ANTIMICROBIAL ACTIVITIES OF HONEY FROM DIFFERENT GEOGRAPHICAL LOCATIONS ON GRAM NEGATIVE AND POSITIVE ORGANISMS

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SUMMARY

Honey samples were obtained from the different agro-ecological zones of Nigeria including: the tropical rainforest, mangrove swamp, plateau grassland, guinea savannah and sudan savannah. The antimicrobial activities of these differently sourced honeys were assessed against six bacteria organisms (*Staphylococcus aureaus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Klebsiella aerogenes* and *Proteus mirabilis)*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were studied using standard methods. Results obtained showed that geographical locations had effects on the antibacterial activities of the different honeys at different concentrations (6.25-100%). Honey originating from Vom, Plateau State showed the highest antimicrobial activity. All the honeys showed varied bacteriostatic and bactericidal activities. None of the honeys produced any effect on *Klebsiella aerogenes* and *Proteus mirabilis.* Further work is encouraged.

KEYWORDS: Honey, Nigeria, antimicrobial, geographical variation.

INTRODUCTION

Developing countries especially Africa has some of the highest records of human fatalities due to preventable diseases (Sofowora, 1987). Despite the enormous advances in healthcare during the last half century, infectious diseases still account for 25% of total mortality worldwide and 45% in low income food deficient countries (LIFDC) (WHO, 1999). Antimicrobial drugs are critically important in reducing the global burden of infectious diseases. However, as resistant microbes develop and spread the effectiveness of such drugs also diminish (WHO, 1999). The growing resistance of micro-organisms to conventional antimicrobial agents is the concern to clinical microbiologist all over the world (Boonmar *et al*., 1998; Goncagul *et al*., 2004). As a result, efforts are being intensified to develop antimicrobial agents from other sources

for better chemotherapeutic effects (Shears, 2000).

Honey, a bye-product of honeybees (*Apis mellifera*) is one of the ancient traditional medicines used for the treatment and prevention of various diseases. Ancient records confirmed the usage of honey as medicaments (Aristotle, 350 BC; Van Ketel, 1892; Gunther, 1959). In more recent times, the contemporary medical practitioners have rediscovered the use of honey as a therapeutic substance and it is gaining widespread acceptance as an antibacterial agent for the treatment of topical infections resulting from surgical wounds and burns (Kandil *et al*., 1987; Efem, 1988). Similarly, peptic ulcers and gastritis have been treated with honey while the antibacterial and antifungal properties of honey have been well documented (Haffejee and Moosa, 1985; Molan, 1992a; Al Somai *et al*., 1994; Abuharfeil *et al*., 1999). It was also confirmed that honey from a particular regions may have better therapeutic activities (Allen *et al.*, 1991a).

Antimicrobial activity of honey has been attributed to a heat and light labile substance called inhibine, a product of honey glucose oxidase (White *et al*., 1963). Other workers attributed these bacteriostatic/bactericidal ability to its high osmotic effect, high acidic nature (pH 3.2-4.5), high level of hydrogen peroxide concentration and its phytochemical nature which include the following chemicals; tetracycline derivatives, peroxides, amylase, fatty acid, phenols, ascorbic acid, flavonoids, streptomycin, sulfathiazole, triterpenes, benzyl alcohol and benzoic acids.(Molan, 1992a, 1992b).

Although, honey is widely produced and used as food and medicaments in tropical Africa and in Nigeria in particular, their antibacterial activity with reference to geographical origin has never been documented.

This work compared the antimicrobial activities of wet-season/rainy season (May-July) honeys derived from different geographical ecosystems of Nigeria {Ogbomoso, Oyo State (Tropical rainforest); Semenkpe bush, Bayelsa State (Mangrove swamp); Vom, Plateau State (Plateau grassland); Angwan Mailafia, Kaduna State (Guinea savannah) and Takum, Taraba State (Sudan savannah)}, against selected Gram negative and positive bacterial organisms (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella aerogenes* and *Proteus mirabilis*). We also studied the effect of climatic conditions/geography on the antimicrobial properties of the honey collected from the various locations. The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these honeys were determined using standardized methods (Barrow and Feltham, 2004a).

MATERIALS AND METHODS

Samples collection

The following locations were visited for sample collections: Ogbomoso, Oyo state-tropical rainforest; Semenkpe bush, Bayelsa state-mangrove swamp, Takum, Taraba state-sudan savannah, Angwan Mailafia, Kaduna state-guinea savannah and Vom, Plateau state- plateau grassland (Table I). Honeycombs were collected and processed according to the standards in the traditional industry. The honeys were melted from the honeycombs and collected into sterile laboratory glassware, labelled as **Ogb-TR** (Ogbomoso-tropical rainforest), **Se-**

MS (Semenkpe-mangrove swamp), **Ta-SS** (Takum-sudan savannah), **Am-GS** (Angwan Mailafia-guinea savannah), and **Vo-PG** (Vom-plateau grassland) and kept at +2 to +8^{0}C until used (Table 1). The samples were tested for microbial purity by plating each honey on McConkey and blood agar plates and incubation at 37^{0}C for 24 hours. Some undiluted portions of the honeys were reserved. Other portions were diluted by thorough homogenization using sterile distilled water to 1:2 (50%), 1:3 (33.3%), 1:4 (25%) and 1:5 (20%) of their original concentrations.

Selection of bacteria isolates

The clinical isolates of bacterial species most commonly involved in causing gastroenteritis, wound infections, and urinary tract infections were obtained from the culture bank of the Bacteriology Department, Federal College of Veterinary and Medical Laboratory Technology, Vom, Nigeria. Pure culture of each of the isolates was obtained by sub-culturing the isolates on their selective media. Briefly, a colony was picked from the nutrient agar slant containing *Staphylococus aureaus* obtained from the culture bank and streaked onto Mannitol salt agar. This was incubated at 37^{0}C for 24 hours. The organism fermented the Mannitol. Similarly, a colony was picked from nutrient agar slant containing *Escherichia coli*. This was introduced into MacConkey agar and incubated at 37^{0}C for 24 hours. Smooth pink colonies were seen on the plate post incubation, this was confirmed using Eosin Methylene Blue (EMB) agar on which the organism gave a blue-black, dark centred colony with green, metallic sheen; an indication of *E. coli*.

Furthermore on a blood agar plate, *Pseudomonas aeruginosa* was cultured which resulted in large flat haemolytic colonies on the plate 24 hours post-incubation. We observed a dark greenish-blue coloration which indicated the diffusing of pigment into the medium. Standard morphological techniques were similarly carried out for the other three organisms (*Salmonella typhi, Klebsiella aerogenes* and *Proteus mirabilis*) (Cheesbrough, 2000a). Biochemical tests were performed on each isolate to confirm the identity of the organisms according to standard procedures (Cheesbrough, 2000b; Barrow and Feltham, 2004b).

Preparation of selected bacterial isolates

Fresh isolates of the morphologically identical pure culture of each test organism was picked from the selective medium. Specifically, three colonies of each of the isolates were picked with an inoculating wire loop, suspended in 5 ml of nutrient broth and incubated at 37^{0}C for 3 hours, to reactivate the organisms. This was then diluted with sterile distilled water until a turbidity that matched 0.5 McFarland Standards (10^6 CFU/ml) was reached. The resulting suspension was further diluted 1:100 in sterile nutrient broth to set an inoculum density of 10^4 CFU/ml used in the experiment (Woods and Washington, 1995; Miles and Amyes, 1996). The process was repeated until all the isolates were similarly prepared. These dilutions were stored at +4^{0}C until used same day.

Table I. Geographical locations of honey and their descriptive features.

Honey (Code)	Source	Description of Source
Ogbomoso, Oyo State (Ogb-TR)	South-West, Nigeria	The landscape consists of old hard rocks and dome shaped hills, which rise gently from about 500 meters in the southern part to about 1,219 metre above sea level in the northern part. The mean annual temperature is 25-35°C with relatively high humidity. Annual rainfall is 1400-1800mm per annum. Tropical rainforest are the usual fauna.
Angwan Mailafia, Kaduna State (Am-GS)	North-West, Nigeria	The State extends from the tropical grassland known as the Guinea Savannah to Sudan Savannah. Vegetation is thick and grasses grow to a height of about 3.6 meters tall with big trees, which grow shorter as one approaches the Sudan Savannah. Mean annual temperature is 27-30°C and the annual rainfall is 1000-1400mm per annum.
Vom, Plateau State (Vo-PG)	North-Central, Nigeria	Mountainous areas with many rock formations. The grassland altitude ranges from around 1,200metres (4000 feet) to a peak of 1,829metres. The average temperature is 18-22°C and the annual rainfall varies from 1800-2200mm per annum.
Semenkpe Bush, Bayelsa State (Se-MS)	South-South, Nigeria	Riverine and estuarine settings. The terrains are difficult due to large pools of water and often inaccessible roads. Mangrove plants thrive well in this state. Mean annual temperature is 24-27°C and the mean annual rainfall is >4000mm per annum
Takum, Taraba State (Ta-SS)	North-East, Nigeria	It consists of undulating landscape dotted with a few mountainous features and lies largely within the tropical zone. It has a vegetation of low forest in the southern part and grassland in the northern part. The Mambilla Plateau (located in the state has an altitude of 1,800 meters (6000 ft) above sea level and a temperate climate all year round. The mean annual temperature is 27-30°C and the annual rainfall is 1000-1400mm per annum

Antimicrobial assay

Nutrient agar plates were prepared asceptically, allowed to set and dried. One millilitre (1ml) of the standard inoculum of the previously prepared bacterial isolates was used to flood nutrient agar plates in the agar diffusion method of in-vitro antimicrobial sensitivity test (Bauer *et al.,* 1966). With the aid of the sterile standard cork borer, 7 radial wells of 6 mm diameter were punched equidistantly at different sites on the plates and the bottoms of the wells were sealed with one drop of the sterile molten nutrient agar. The 6^{th} and 7^{th} wells served as negative (Distilled water) and positive (40 mg/ml of Ciprofloxacin) controls respectively.

Fifty microlitres (50 µl) of each of the honey: undiluted and diluted with sterile distilled water (undiluted-100%, 50%, 33%, 25% and 20%) from each location were placed in five of the bored wells. The 6^{th} and 7^{th} wells were filled with sterile distilled water (negative control) and Ciprofloxacin (positive control) respectively. This processes were repeated for each honey sample viz Ogb-TR, Se-MS, Ta-SS, Am-GS and Vo-PG; and for each of the organisms (Table 2).

The plates were left on the bench for 40 minutes for pre-diffusion and then

incubated at 37^{o}C overnight. The resulting zones of inhibition (measured in millimetres) were observed for the different concentrations of the honeys.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Following the initial antimicrobial screening tests, the minimum inhibitory concentration of each honey was determined by using the broth tube dilution method as described by Barrow and Feltham (2004b).

Twelve sterile test tubes were set for each honey sample. 1 ml of freshly prepared nutrient broth was added to eleven sets of sterile tubes (number 2-12). This process was repeated seven times to accommodate all the five honeys and the organisms susceptible in the antimicrobial screening assay. 1 ml of the undiluted honeys was transferred into the first set of tubes. Two-fold serial dilutions were performed by transferring 1ml each of the different undiluted honeys into the second tubes and vortexed for homogenization to give a dilution of 1:2. The process continued until eleventh tubes with a dilution of 1:1024 was reached and 1ml was discarded. Nothing was added to the last tubes which contained only nutrient broth (controls). One (1 ml) of the standard inoculum of each of the organisms was then added to all tubes.

The entire procedure was repeated for all the organisms that were susceptible to each of the honey sample. The tubes were incubated at 37^{0}C for 24 hours and observed visually for turbidity post-incubation (matching the nutrient broth negative control). Cultures from the incubated tubes were sub-cultured onto fresh sterile nutrient agar plates and incubated at 37^{0}C for 48hours. The plates were examined for growth to determine the minimum bactericidal concentration (MBC). The MBC is the lowest concentration of honey required to produce a sterile culture.

The plates were scored according to the assessment of Payveld, (1986). These include; no growth (bactericidal); light to moderate growth (bacteriostatic); heavy/luxuriant growth (no effect).

RESULTS

With the exception of the honey from Vom, spores of *Clostridia species* were isolated from the other honeys when plated on blood agar and McConkey agar plates. Some of the honeys tested had antimicrobial activity against all the bacteria isolates except *Proteus mirabilis and Klebsiella aerogenes* (Table II).

Honeys from Vom, Plateau (Vo-PG) and Angwan-Mailafia, Kaduna States (Am-GS) showed inhibitory effect against *Staphylococcus aureus*. *Pseudomonas aeruginosa* was inhibited by honeys from Vom, Plateau and Takum, Taraba States (Ta-SS). *Salmonella typhi* was inhibited by honeys from Angwan-Mailafia, Kaduna and Takum, Taraba States. Only honey from Vom, Plateau State inhibited *Escherichia coli*. Other honeys showed only partial inhibitory activities (Table II).

For the minimum inhibitory concentration, Vo-PG inhibited *P. aeruginosa* at 6.25%, *E. coli* at 6.25% and *S. aureus* at 12.5% concentrations. Ta-SS inhibited *S. typhi* at 25% and *P. aeruginosa* at 12.5% concentrations. Am-GS was active against *S. typhi* at 12.5% and *S. aureus* at 6.25% concentrations (Table III).

Vo-PG, Ta-SS and Am-GS all showed bactericidal effects at 100% concentration against the inhibited organisms. However, Am-GS have no

bactericidal effect on *S. typhi* after 48hours of incubation. Vo-PG was bactericidal against *Pseudomonas aeruginosa* and *Staphylococcus aureaus* at 50% concentration; and Am-GS produced similar effect on *Staphylococcus aureaus* at 50% concentration too. None of the honey was effective at concentration below 50% (Table IV).

Table II. Antimicrobial activities of honeys from different geographical locations in Nigeria against Gram positive and negative bacteria organisms

Honey dilutions	*Staphylococcus aureus* Zone of inhibition (mm)					*Pseudomonas aeruginosa* Zone of inhibition (mm)					*Escherichia coli* Zone of inhibition (mm)					*Salmonella typhi* Zone of inhibition (mm)				
	Ogb-TR	Am-GS	Vo-PG	Se-MS	Ta-SS	Ogb-TR	Am-GS	Vo-PG	Se-MS	Ta-SS	Ogb-TR	Am-GS	Vo-PG	Se-MS	Ta-SS	Ogb-TR	Am-GS	Vo-PG	Se-MS	Ta-SS
Undiluted	16	**10***	**8***	18	25	2	5	**9***	0	**14***	30	20	**14***	20	0	21	**8***	9	20	**9***
1/2(50%)	18	18	17	16	18	0	0	**7***	0	10	20	20	17	20	0	20	00	0	18	11.2
1/3(33%)	16	18	15	16	14	0	0	**7***	0	7	20	15	17	15	0	13	10	0	8	8
1/4(25%)	16	0	15	15	10	0	0	9	0	0	18	15	16	14.5	0	10	0	5	8	0
1/5(20%)	15	0	10	15	10	0	0	0	0	0	16	0	0	0	0	0	0	0	6	0
Ciprofloxacin (+)	**15***	**15***	**17***	**15***	**16***	**20***	**00***	**1***	**00***	**2***	**25***	**00***	**25***	**25***	**25***	**10***	**00***	**00***	**1***	**10***
Distilled water (-)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*complete zone of inhibition; All zones of inhibitions were measured in millimetres; Ogb-TR= Ogbomoso (Tropical rainforest); Am-GS= Angwan Mailafia (Guinea savannah); Vo-PG= Vom (Plateau grassland); Se-MS= Semenkpe Bush (Mangrove swamp); Ta-SS= Takum (Sudan savannah); Only honeys from Kaduna (Am-GS), Vom (Vo-PG) and Taraba (Ta-SS) showed clear antimicrobial activities. Bold numbers indicated clear antimicrobial activities. Other values indicated partial inhibitory activities. No effect of the honeys was observed on *Proteus mirabilis* and *Klebsiella aerogenes*.

Table III: Minimum Inhibitory Concentration (MIC) of honey on selected organisms on nutrient broth culture

Isolates	Dilutions of the honeys											Honey	MIC
	Undiluted	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024		
Pseudomonas aeruginosa	-	-	-	-	-	+	+	+	+	+	+	Vo-PG	1/16(6.25%)
Escherichia coli	-	-	-	-	-	+	+	+	+	+	+	Vo-PG	1/16(6.25%)
Staphylococcus aureus	-	-	-	-	+	+	+	+	+	+	+	Vo-PG	1/8(12.5%)
Salmonella typhi	-	-	-	+	+	+	+	+	+	+	+	Ta-SS	1/4(25%)
Pseudomonas aeruginosa	-	-	-	-	+	+	+	+	+	+	+	Ta-SS	1/8(12.5%)
Salmonella typhi	-	-	-	-	+	+	+	+	+	+	+	Am-GS	1/8(12.5%)
Staphylococcus aureus	-	-	-	-	-	+	+	+	+	+	+	Am-GS	1/16(6.25%)

- = No visible growth (not turbid); + = visible growth (turbid)
Vo-PG= Vom (Plateau grassland); Ta-SS= Takum (Sudan savannah); Am-GS= Angwan Mailafia (Guinea savannah).

Table IV. Minimum Bactericidal Concentration (MBC) of honey on selected organisms on nutrient agar culture

Isolates	Dilutions of honeys					Honey	MBC
	Undiluted	1/2	1/4	1/8	1/16		
Pseudomonas aeruginosa	-	-	++	+++	+++	Vo-PG	1/2(50%)
Escherichia coli	-	+	++	+++	+++	Vo-PG	1(100%)
Staphylococcus aureus	-	-	++	+++	+++	Vo-PG	1/2(50%)
Pseudomonas aeruginosa	-	+	+++	+++	+++	Ta-SS	1(100%)
Salmonella typhi	-	+++	+++	+++	+++	Ta-SS	1(100%)
Salmonella typhi	++	++	+++	+++	+++	Am-GS	0(0%)
Staphylococcus aureus	-	-	+	+++	+++	Am-GS	1/2(50%)

- = Absence of growth (bactericidal); + = Light growth (bacteriostatic); ++ = Moderate growth (bacterostatic); +++ = Heavy growth (No antimicrobial activity)

DISCUSSION

In this study five honey samples from the different agro ecological zones of Nigeria were tested for their antimicrobial activities on some selected bacteria organisms. Honeys from Vom, Takum and Angwan-Mailafia displayed both bacteriostatic and bactericidal properties on both Gram positive and Gram negative bacteria depending on their dilutions. The honey samples showed bactericidal activities against the tested organisms up to the dilutions of 50%. This result is similar to those reported by Nzeako and Hamdi (2000) but is at variance with the report of Willix *et al.* (1992). Since honey's antimicrobial property is dependent on its water activity – (the free water molecules in honey which is usually between 15 and 21%) in part, this will directly affect its osmotic effect. It is then rational to assume that a more diluted honey may have lost its antibacterial ability.

This increased dilution factor may also partially explain why honeys from Ogbomoso, Oyo State and Semenkpe bush, Bayelsa State, locations with heavy rainfalls (over 1400mm per annum) does not show full antibacterial activity against any of the organisms tested, however, Vom, Plateau State also have high level of rainfall per annum yet its honey was effective against the tested bacterial organisms. The reason for this is not clearly evident in this study.

Of all the honey samples tested, Vom honey had the highest antimicrobial activity followed by Angwan-Mailafia and Takum. These latter two honeys originated from drier parts of Nigeria that receives less annual rainfalls compared to the other two locations (Table I).

The bactericidal activity of the honeys on *Pseudomonas aeruginosa, Salmonella typhi and Escherichia coli* was found to be between 50 and 100% concentration for all the three active honey samples. This agrees with the reports of Kingsley (2001) which found honeys to be effective in wound treatment at higher concentrations. The honeys' bactericidal effect against *Staphylococcus aureaus* was at 50% concentration. This organism has been reported to be one of the most sensitive to the effects of honey (Molan, 1992a). This factor can therefore be positively annexed in hospital infection controls in view of the development of many methicillin-resistant *Staphylococcus aureus* (MRSA) species. Other workers had described the complete inhibition of MRSA at honey concentration of as low as 10% (Hancock B.M., unpublished findings; Willix *et al.*, 1992; Molan and Betts, 2000). It will be necessary to conduct further study to assess the potentials of Nigerian honeys against MRSA agent.

Although Efem, (1988) reported that *Pseudononas aeruginosa* was resistant to honey, results from this study contradict this assertion since honeys from Vom and Taraba exerted antimicrobial activity on *Pseudomonas aeruginosa* which have been known to be resistant to some common antibiotics such as penicillin, chloramphenicol, ampicillin etc due to a combination of factors including: low permeability of its cell wall, genetic capacity to express resistant

mechanisms, mutation in chromosomal genes which regulate resistance genes, and ability to acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages (Molan and Betts, 2000; Lambert, 2002).

The variation in the antimicrobial potentials of honeys used in the present study as compared with previous similar studies confirms that the source of the nectars may have contributed to the difference in the antimicrobial activities of honey. The different vegetative zones assessed in this study present with different fauna as well as nectars and pollens available for the honeybees (*Apis mellifera*) found in such locations. Other reports have confirmed that variation in antibacterial activities of honey can be traced to available nectars and pollens (Allen *et al.,* 1991a, 1991b).

In this study, the chemical components of the antimicrobial substances in the honeys were not estimated except for pH. The pH which was 4.5, 5.0, 5.5, 6.0, and 6.0, for honeys from Vom, Ogbomoso, Angwan Mailafia, Semenkpe and Takum respectively. These values were low enough to be inhibitory to many pathogens whose pH for normal growth falls between 7.2 and 7.4. The mimimum pH required for the growth of *E. coli*, *S. typhi* and *P. aeruginosa* is 4.3, 4.0 and 4.4 respectively. This unsuitable pH may have contributed to the antimicrobial effects noticed in this experiment.

That the honeys used in this work had no effect on *Klebsiella aerogenes* and *Proteus mirabilis* is in contrast with the report by Molan (1992a) and Subrahmanyan (1991). Reasons for this variance will need to be further explored. Spores of *Clostridium species* were isolated from the honeys from the various locations with the exception of Vo-PG honey. Hauschild and colleagues (1988), had previously reported that spores of *Clostridium botulinum* were found in honey. This finding serves as a major limitation to the use of some honeys. However, this limitation is nullified in view of a recent study that use gamma-irradiation to sterilize honey; in that work, the antibacterial activity was intact while Clostridia spores, earlier seeded in the honey before sterilization was completely destroyed (Molan and Allen, 1996). Honeys from Vom, Plateau State therefore hold potentials as a better antimicrobial agent since it presented without Clostridia spores and has the least pH.

In this experiment, attempt was made to assess the value of honeys from some geographical locations within Nigeria as antimicrobial therapeutic agents. It has established that some Nigerian honeys produced by honey bees (*Apis mellifera*) has both bacteriostatic and bactericidal activity when tested in-vitro.

It will be important to carefully assess the types of flowering plants and trees present in each of the study locations, from which the bees obtain their nectar to produce the honeys. It will also be important to do further study on the dry-season honeys from these locations as well as study the phytochemical components and phenolic contents of honeys from these various locations. These studies will be necessary for purposes of pharmacological standardization and clinical evaluation before these honeys can be recommended for preventative and curative measures against common diseases related to the tested bacterial isolates.

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