



INVESTIGATION OF PHYTOCHEMICAL PROPERTIES AND ANTIBACTERIAL ACTIVITIES OF HONEY EXTRACTS FROM SOUTH WEST NIGERIA ON *Staphylococcus aureus* AND *Escherichia coli*



O. F. Obidi* and O. R. Ogunse

Department of Microbiology, University of Lagos, Nigeria

*Corresponding author: laideob@yahoo.com

Received: January 10, 2017

Accepted: March 27, 2017

Abstract: In Africa, microbial resistance to synthetic antimicrobial drugs has created growing interest and dependence on the use of natural products for medicinal purposes. Honey samples obtained from Oyo, Ogun, Edo, Lagos and Ondo States in south western Nigeria were investigated for phytochemical constituents and antibacterial effects on *Staphylococcus aureus* ATCC 700699 and *Escherichia coli* ATCC 11229. Raw honey samples, water extracts of honey (WE) and ethanol extracts of honey (EE) showed antibacterial activity against the tested organisms with zones of inhibition ranging from 12 – 32 mm. The minimum inhibitory concentration (MIC) was determined with concentrations of 5, 10, 15, 20 and 25 mg/ml of WE and EE. These were then compared with 20%, 40%, 60%, 80% and 100% of raw honey samples. MIC of WE on *S. aureus* were 20, 10, 20, 25 and 10 mg/ml while those of EE were 25, 10, 15, 20 and 10 mg/ml. For *E. coli*, MIC of WE were <5, 5, 10, 25, 15 mg/ml while those of EE were 5, 5, 10, 20, 20 mg/ml for Oyo, Lagos, Ogun, Edo and Ondo samples, respectively. The minimum bactericidal concentration (MBC) for the raw honey was 80% for *S. aureus* in Lagos, Ogun and Edo while it was 100% for Oyo and >100 for Ondo state. For *E. coli*, MBC was 100% for samples from Lagos, Edo and >100 for Oyo and Ogun states. Phytochemical screening revealed the presence of reducing sugar, saponins, flavonoids, steroids, glycosides, alkaloids, phenols and tannins. Overall, antibacterial activity increased in the order: Lagos > Ogun > Edo > Oyo > Ondo with the extracts comparing favorably with conventional amoxicillin/clavulanic acid.

Keywords: *Escherichia coli*, Honey, MBC, MIC, phytochemical constituents, *Staphylococcus aureus*

Introduction

The screening of natural products for antimicrobial, phytochemical and curative properties has contributed immensely to the discovery of new drugs in view of the growing resistance of antibiotics against medically important pathogens. In recent times, the scope of herbal medicine has extended to include mushrooms and bee products like honey as well as animals, shells and certain animal parts (Akinpelu, 2000). At present, natural products and their various derivatives are reported to constitute more than 50 % of drugs used in the world at large for clinical purposes (Mohapatra *et al.*, 2010). Honey, a natural product with very high nutritive and therapeutic value is produced from the nectar and sweet deposits from plants which are gathered, reconstituted and stored in the honeycombs by honeybees of the genera *Apis* and *Meliponini* (Namias, 2003; Al-jabri, 2005). The composition and nutritional quality of honey vary greatly with the botanical source of nectar as well as environmental and climatic conditions. This variation obviously goes a long way in determining the antimicrobial, an anti-inflammatory and anti-oxidant potential of the honey (Hammer *et al.*, 1999; Gulluce *et al.*, 2003).

The variation in colour (from nearly colourless to dark brown) and consistency (from fluid to viscous or partly to entirely crystallized) as well as flavor and aroma has been linked with the botanical spectrum (NHB, 1994). Indeed, honey has been successfully used in the treatment of several diseases where synthetic drugs have failed (Molan, 2001) such as preventing infection in postoperative wounds (Al-Waili and Saloom, 1999; Boukraa *et al.*, 2008); treatment of decubitus ulcers (bedsores) (Mohapatra *et al.*, 2010); inhibition of *Salmonella* and *Escherichia coli* growth (Radwan *et al.*, 1984); broad-spectrum activities against many different kinds of bacteria including aerobes and anaerobes, Gram-positives and Gram-negatives (Molan, 1992); inhibition of many enteric bacteria (Tan *et al.*, 2009); treatment of burns, Fournier's gangrene, radiation-induced mucositis, other dermatitis conditions such as skin graft (Willix *et al.*, 1992); stimulation and proliferation of peripheral blood lymphocytic and phagocytic activity (Mohapatra *et al.*, 2010). The mild acidity and low

level hydrogen peroxide release of honey has been reported to contribute to tissue repair and antibacterial activity (Mullai and Menon, 2007).

Previous studies have shown that in addition to its antimicrobial activities, honey is capable of clearing infection in a number of ways, including boosting the immune system, having anti-inflammatory and antioxidant activities via stimulation of cell growth (Al-jabri, 2005). The bactericidal effect of honey has been traced to the release of hydrogen peroxide when diluted due to the activation of the enzyme glucose oxidase, which oxidizes glucose to gluconic acid and hydrogen peroxide (Mandal and Mandal, 2011; Lusby *et al.*, 2005). Although, according to Ng *et al.* (2014) the presence of non-peroxide compounds in the honey can also inhibit an extensive range of bacteria. Another factor responsible for honey's bactericidal property is its high sugar content, a low water content and acidity (pH of 3.2-4.5) which is not favorable to microbial growth (Haniyeh *et al.*, 2010; Molan, 1992; Mohapatra, 2010; Mandal and Mandal, 2011). Beyond its therapeutic and medicinal attributes (Mohapatra *et al.*, 2011; Conti *et al.*, 2007), honey has been reported to have potential use as bio-indicators for environmental contamination (Celechovska and Vorlova, 2001). According to Taormina *et al.* (2001), honey from different sources possesses different levels of antibacterial activity, which may be due to varied geographical distribution and floral content as well as spatial and temporal variation in sources of nectar. Therefore, the present study sets out to assess the bacteriostatic and bactericidal properties of honey from selected south western states in Nigeria against *Staphylococcus aureus* ATCC 700699 and *Escherichia coli* ATCC 11229 as well as to evaluate bioactive agents which may contribute to the curative potential of honey samples from Nigeria

Materials and Methods

Test organisms and sample collection

Pathogenic bacterial cultures that included *Staphylococcus aureus* ATCC 700699 and *Escherichia coli* ATCC 11229 were obtained from the stock culture collection of the Department of Microbiology, University of Lagos, Nigeria for

in vitro antibacterial activities of undiluted raw honey, water extracts and ethanol extracts of honey. Confirmatory identification procedures of the test organisms were routinely carried out based on biochemical tests. Raw honey samples were obtained commercially from five south western Nigerian states including Oyo, Ogun, Edo, Lagos and Ondo States, respectively.

Preparation of standardized inoculums

The inocula were prepared from freeze-dried cell cultures of *Staphylococcus aureus* ATCC 700699 and *Escherichia coli* ATCC 11229 which were maintained at 4°C. The typed cultures were revived by routine culturing on freshly prepared nutrient agar (Biomark Laboratories, India) and incubated aerobically at 37°C for 24 h. Developed colonies were harvested into nutrient broth (Biomark Laboratories, India) and incubated overnight at 37°C. Aliquots (0.1 ml) of each broth culture was introduced into 9.9 ml of sterile peptone water in sterile test-tubes. The turbidity of the suspension was adjusted to match 0.5 McFarland standard to achieve a final concentration of 1×10^8 CFU/ml with the absorbance range of 0.08 to 0.10 by colorimeter at wavelength of 540 nm (Ogah and Osundare, 2015).

Preparation of water and ethanol extracts

Raw honey samples were extracted as described by Mohapatra *et al.* (2011). Ten (10) grams of raw honey sample from each State was placed in test-tubes and 25 ml solvent (water and/or ethanol) was added. Subsequently, the solutions were mixed by vortexing and centrifuged at 3000 rpm for 10 min at 25°C. The resulting supernatant was evaporated to dryness with a gentle stream of nitrogen and reconstituted with 10 ml dimethyl sulphoxide. The solution was mixed thoroughly by vortexing and transferred to stopper test-tubes for phytochemical analysis.

Antibacterial assay

Determination of antibacterial activity

Determination of antibacterial activities of raw honey, water extract and ethanol extracts of honey were performed using the agar well-diffusion assay (Ochei and Kolhatkar, 2004) with Mueller Hinton Agar (MHA) (Biotech) plates. Aliquots (0.1 ml) of the standardized bacterial suspensions were seeded evenly onto the surface of Mueller Hinton agar plates with a sterile hockey stick. Subsequently, plates were punched to make wells of 7 mm diameter using a sterile cork borer. Raw honey was diluted in sterile distilled water to different concentrations of 20%, 40%, 60%, 80% and 100% (v/v) undiluted honey while water and ethanol extracts were prepared in different concentrations of 5, 10, 15, 20 and 25 mg/ml. Wells were filled with 0.1 ml each of raw honey, ethanol and water extracts and labeled appropriately. The inoculated plates were allowed to stand for 30 minutes to 1 hour for proper diffusion of honey and extracts into the medium. The inoculated plates were then incubated at 37°C for 24 h and subsequently, examined for zones of inhibition. Each zone of inhibition was measured in millimeter (mm) with a measuring ruler. The experiment was done in duplicates and the mean was obtained. The effects were compared with those of commercially obtained antibiotic

discs (Amoxicillin/clavulanic acid 30 µg) (Ogah and Osundare, 2015).

Minimal inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) of the extracts and raw honey were determined for the test organisms in broth culture and on MHA plates. Aliquots (0.1 ml) of standardized test inoculum was inoculated onto different concentrations of extracts in sterile Muller Hinton broth and agar plates. Following overnight incubation for 24 h at 37°C, the tubes and plates were examined macroscopically for visible growth. MIC was defined as the lowest concentrations able to inhibit bacterial growth in the culture tubes and plates. The MIC according to Mandal and Mandal (2011) reflects the quantity needed for the test bacteria inhibition.

Minimal bactericidal concentration (MBC)

The minimum bactericidal concentration was determined by sub culturing to detect microbial growth after 24 h from the tubes without visible growth or turbidity in MIC into sterile molten nutrient agar plates. The plates were incubated at 37°C for 24 h and minimum bactericidal concentration (MBC) was taken as the lowest concentration that prevented any visible growth on plate (Mohapatra *et al.*, 2011).

Phytochemical screening

Ethanol and water extracts of the different honey samples were subjected to qualitative phytochemical analysis to evaluate the presence of various phyto-constituents. The phytochemical screening included tests for flavonoids, sterols, glycosides, alkaloids, reducing sugars, saponins, tannins and phenols (Harborne, 1992; Sofowora, 1993).

Results and Discussion

The water and ethanol honey extracts as well as the raw honey tested showed varying degree of antibacterial activities against *Staphylococcus aureus* ATCC 700699 and *Escherichia coli* ATCC 11229 as shown in Table 1. The raw honey from Lagos and Ogun states showed higher antibacterial activities (with inhibition zones ranging from 30-32 and 15-32 mm for *S. aureus* and *E. coli*, respectively) than that of the standard antibiotic (amoxicillin/clavulanic acid which ranged from 18-22 mm). Also, the antibacterial activity of raw honey from all the States except Ondo State were also higher (with inhibition zones ranging from 30-32 mm, respectively) than that of amoxicillin (22 mm) against *E. coli*. This result corroborates the findings of Nwankwo *et al.* (2014) who reported the inhibitory effects of honey produced from *Apis mellifera* with inhibition zones of 45 and 34 mm for *S. aureus* and *E. coli*, respectively. The differences in the zones of inhibition obtained can be attributed to the variations in the geographical locations of the samples which have been reported to be directly related to the antibacterial properties of honey (Hammer *et al.*, 1999; Gulluce *et al.*, 2003). The water extracts compared favourably with amoxicillin/clavulanic acid having inhibition zones ranging from 16-20 mm for *S. aureus* and 16-24 mm for *E. coli*. The ethanol extract also showed good antibacterial activity with inhibition zones ranging from 12-24 mm and 5-21 mm for *S. aureus* and *E. coli*, respectively.

Table 1: Antibacterial activities of raw honey, water extract and ethanol extract

Bacterial Isolates	Diameter of zone of inhibition in millimeter (mm)															Amoxicillin/ clavulanic acid 30 µg
	Oyo State			Lagos State			Ogun State			Edo State			Ondo State			
	WE	EE	RH	WE	EE	RH	WE	EE	RH	WE	EE	RH	WE	EE	RH	
<i>S. aureus</i>	18	15	13.5	20	24	30	20	14	15	16	12	17	18	16	20	18
<i>E. coli</i>	22	21	30	24	20	32	23	17	32	22	18	30	16	20	15	22

WE = water extract, EE = ethanol extract, RH = raw honey

Table 2: Minimum inhibitory concentration of water extract, ethanol extract and raw honey

Bacterial Isolates	MIC														
	Oyo State			Lagos State			Ogun State			Edo State			Ondo State		
	WE (mg/ml)	EE (%)	RH (%)	WE (mg/ml)	EE (%)	RH (%)	WE (mg/ml)	EE (%)	RH (%)	WE (mg/ml)	EE (%)	RH (%)	WE (mg/ml)	EE (%)	RH (%)
<i>S. aureus</i>	20	25	100	10	10	40	20	15	80	25	20	100	10	10	60
<i>E. coli</i>	<5	5	40	5	5	10	10	40	25		20	80	15	20	60

WE = water extract, EE = ethanol extract, RH = raw honey

The minimum inhibitory concentration (MIC) of the water extract ranged between 10 and 25 mg/ml for *S. aureus* and between <5 and 25 mg/ml for *E. coli*. For the ethanol extract, MIC ranged from 10-25 mg/ml and from 5-20 mg/ml for *S. aureus* and *E. coli* respectively (Table 2). The MIC of raw honey ranged between 40 and 100% for *S. aureus* with Lagos State samples having highest effectiveness (MIC 40%) and Oyo State having the least effectiveness (MIC 100%). The minimal bactericidal concentration (MBC) of the raw honey ranged from 100 to >100% for *S. aureus* and from 80 to > 100 for *E. coli*, respectively (Table 3). Phytochemical screening of ethanol extracts, water extracts and raw honey revealed the presence of most important phytoconstituents including flavonoids, steroids, glycosides, alkaloids, reducing sugars, saponins, phenols, and tannins. These bioactive compounds have been reported to exhibit antimicrobial activities (Shimada, 2006). Flavonoids and glycosides were observed in WE and EE from all the states. Flavonoids have been reported to exhibit antimicrobial, anti-inflammatory, analgesic, anti-allergic, cytostatic and antioxidant properties (Hodek *et al.*, 2002). Similarly, alkaloids were detected in all WE and EE except samples from Oyo state. It is noteworthy to observe that reducing sugars were present in all EE but not in all WE. The WE from Ogun, Edo and Ondo lacked reducing sugars. The EE were generally more active on *S. aureus* than the WE having a zone of inhibition ranging from 12-24 mm while WE had a zone of inhibition ranging from 16-20 mm. This is in agreement with previous reports that ethanolic extracts are more active than water extracts (Parekh and Chanda, 2001) and could be attributed to the presence of reducing sugars and higher levels of steroids compared to the WE. This also suggests that the bioactivity of an extract could depend largely on the solvent used in the extraction process as well as the microorganism in question. Kowalski and Kedzia (2007) also reported a higher activity of methanol extract than water extract of *Jathropha curcas* (Linn) on pathogenic strains which may be attributed to the presence of soluble polyphenolic compounds.

Table 3: Minimum bactericidal concentrations (MBC) of raw honey

Bacterial Isolates	MBC (%)				
	Oyo	Lagos	Ogun	Edo	Ondo
<i>S. aureus</i> ATCC700699	>100	100	>100	100	100
<i>E. coli</i> ATCC11229	100	80	80	80	>100

Medicinal and therapeutic properties of the extracts and raw honey can be deduced from the presence of various bioactive chemical constituents as shown in (Table 4). The antibacterial properties of these bioactive compounds is well documented (Quinlan *et al.*, 2000; Igbinsosa *et al.*, 2009). Alkaloids were present in all the extracts except samples from Oyo state. Generally, alkaloids have been known to be toxic to microbial cells as a result, they have been considered for their potential use in cancer treatment and pain relief (Igbinsosa *et al.*, 2009). The presence of saponins in the extracts suggests the potential of honey as anti-inflammatory agents as reported by Igbinsosa *et al.* (2009). The presence of steroids detected in the honey extract could also contribute to its antibacterial activities as suggested by Quinlan *et al.* (2000) who reported the antibacterial activities of steroidal extracts on important human pathogens. In the present study, antibacterial activity increased in the order of Lagos state > Ogun State > Edo State > Oyo State > and Ondo State. The variations observed in the inhibitory activities of these samples could be attributed to the geographical differences and floral origin which in turn contribute to the chemical composition, physicochemical properties and sugar and glycerol content (Molan, 1992b; Nwankwo *et al.*, 2014). Overall, *E. coli* had a higher susceptibility than *S. aureus*. This also agrees with work of Ng *et al.* (2014) who reported a higher susceptibility of Gram negative organisms to honey than Gram positive organisms. This higher inhibitory effect on Gram negative organisms has been linked to the fact that the cell wall of Gram-negative bacteria is more susceptible to mechanical damage because of the low amount of peptidoglycan compared to Gram-positives (Tortora *et al.*, 2013).

Table 4: Phytochemical analysis of water and ethanol extract of honey samples

Phytoconstituents	Oyo state		Lagos state		Ogun state		Edo state		Ondo state	
	WE	EE	WE	EE	WE	EE	WE	EE	WE	EE
Flavonoids	+	+	+	++	+	+	+	++	++	+
Steroids	+++	+++	-	+	-	+	+++	+	+++	+
Glycosides	+	+	+	++	+	+	++	++	+	+
Alkaloids	-	-	+++	++	+	+	+	+	+	+
Reducing sugar	++	++	+	+	-	++	-	++	-	+
Saponins	+	-	+++	++	++	++	-	+	+	++
Phenols	-	-	-	-	-	-	-	-	-	-
Tannins	-	-	-	-	-	-	-	-	-	-

WE = water extract; EE= ethanol extract; - = absent + = slightly present; ++ = moderate; +++ = abundant

It is noteworthy to observe that the antibacterial properties of raw honey is far higher (13.5-30 mg/ml and 15-32 mg/ml) than the ethanol extract (12-24 mg/ml and 17-21 mg/ml); water extracts (16-20 mg/ml and 16-24 mg/ml) and amoxicillin/clavulanic acid (18 and 22 mg/ml) for *S. aureus* and *E. coli*, respectively. This is in line with the findings of Molan (2001) and Badaway *et al.* (2004) who reported that the antibacterial effect of honey is best in its undiluted form, where the conditions of antibacterial properties like acidity, osmolarity, and phytochemical components including flavonoids and phenolic content are intact, well preserved and undiluted.

Conclusion

Conventionally, honey has been proved to be a therapeutic agent. It has various physicochemical properties and bioactive compounds which can be harnessed for the development of synthetic drugs against common human pathogens such as *S. aureus* and *E. coli*. These findings suggest the potential usefulness of honey extracts in development of alternative medicines for therapeutic and medical uses.

Conflict of Interest

The authors declare that there is no conflict of interest.

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