



Screening of Honeys for Antibacterial Potential against Multi-Drug Resistant Clinical *Salmonella* Species

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ABSTRACT

The development of resistance to conventional antibiotics, fluoroquinolone and emergence of multidrug resistant (MDR) bacteria of medical importance had prompted us to investigate the antibacterial activities of honeys on MDR *Salmonella* isolates. A total of 8 clinical *Salmonella* isolates were obtained from Aminu Kano Teaching Hospital, Kano-Nigeria. The isolates were reconfirmed through cultural and biochemical tests. Antibiotic susceptibility test was carried out to determine the isolates susceptibility patterns and multidrug resistant isolates using agar disk diffusion method. Sensitivities of the MDR isolates to light and dark honeys were determined using agar well diffusion method. Minimum inhibitory concentration and minimum bactericidal concentration of the honeys against the isolates were also determined. The isolates collected were 100% and 50% susceptible to ciprofloxacin and chloramphenicol respectively. However, they were 100% and 87.5% resistant to ampicillin and trimethoprim-sulfamethoxazol respectively. Seven (87.5%) of the isolates collected were found to be multi-drug resistant. This study indicated that both honeys had growth inhibitory effect on all the tested MDR *Salmonella* isolates. The isolates were sensitive to 20-75% concentrations of both honeys. Increasing the honey concentration significantly ($p < 0.05$) increased the zone diameter of inhibition. Dark honey was more potent than light honey in inhibitory effect. Minimum inhibitory concentration (MIC) of dark honey was 6.25%, while that of light honey was 12.5%. The Minimum bactericidal concentration (MBC) of dark honey was 12.5%, while that of light honey was 25.0%. Since both honeys were found to have inhibitory effect on the MDR *Salmonella* isolates, they could be used as alternative therapeutic agents against *Salmonella* isolates.

Keywords: *Salmonella*; Multidrug Resistant; Honey; Antibacterial Activity

INTRODUCTION

Honey, a natural product of very high nutritive value is made when the nectar (flora) and sweet deposits from plants (non-floral) are gathered, modified and stored in the honey combs by honeybees of the genera *Apis* and *Meliponini* (Al-jabri, 2005). Depending on its quality, honey can contribute to the health and nutritional status of humans. This beneficial action has been ascribed to its antimicrobial, anti-inflammatory and anti-oxidant potential. Interestingly, honey is gradually receiving attention as a complementary and an alternative source of treatment in modern medicines. The use of honey as a drug for the treatment of disease dates back to 2100-2000 BC (Vallianou *et al.*, 2014). The antimicrobial

properties of honey have been well documented, and honey has been used from ancient times as a method of accelerating healing. A possible reason behind its activity relies on its ability to generate hydrogen peroxide by the bee-derived enzyme glucose oxidase (Jing *et al.*, 2014). Another possibility is the composition of honey, which has more than 181 constituents (Mandal and Mandal, 2011). It is well established that honey inhibits a broad spectrum of bacterial species. More recently, honey has been reported to have an inhibitory effect to around 60 species of

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bacteria including aerobes and anaerobes, Gram positives, and Gram negatives (Hannan *et al.*, 2004).

Salmonella is a Gram negative facultative rod shaped bacterium, trivially known as “enteric bacteria”. *Salmonella* is the etiologic agent of salmonellosis. Typhoid salmonellosis is a global problem with an estimated 12-33 million people infected in the world (Kohinur *et al.*, 2010). Non-typhoidal salmonellosis is self-limiting; however, it may lead to systemic symptoms in children, the elderly and immunocompromised (Rayamajhi *et al.*, 2008). Bacteremia is reported to occur in 3 to 10 percent of cases of non-typhoidal salmonellosis and in such situations, antimicrobial therapy is lifesaving (White *et al.*, 2001).

Increasing number of antimicrobial-resistant *Salmonella* strains has led to a decrease in the efficacy in the treatment of salmonellosis by the use of antibiotics (Angulo *et al.*, 2000). The implication of therapeutic failure in public health due to multidrug resistance is particularly important, given that *Salmonella* is one of the leading causes of illness infection in the world (Mead *et al.*, 1999). Therefore, alternative antimicrobial strategies are urgently needed. This situation had led to a re-evaluation of the therapeutic use of ancient remedies, such as plants, plant-based products and honey.

The aim of this study was therefore to evaluate the antibacterial potential of light and dark honey on multi-drug resistant *Salmonella* species.

METHODOLOGY

Collection of *Salmonella* Isolates and Honeys

Eight clinical *Salmonella* isolates were collected from Department of Medical Microbiology Laboratory, Aminu Kano Teaching Hospital, Kano, Nigeria on sterile nutrient agar slants and transported to Department of Microbiology, Ahmadu Bello University, Zaria-Nigeria, for microbiological analysis.

Also, Fresh light and dark honey samples were obtained from a local bee keeper in Kano Metropolis, Kano State, Nigeria and transported to Department of Microbiology,

Ahmadu Bello University, Zaria, for screening of their antibacterial activities.

Reconfirmation of Clinical *Salmonella* Isolates

The clinical *Salmonella* isolates collected from Department of Medical Microbiology Laboratory, Aminu Kano Teaching Hospital, Kano, Nigeria were reconfirmed by cultural and biochemical tests.

A loop full of the isolate collected on nutrient agar slant was streaked on Xylose Lysine Deoxychocolate (XLD) agar (Oxoid, UK) and incubated over night at 37°C. Red colonies with or without black centres on XLD were suspected to be *Salmonella* species. The suspected colonies were then inoculated on to nutrient agar slants and stored in a refrigerator for biochemical reconfirmations.

The suspected *Salmonella* isolates were first purified, by streaking on XLD agar plate from nutrient agar slant and incubated overnight at 37°C. The pure isolated colonies were then used to characterized the organisms biochemically. Urease, Lysine Iron Agar (LIA), Triple Sugar Iron (TSI) agar tests and the commercially-available identification system-API 20E (bio Mereux, France) were used to reconfirmed the isolates.

After reconfirming the *Salmonella* spp. biochemically, the pure cultures were further streak on nutrient agar slants and stored at 4°C until needed for antibiotics susceptibility test and antimicrobial activity of honey.

Determination of Multi-Drug Resistant *Salmonella* Isolates

Each of the isolate was standardized by emulsifying 4–5 colonies of the isolate from nutrient agar plate into sterile normal saline and the turbidity adjusted to 1.5×10^8 CFU/mL (corresponding to 0.5 McFarland standards). The antibiotics susceptibility patterns of each of the isolate was determined by using Kirby-Bauer disk agar diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2010) using commercially available antimicrobial discs; Ampicillin (10µg), Cefotaxime (30µg), Trimethoprim-Sulfamethoxazole (1.25+23.75µg), Ciprofloxacin (5µg) and Chloramphenicol (30µg), (all Oxoid, UK) . Mueller Hinton (MH) agar (Oxoid, UK) was prepared according to the manufacturer's

instructions and poured on the plates and allowed to solidify. A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the MH agar plates. The plates were allowed to dry for 3–5 minutes. Thereafter, the antibiotic discs were aseptically placed on the plates and pressed gently to ensure complete contact with the agar. A distance of at least 15mm was maintained from the edges of the plates to prevent overlapping of inhibition. It was then incubated for 24 hours at 37°C. After 24hrs, the zones of inhibitions were measured and interpreted according to (CLSI, 2010) guidelines. Isolates that were resistance to two or more classes of antibiotics were said to be multi-drug resistant and were further inoculated onto nutrient agar slants and stored at 4°C until needed for their sensitivity to honey.

Honey Sterility Test

The honey samples were first inoculated each separately on a nutrient agar and incubated for 24hours at 37°C, to observe any microbial growth and count colonies.

Honey intended to be used for medical use should contain less than 10cfu/g before sterilization and 0cfu/g after sterilization (Molan and Allen, 1996). Sterilization of the honey samples were carried out using gamma irradiation, a method of sterilization that does not affect the non-peroxide activity of honey. After sterilization, the honey samples were kept in dark room at 20-30°C. Light and heat affect the antibacterial activity of honey (Molan, 1992).

Preparation of Different Concentrations of Honey

Two honey varieties were used; dark and light honey. Different concentrations of each honey constituting, 10% v/v, 20% v/v, 50% v/v and 75% v/v were made in sterile distilled water. This was done by dissolving the respective volumes: 0.1mL, 0.2mL, 0.5mL, 0.75mL of each honey into corresponding volumes of sterile distilled water to give a 1mL preparation.

Agar Well Diffusion Method

The antibacterial of activities of the honeys were tested using agar well diffusion method. A Muller Hinton Agar (MHA) was prepared and sterilized. Twenty five milliliter each of the MHA was poured aseptically into sterile

petri-dishes and allowed to solidify for 30minutes. A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly to inoculate the MH agar plates. The plates were allowed to dry for 5-10 minutes. A sterile cork borer was used to create wells of 6mm in the Mueller Hinton agar and a distance of at least 15mm was maintained from the edges of the plates to prevent overlapping of inhibition zones. Then, the wells were aseptically filled with different concentration of the honey using a micro pipette and the plates incubated at 37°C for 2–5 days. They were then examined and the diameters of the zones of inhibition were measured.

Determination of Minimum Inhibitory Concentrations (MIC)

The MIC is the lowest concentration that is able to inhibit any visible bacterial growth on the culture tube (Prescott *et al.*, 2008). MH broth was employed for MIC determination. A stock solution of 50% concentration (being the lowest significant concentration of the zones of inhibitions) of the honey sample was made by dissolving 5ml of honey in 5ml of sterile Mueller Hinton broth in test tube making 10ml. A 5ml from the stock solution was serially diluted into five-fold, other test tubes each containing 5ml of MH broth to made a final concentration of 25%, 12.5%, 6.25%, 3.125% and 1.562. A loopful of a standardized inoculum were dispense into each serially diluted test tube. A negative and positive control tubes were prepared containing 5ml honey with no organism, and 5ml broth medium with the test organism but no honey respectively. The tubes were incubated at 37°C for 24hours. After incubation, turbidity of each test tube was visually inspected. The clear tubes were indication of no growth. The lowest concentration that showed no growth was the MIC break point i.e. the lowest concentration of the honey sample that shows no visible sign of growth/turbidity.

Determination of Minimum Bactericidal Concentration (MBC)

MBC is the lowest concentration of the sample that prevents bacteria growth after incubation or required to kill the organism (Prescott *et al.*, 2008). This was obtained by streaking out the samples from the MIC tubes that showed no visible growth on nutrient agar plates.

However, MBC were indicated by failure of the organism to grow on the media plates after 24hrs incubation at 37°C.

RESULTS AND DISCUSSION

High rate of multi-drug resistance among *Salmonella* spp. was observed in this study. The major reason might be due to the inappropriate and irrational use of antimicrobials which provides favourable conditions for resistant organisms to emerge and spread. For instance, when patients do not take the full course of prescribed antibiotics, or when substandard antimicrobials are used; resistant organisms can emerge and spread (Simpson *et al.*, 2006; WHO, 2011). In the present study, the isolates showing resistance against 2 or more group of antibiotics were considered MDR strains. Among the 8 isolates, 7(87.5%) were multi-drug resistant. In recent years, the prevalence of antimicrobial resistant bacteria pathogens has become a major public health concern and increasing antimicrobial resistance in *Salmonella enterica* is a serious clinical problem worldwide (Therlfall *et al.*, 1994). During the last decade, a single clone of multi resistant *S. enterica* serovar Typhimurium of phage type DT104 has spread worldwide. This *Salmonella* type has emerged within a few years as one of the most common causes of human salmonellosis in several countries. The majority of the DT104 isolates have a multidrug resistance phenotype called ACSSUT; which means that they are resistant to ampicillin,

chloramphenicol, streptomycin, sulphonamides and tetracycline (Therlfall *et al.*, 1994).

In the present study, two honey samples (Dark and Light honeys) were tested for their antibacterial activity against multi-drug resistant *Salmonella* isolates. Three of the four different concentrations of both honey samples (20, 50 and 75vv%) showed growth inhibitory activity against MDR *Salmonella* isolates. This contrasts with the result reported by Hegazi (2011) and Hegazi and Fyrouz (2012), who reported that the different types of Saudi honey were less inhibitory against *Salmonella*. The study showed different value of zone of inhibitions of Dark and Light honeys against the tested Isolates. These might be due to the followings: osmotic effect, effect of pH, and sensitivity of these organisms to hydrogen peroxide, these are unsuitable for bacterial growth and represented as an inhibition factor in honey (Minisha and Shyamapada, 2011).

In this study, the isolates were more susceptible to the dark honey than light honey. This was in agreement with the research carried out by (UIUC, 1998), that dark honey has more illness-fighting agents than light honey which is because, darker honey has less water content and more antioxidants than light-colored honey. Antioxidants are substance that slow the oxidation of other substances, counter the toxic effects of free radicals, which can cause DNA damage that can lead to age-related problems such as arthritis, strokes and cancer. Free radicals are atoms or molecules that are usually reactive or unstable. (UIUC, 1998).

Table 1: Zone of Inhibition of Antibiotics used against *Salmonella* Isolates

ID	Zone of Inhibition (mm)				
	AMP(10µg)	C(30µg)	CFS(30µg)	SXT(25µg)	CIP(5µg)
AKTH1	06 R	20 S	24 S	06 R	32 S
AKTH2	06 R	06 R	24 S	06 R	34 S
AKTH3	06 R	19 S	24 S	06 R	31 S
AKTH4	06 R	22 S	25 S	06 R	32 S
AKTH5	06 R	06 R	14 R	06 R	32 S
AKTH6	06 R	15 I	15 I	06 R	33 S
AKTH7	12 R	23 S	23 S	17 S	37 S
AKTH8	06 R	06 R	25 S	06 R	31 S

Key: ID = Isolate Number; AMP = Ampicillin; C = Chloramphenicol; CFS = Cefotaxime; SXT = Trimethoprim-Sulfamethoxazol; CIP = Ciprofloxacin; S = Susceptible; I = Intermediate; R = Resistant

Table 2: Percentages of Antibiotic Resistant, Intermediate and Susceptible *Salmonella* Isolates

Antibiotics	R (%)	I (%)	S (%)
AMP (10µg)	100.0	0.0	0.0
C(30µg)	37.5	12.5	50.0
CFS (30µg)	12.5	12.5	75.0
SXT(25µg)	87.5	0.0	12.5
CIP (5µg)	0.0	0.0	100

Key: AMP= Amoxicillin; C= Chloramphenicol; CFS= Cefotaxim; SXT= Trimethoprim-Sulphamethoxazol; CIP: Ciprofloxacin; R = Resistant; I = Intermediate; S = Susceptible

Table 3: Phenotypic Susceptibility Patterns of *Salmonella* Isolates

ID	Resistance	MDR
AKTH1	AMP, SXT	+
AKTH5	AMP, C, SXT	+
AKTH8	AMP, SXT	+
AKTH13	AMP, SXT	+
AKTH16	AMP, C, CTX, SXT	+
AKTH17	AMP, CTX, SXT	+
AKTH19	AMP	-
AKTH20	AMP, C, SXT	+
	MDR	(87.5%)

Key: ID= Isolate Number; AMP = Ampicillin; C = Chloramphenicol; CTX =Cefotaxime; SXT = Trimethoprim-Sulfamethoxazole and CIP = Ciprofloxacin; MDR = Multi-Drug Resistant

Table 4: Growth Inhibitory Activity of Dark Honey against Multi-drug Resistant *Salmonella* Species

ID	Concentration (vv%)			
	10	20	50	75
AKTH1	0	10	25	37
AKTH5	0	11	27	38
AKTH8	0	11	26	38
AKTH13	0	11	26	37
AKTH16	0	12	28	40
AKTH17	0	15	30	43
AKTH20	0	10	26	37

Key: ID = Isolate Number

Table 5: Growth Inhibitory Activity of Light Honey against Multi-drug Resistant *Salmonella* Species

ID	Concentration (vv%)			
	10	20	50	75
AKTH1	0	05	12	20
AKTH5	0	06	14	19
AKTH8	0	06	14	20
AKTH13	0	05	12	18
AKTH16	0	05	13	17
AKTH17	0	06	15	20
AKTH20	0	06	15	19

Key: ID = Isolate Number

Table 6: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Dark and Light Honeys against Multi-Drug Resistant *Salmonella* Species

ID	MIC (%v/v)		MBC (%v/v)	
	Dark Honey	Light Honey	Dark Honey	Light Honey
AKTH1	6.25	12.5	12.5	25.0
AKTH5	6.25	12.5	12.5	25.0
AKTH8	6.25	12.5	12.5	25.0
AKTH13	6.25	12.5	12.5	25.0
AKTH16	6.25	12.5	12.5	25.0
AKTH17	6.25	12.5	12.5	25.0
AKTH20	6.25	12.5	12.5	25.0

Key: ID= Isolate Number; MIC=Minimum Inhibitory Concentration; MBC= Minimum Bactericidal Concentration.

Conclusion

High rate of MDR 7(87.5%) was noticed against the 8 *Salmonella* isolates. Furthermore, the results in the present study revealed that dark honey as well as light honey have both bacteriostatic as well as bactericidal activity against multi-drug resistant-*Salmonella* species. It was observed that, dark honey inhibited all MDR *Salmonella* isolates at quite lower concentration 12.5% (v/v) as compared to light honey 25% (v/v).

These results indicate the effectiveness of honey particularly dark honey against multi-drug resistant *Salmonella* species. Nevertheless, the results in the present study support the notion that there is marked variation in the antibacterial activity of honey between their different colours. Therefore, it is extremely important to evaluate honeys antibacterial activity before therapeutic applications.

In overcoming the issue of drug resistant, further research can be carried out in vivo to evaluate the potential of honey as an alternative to antibiotics as therapeutic agent in certain medical conditions, particularly typhoid fever.

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