bacteria isolated from diabetic foot ulcer patients.

MATERIALS AND METHODS

Sample Collection and Mode of Identification of Pure/Original Honey

The honey samples used in this study were purchased from Umudike research Institute Umuahia, in Abia State, Southeastern Nigeria. Several experiments were conducted to ascertain that the honey samples were pure and original using the methods of (Mohapatra *et al.,* 2011). These include:

I. Dropping some of the sample onto sand: if it is a pure honey, it will not sink immediately.

II. Pouring a small quantity into a cup of water: if pure, it will go down to the bottom of the cup without mixing up with the water except when stirred.

III. Dipping a finger into the honey sample, dropping one or two drops on the ground: if it is pure, it will go down like a thread without breaking.

IV. Dipping a match stick into the honey and striking it: the matches will burn if its pure honey and the honey will even act as fuel while the match is burning (Mohapatra *et al.,* 2011).

The samples were then collected in sterile screw-caped container and were kept in a dark, cool and dry place (at room temperature) overnight before they were finally transported to the laboratory for processing.

Processing of Honey Samples

This was done using the methods of (Mohapatra *et al.,* 2011). Each sample was first filtered with a sterile mesh to remove debris; viscosity was reduced by heating honey at 30°C for 30 minutes.

The samples were checked for sterility by inoculating on blood agar plates and incubated overnight. Uncontaminated samples were stored at refrigeration temperature of about 4°C until used (Mohapatra *et al.,* 2011).

Study Area

This study was conducted at Braith-whyte Memorial Hospital , Port-Harcourt, Rivers State.

Sample collection

A total of one hundred and fifty (150) swabs were obtained from diabetic foot ulcer patients in the hospital and the subject's age were between 20 – 79 years.

Sources of Bacteria

The bacteria used in this study were isolated from swabs obtained from diabetic foot ulcer from Braithwhyte memorial Hospital Port-Harcourt, Rivers State, Nigeria. They include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella* spp, *Bacillus subtilis* and *Proteus vulgaris*.

Biochemical tests such as Gram stain, catalase, oxidase, indole, coagulase, methyl red, Vogues Proskauer, citrate, nitrate, H_2S, lactose, glucose, xylose, fructose sucrose and lactophenol test were carried out to confirm the identity of the organisms using standard laboratory techniques (Gaill and Jon, 1995). These organisms were maintained in agar slants at 4°C until used.

Phytochemical Screening

The sample was screened for the following compounds: reducing sugar, saponins, glycosides, alkaloids, phenols, flavonoids and tannins using standard laboratory techniques (Gaill and Jon, 1995).

Antibacterial Susceptibility Testing

The antibacterial activity of apiary honey in comparison with standard antibiotic gentamicin (80mg/ml) *in vitro* on the isolates was determined by the agar well diffusion method as described by (Osho and Bello, 2010; Olakunle *et al.,* 2013). Gentamicin was used as it belongs to a class of aminoglycoside antibiotics which have bactericidal effect on bacteria. Pure culture of micro-organisms was grown on nutrient agar. Three colonies of each organism were picked using an inoculation loop into the Mueller Hinton broth (Oxoid, England) incubated for 4 hours at 37°C, diluted with sterile saline to a density visually equivalent to 10^6 cfu/ml, which corresponded to MacFarland standard. Using a sterile 6 mm diameter cock borer, four wells were cut in the agar to which 2.5ml concentrations of honey were added, as well as the standard drug, gentamicin (2.5ml) separately, which served as the controls. The plates were incubated at 37°C for 48 hours. The zones of inhibition were measured with the use of a metric rule.

Determination of Minimum Inhibitory Concentration (MIC)

The apiary honey was used to determine the MIC of the honey against the bacteria in broth culture. The MIC is the lowest concentration of the sample that is able to inhibit any visible bacterial growth in the culture tube (Nwankwo *et al.,* 2014). The following concentrations of the honey samples 1, 2.5, 7.5, 10, 15, 20 and 30%v/v corresponding to the following volumes 0.1, 0.25, 0.75, 1.0, 1.5, 2.0, and 3.0ml were made in a test tube. To prepare 30%, 3ml of stock honey was diluted in 7ml of the sterile nutrient broth making 10ml solution; and to prepare 20%, 2ml of stock honey was diluted in 8ml of sterile nutrient broth making 10ml solution. They tubes were examined for visible growth after 24hours incubation.

Determination of Minimum Bactericidal Concentration (MBC)

MBC is the lowest concentration of the sample that is required to kill the organism (Nwankwo *et al.,* 2014). Briefly, 1ml bacterial culture was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and were sub-cultured onto nutrient agar and incubated at 37°C for 24hours.This was obtained by streaking out the samples from the MIC tubes that showed no visible growth on nutrient agar plates. The lowest concentration of the

sample that showed no growth was noted and recorded as the minimum bactericidal concentration.

RESULT

Table 1 shows the Phytochemical screening of Apiary honey. Flavonoids, saponins, reducing sugar, alkaloids and glycosides were found to be present in varying concentrations while phenol, tanins, terpenoids and phlobatanin were absent

Table 1: Phytochemical screening of Apiary Honey

Phytochemical Component	Remarks
Flavonoids	+++
Phenols	-
Saponins	+
Reducing sugar	++
Alkaloids	++
Tanins	-
Glycosides	+
Terpenoids	-
Phlobatanins	-

Keys: +++ = Highly present; ++ = Moderately present; + = Slightly present
- = Absence of constituents

The antibacterial activity of gentamicin against bacteria isolates is indicated in Table 2. The highest zone of inhibition was discovered against *Pseudomonas aeruginosa* 28mm, *Klebsiella* spp 26mm, *Staphylococcus aureus* 23mm and *E coli* 21mm.

The antibacterial activity of Apiary honey observed against bacteria isolates is indicated in Table 3. The highest zone of inhibition was observed against *Pseudomonas aeruginosa* 26mm, *Klebsiella* spp 24mm, *Staphylococcus aureus* 21mm *and E coli* 20mm.

Table 2: Antibacterial Activity of Gentamicin against bacterial isolates

Bacterial Isolates	Conc (v/v)	Zone of Inhibition (mm)
Pseudomonas aeruginosa	2.5	28
Staphylococcus aureus	2.5	23
Escherichia coli	2.5	21
Klebsiella spp	2.5	26
Bacillus subtilis	2.5	19
Proteus vulgaris	2.5	16

Key: v/v = Volume to volume ratio
Conc. = Concentration

Table 3: Antibacterial activity of Apiary Honey

Bacterial Isolates	Conc. (v/v)	Zone of Inhibition (mm)
Pseudomonas aeruginosa	2.5	26
Staphylococcus aureus	2.5	21
Escherichia coli	2.5	20
Klebsiella spp	2.5	24
Bacillus subtilis	2.5	17
Proteus vulgaris	2.5	18

The MIC and MBC of Apiary honey is indicated in Table 4. The MIC for *Pseudomonas aeruginosa* is 32v/v while its MBC is 64v/v. Other bacteria MIC and MBC are as indicated in the Table.

Table 4: MIC and MBC of Apiary honey.

Bacterial Isolates	MIC (v/v)	MBC(v/v)
Pseudomonas aeruginosa	32	64
Staphylococcus aureus	28	56
Escherichia coli	104	208
Klebsiella spp	104	104
Bacillus subtilis	50	50
Proteus vulgaris	ND	ND

N/D = not done

DISCUSSION

The phytochemical screening from this study is similar to the results of (Nwankwo *et al.,* 2014) and (Elijah *et al.,* 2015). These classes of compounds are known to possess therapeutic properties against several pathogens and could therefore be responsible for its antibacterial activity.

Flavonoids have been documented to possess potent antioxidant and free radical scavenging effect (Ganapth *et al.,* 2011) and have strong anticancer activity (Salah *et al.,* 1995, Del-Rio *et al.,* 1997). They are also known to have anti-inflammatory, anti-allergic and anti-viral properties and help in the healing of wounds and treatment of skin diseases due to their ability to neutralize the acidity of wounds, and inflammation (Okwu, 2004; Nwankwo *et al.,* 2014).The honey samples were also revealed to contain saponins. Saponin has the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness (Sodipo *et al.,* 2000; Okwu, 2004). More so, saponins have been found to be an antibacterial substance on the cell wall of many organisms (Harbron, 1992). Saponins cause a reduction of blood cholesterol by preventing its re-absorption. The presence of alkaloid indicates that honey can be used as basic medicinal agents for their analgesic, antispasmodic and bactericidal effects (Stray, 1998;, Okwu and Okwu, 2004). Glycosides are known to lower blood pressure according to many reports (Nyarko and Addy 1990). Plants containing reducing sugar are of great value to living organisms since they are primary metabolites and are directly involved in their growth, development and reproduction. Absence of other phyto-constituents (Phenols and tannins) may be due to certain factors such as climatic conditions, temperature and soil fertility

Agar well diffusion test showed that the apiary honey has antibacterial activity against both gram positive and gram negative organisms. This is an indication that honey can be used as a potential antibacterial substance. Similarly, previous work has shown that honey has been used to heal recalcitrant wounds whereby it was found to be effective *in vitro* against a wide range of multi-resistant organisms including methicillin resistant *S. aureus* (MRSA), vancomycin –resistant Enterococci (VRE) and multi-resistant *Pseudomonas aeruginosa* (Cooper *et al.,* 2002; George and Cutting, 2007). The findings in this work is also in agreement with the work of (Nzeakor and Hamdi, 2000). Their study has shown that apiary honey has an antimicrobial activity against *S. aureus* and *E coli.* Another study by Kingsley also reported that honey completely inhibited major wound infectious pathogens such as *S. pyogenes* and *S. aureus*. The result of this study is consistent with the above studies.

In this study, the minimum inhibitory concentration (MIC) observed for *S. aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *E. coli* contrast the report of Molan, 2001, who observed that honey produced by honeybees (*A. mellifera*) could inhibit most of the test organisms including *S. aureus* and *E coli* at a very low concentration (2.5 to 7.5 v/v). Another study by (Molan and Betts, 2000) reported that the MIC and MBC for *E coli* were to be 7 and 10% respectively. The variations in the antimicrobial potential of honey used in this present study as compared to previous studies highlights the difference in the antimicrobial activities of honey; that is, the flowers from which bees gather nectar to produce the honey, since flora source determine many of the attributes of honey; for example flavor, aroma, color and composition of honey is highly variable as demonstrated by (Mogessie, 1994).

The presence of antibacterial substances as demonstrated by zone of inhibition showed distinctly the efficacy of apiary honey as an antibacterial agent.

CONCLUSION

Phytochemical screening revealed the presence of reducing sugar, saponins, glycosides, alkaloids and flavonoids. Antibacterial activity of apiary honey on some medically important bacteria shows that apiary honey exhibit strong antibacterial activity producing zones of inhibition against the tested bacteria. Also, apiary honey used in this study showed antibacterial efficacy more than the commercially available antibiotics.

This implies that apiary honey can be used as a therapeutic agent to treat infections as they may be an excellent alternative to curtail the further spreading of drug resistant bacteria in Nigeria.

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