PHYTOCHEMICAL COMPOSITION AND COMPARATIVE ASSESSMENT OF ANTIBACTERIAL ACTIVITY OF HONEY AGAINST CLINICAL BACTERIAL ISOLATES BY AGAR DIFFUSION TECHNIQUES.

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ABSTRACT

Phytochemical screening and antibacterial activities of a South-Eastern Nigeria honey against clinical bacterial isolates were carried-out using standard phytochemical and microbiological methods. Phytochemical screening indicated the presence of alkaloids, saponins, flavonoids, cardiac-glycosides and terpenes. The bacteria which were isolated from clinical samples-stool, sputum, urine as well as nasal, throat and high vaginal swabs were characterized and identified as *Staphylococcus aureus* (SA); *Staphylococcus epidermidis* (SE); *Staphylococcus saprophyticus* (SS); *Streptococcus pyogenes* (SP); *Escherichia coli* (EC); *Pseudomonas aeruginosa* (PA); *Salmonella typhi* (ST); *Proteus mirabilis* (PM); *Shigella dysentariae* (SD); and *Vibrio cholerae* (VC). The antibacterial activity of the Nigerian honey comparatively assayed by the Agar-Well Diffusion (AWD) and Agar-Disc Diffusion (ADD) techniques, indicated broad antibacterial activity, which was significantly ($P < 0.05$) greater for the ADD than AWD; but the potencies comparatively assessed by activity –index was non-significant ($P > 0.05$) between AWD and ADD. The antibacterial activity of the Nigerian honey, against the clinical bacterial isolates was concentration-dependent. The Minimum Inhibitory Concentration (MIC) assayed by the macrobroth dilution technique, indicated MIC range of 6.25 – 25.0 mg/ml, which was lowest for SS, SE, SD and VC (6.25 mg/ml) and highest for both PA and ST (25.0 mg/ml). The Minimum Biocidal Concentration (MBC) and the establishment of the mode of activity, assayed by the macrobroth dilution technique, indicated basically static activity at elevated concentrations against the clinical bacterial isolates.

INTRODUCTION

Honey is the natural sweet substance produced by honey-bees (*Apis mellifera*), from nectar blossom of wild and cultivated flowers; or as secretory and excretory products of plants which honey-bees collect, process in combination with some of their specific substances and store as ripened-matured honey (Crane, 1980; Isaac 2006; Udoidem *et al.,* 2012). Since honey is a plant based product, and man has explored and exploited plants derivatives from antiquity era for food and medicaments, etc; and as such honey used and production has a long and varied history. In many cultures, the use of honey has gone beyond its use as food. However, honey is secreted by bees as a food source; and during scarcity or adverse weather conditions, bees use their stored honey as their source of energy (Standifier 2007).

Meanwhile, people have been able to either semi-or wholly-domesticated bees by contriving them to nest in artificial hives to enhance efficient production and collection of the honey (Udoidem *et al*; 2012). The classification of honey has been extensively reviewed by Udoidem *et al*. (2012). This was based on such previously reported parameters as floral source, the packaging and processing techniques used (Isaac, 2006; Gounari, 2006). Accordingly, based

on floral source, there are blended honey, polyfloral honey, monofloral honey and honey-dew honey. Also, based on packaging and processing, honey is classified as crystallized, pasteurized, raw, strained, ultra-filtered, ultrasonicated, whipped, dried, comb and chunk honey.

Honey has been widely reported to posses, antimicrobial activities including its used for the treatment of ulcer, diarrhea and prevention of cancer and as wound- healing agent (Jeddar *et al.*, 1985; Efen, 1988; Green, 1988; Allen *et al.*, 1991; Greenwood, 1993; Allen *et al,* 2000; Batista *et al.,* 2004; Kucuk *et al.*, 2007; Udoidem *et al.*, 2012). Thus, this study reports the phytochemical composition and antibacterial activity of natural honey from South-Eastern Nigeria on clinical bacterial isolates assessed by the agar-diffusion techniques.

MATERIALS AND METHOD

Honey Collection, Pretreatment and Solubility

The natural honey used for the study was collected *in situ* from a homogenous apiculture at the Michael Okpara University of Agriculture, Umudike, Abia State, South Eastern Nigeria. The honey was aseptically filtered to remove dead-bees, honey-wax and other debris, to reduce any residual or associated microbial load and stored in closed containers at 4 ^{o}C for subsequent assays. The honey was solubilized by mixing equimolar volumes with 1 % $(^{w}/_{v})$ polysorbate 80, devoid of any antimicrobial activity under the conditions of the test and maintained in a water-bath at 40 ^{o}C for all assays.

Phytochemical Screening

Phytochemical screening of the natural honey was carried-out, according to the standard procedures (Harborne, 1973, Trease and Evans, 1989, Sofowora, 1993).

Isolation and Characterization of Clinical Bacterial Isolates

Clinical samples comprising stool, sputum, urine as well as nasal, throat and high vaginal swabs were aseptically and respectively collected *in situ* from patients at the Out-Patient Department (OPD) of the University of Uyo Teaching Hospital, (UUTH) into samples-stoppered bottles containing 10 ml of Nutrient Broth (NB). They were immediately taken to the laboratory for processing and cultivation. The isolation and enumeration of bacterial types in the samples were aseptically carried out by using 1.0 ml aliquots of ten-fold serially diluted samples in general purposes and selective/differential media (Nutrient-Agar (NA), MaConkey agar (MCA), Salmonella-Shigella Agar (SSA) Cystine Lactose Electrolyte Deficient agar (CLED), Mannitol-Salt Agar (MSA); Blood Agar (BA); Eosin-Methylene Blue agar (EMB); Triple Sugar Iron agar (TSI) and Thiosulphate Citrate sucrose Bile Salt agar (TCBS)) by the standard pour-plate technique and incubated at 37 ^{o}C for 48 h (Collins and Lyne, 1979). Pure cultures of the isolates were obtained by repeated streaking on freshly prepared NA plates and stored at 4 ^{o}C as agar slants cultures. The isolates were subsequently characterized and identified based on standard biochemical and physiological properties.

Antibacterial potency and Activity spectrum of Honey on Clinical bacterial isolates.

The antibacterial activity spectrum of the natural honey against the clinical bacterial isolates, was comparatively evaluated by the modified agar-well diffusion and disc-agar diffusion techniques, on NA plates incubated at 37 ^{o}C for 24 h (Ekong *et.al*, 2004). Thereafter, the Inhibition Zone Diameters (IZD), denoting the potency of the natural honey against the clinical bacterial isolates was measured in duplicates. Standard antibiotic: Gentamicin 30µg/ml served as positive control. The potency of the natural honey with respect to the control was assessed by activity-index and calculated as the ratio of antibacterial activity of honey to that of the control. The higher the activity index value, the more potent the honey against the clinical bacterial isolates.

Effects of Honey Concentration on Antibacterial Activity

Different concentrations of the honey natural 50 % ($^{v}/_{v}$) were aseptically prepared with sterile 1 % ($^{v}/_{v}$) polysorbate 80 in a water bath at 40 ^{o}C. Thereafter, the antibacterial activity of the respective concentrations was assayed by the agar-well and agar-disc diffusion techniques as previously described in this work.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Biocidal Concentrations (MBC)

The MIC of the honey for each of the clinical bacterial isolates was determined by the macrobroth dilution method (Tilton and Howard, 1987; Baron and Finegold 1990), with some modifications (Ekong *et al.*, 2004), using equivalent volumes of two-fold serially diluted honey concentration in nutrient broth (NB). The tubes were inoculated with 0.1 ml aliquots of the respective clinical bacterial isolates, and incubated at 37 ^{o}C for 24 h. Uninoculated NB served as control. After incubation, the MIC of the honey against the clinical bacterial isolates were taken as the least concentrations of the honey that inhibited the growth of the isolated bacterial cultures, with respect to turbidity of the control. Similarly, the MBC of the honey against the clinical bacterial isolates was determined by the macrobroth dilution technique as previously described, from the further incubation of the non-turbid MIC tubes at 37 ^{o}C for 48 h. The presence or absence of turbidity in the overnight incubated MIC tubes indicated either-static or cidal mode of activity for the honey against the clinical bacterial isolates Ekong *et al.*, 2004).

RESULT AND DISCUSSION

The results of the phytochemical analysis of the South –Eastern Nigeria honey indicated remarkable presence of flavonoids and terpenes, moderate presence of alkaloids, saponins and cardiac-glycosides; while tannins, anthraquinones and phlobatannins were absent (Table 1).

Table 1: Phytochemical Composition of the Nigeria Honey

Properties	Inference
Alkaloids	++
Saponins	++
Tannins	-
Flavonoids	+++
Anthraquionoes	-
Cardiac-glycosides	
(i) Liberman test	++
(ii) Keller kiliani test	+ (deoxy-sugar)
(iii) Salkowsk test	++
Terpenes	+++
Phlobatannins	-

– = absent; + = slightly present; ++ = moderately present; +++ = abundantly present.

These findings are completely in line with that previously reported by Kucuk *et al.,* (2007) and recently confirmed by Udoidem *et al.,* (2012) . The presence of these phytochemicals in honey had been widely reported to have very high and useful antimicrobial activities including anti-ulcer, anti-diarrhoeal, and anti-cancer activities, Greenwood, 1993; Ali, 1995; Wood *et .al* 1997; Allen, 2000; Batista *et al.,* 2004; wound-healing activity, Green 1988; Wood *et al.,* 1997; Cooper *et al*., 2000; 2000; Willix *et al.,* 2000; Belts and Molan, 2001; Subrahamanvan *et al.,* 2003; Alwaili, 2004); and preservative- agent (Jedda *et al.,* 1985*;* Burgget, 1995; Lee 1996; Udoidem *et al.,* 2012). Thus, the laudable antimicrobial activities of the natural Nigeria honey could be attributed to the presence of these phytochemicals (Radwan *et al.,* 1984; Kucuk, 2007). Specifically Alkaloids have been reported to be clinically important and possess anti-cancer, antibacterial, antimalarial, antipyrectic anti-neoplasic, antiasthma, saponins which are basically emulsifying agents have been reported to have potentials as antibacterial, anti-exudative agents for wound treatment, antihyaluronidase; anti-ulcerogenic, anti-inflammatory, antifibrinolytic, antipyrectic, as well as antidermatous activities; flavonoids possess similar

pharmacological effects to alkaloids, plus their antioxidant actvitiy against free-radicals, antiallergic and anti-inflammatory. Nevertheless, besides the phytochemicals. The antimicrobial activity of honey have been attributed to the proximate composition, particularly its low moisture content (17.70 %) which invariably results in low water-activity (a_w) for microbial growth acidity content osmotic-effect as well as hydrogen-peroxide activities, which together are inhibitory to bacterial growth (Kucuk *et al.*, Jedder, 1985; 2007).

The results of the antibacterial activity and potency of the Nigerian honey against the clinical bacterial isolates in the study revealed a broad-spectrum of antibacterial activity which was significantly ($P < 0.05$) higher against the Gram-negative than Gram-positive bacteria (Table 3). The antibacterial activity assessed by the ADD test were significantly ($p<0.05$) higher than those of the AWD. These variations may be predominantly attributed to such factor as depths of the wells in the AWD, (as all other factors were similar), which impeded or slowed the diffusion rate of the honey from the wells to the media compared to the prompt diffusion from the discs in the ADD (Tilton and Howard; 1987; Baron and Finegold, 1990). However, irrespective of the significant variation in antibacterial activity between the two assay techniques, the potency of honey recorded by the two techniques were insignificant ($P > 0.05$). In most cases, some AWD potencies were higher than those of ADD under the conditions of the test and vice-versa. These results are of high clinical significance, since these clinical bacterial isolates are the etiologic agents of many mild, opportunistic and fatal superficial and systemic infections, including Urinary Tract Infections, UTI's (*Staphylococcus saprophyticus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli; Proteus mirabilis; Pseudomonas aeruginosa);* respiratory tract, wound and skin-infections (*Staphylococcus* aureus; *Staphylococcus epidermidis; Streptococcus pyogenes; Pseudomonas aeruginosa*); as well as enteric fever and gastroenteritis such as diarrhea, dysentery and cholera (*Staphylococcus aureus; Escherichia coli, Salmonella typhi, Proteus mirabilis, Shigella dysentariae and Vibrio Cholerae*). Hence, natural honey could be formulated into a herbal remedy for chemotherapy in the containment of these problematic etiologic agents.

The result of honey concentrations on antibacterial activity indicated the direct variations of antibacterial activity of the natural honey with concentration. This was highest with the high concentrations which gradually falls with the decreasing concentrations and stabilized at the low concentrations (Table 4). This may possibly suggest that the antibacterial activity of the natural honey is concentration and pH-dependent. This is expedient in that dilution increases the pH of the natural-undiluted honey from strongly acidic pH (3.2 – 4.3), to weakly acidic and basic pH (4.5 – 9.4) at the concentrations tested in the study. The decline in antibacterial activity of honey at high pH values, due to dilution has been reported (Allen *et al.*, 1991). Hence, in raw and undiluted honey, the high acidity is a significant antibacterial factor, but if diluted, the antibacterial activity and effectiveness are reduced or inactivated.

The Minimum Inhibitory Concentration (MIC) and Minimum Biocidal Concentrations (MBC) of the natural honey against the clinical bacterial isolates are shown in Table 5. The MIC indicated remarkably lower static activity ranging between 6.25 – 12.5 mg/ml against all the Gram-positive and most of the Gram-negatives isolates, except *Pseudomonas aeruginosa* and *Salmonella typhi* which recorded an elevated MIC of 25.0 mg/ml respectively. The lower MIC values recorded for the Gram-positive isolates notably the staphylococci, are clinically interesting and further add credence to the potency of the natural honey. This may be a promissory development in chemotherapy for the containment of these resistant pathogens. Similar low MIC values have been reported for honeys and bacterial isolates from other countries (Jeddar *et.al.*,1985; Molan,1992; Molan and Russel, 1998). The MBC for the establishment of mode of activity were between the range 25.0 – 50.0 mg/ml for all the bacterial isolates, except *Pseudomonas aeruginosa* and *Salmonella typhi*. The presence of turbidity and numerous colonies of the test organisms from the non-turbid MIC tubes indicated predominantly bacteriostatic activity of the natural honey against the bacterial isolates.

Table 2 : Cultural/biochemical characterization and identification of the clinical bacterial isolated

Media/Colony Description	Colony morphology	biochemical tests										sugar fermentation test					Probable organism
		Gram reaction	Motility	Indole	Methyl-red	Voges Proskaeur	Catalase	Coagulase	Citrate	Urease	Oxidase	Man.	Suc	Man	Lac.	Glu	
MSA- yellow colonies BA- yellow colonies with β-haemolysis	cocci in cluster	+	-	-	-	+	+	+	-	-	-	A	A	AG	A	AG	*Staphylococcus aureus*
MSA–yellow colonies BA – white colonies no β- haemolysis	cocci in cluster	+	-	-	-	+	+	-	-	+	-	A	A	A	-	A	*Staphylococcus epidermidis*
MSA – light yellow colonies BA – white colonies, no heamolysis, slow growth	cocci in cluster	+	-	-	-	+	+	-	-	-	-	AG	A	AG	A	A	*Staphylococcus saprophyticus*
BA – white colonies with β- haemolysis	cocci in chains	+	-	+	-	+	-	-	-	-	-	AG	AG	A	A	A	*Streptococcus pyogenies*
MCA – red, non mucoid colonies EMB –green metallic sheen colonies CLED- yellow colonies	short rods	-	+	+	+	+	-	-	-	-	-	A	AG	A	AG	A	*Escherichia coli*
NA – blue –green spreading colonies EMB – non-pigmented or light purple colonies	short rods	-	+	+	+	+	+	-	+	+	-	-	A	A	-	A	*Pseudomonas aerugenosa*
MCA – Light purple colonies SSA – black colonies TSI –black colonies	straights rods	-	+	-	+	+	-	-	-	+	-	A	A	-	-	A	*Salmonella typhi*
MCA –non pigmented colonies EMB – light purples colonies TSI – black colonies	rods	-	+	-	+	-	-	-	-	+	-	A	A	-	-	A	*Proteus mirabilis*
MCA- light purple colonies SSA- black colonies TSI –black colonies	rods	-	-	-	+	±	-	-	+	-	-	AG	A	A	-	A	*Shigella dysentariae*
MCA- colourless colonies TCBS – yellow colonies	curved rods	-	+	-	+	±	-	-	+	-	+	AG	-	A	-	-	*Vibrio cholerae*

NA = Nutrient agar; BA = Blood agar; MSA = Mannitol salt agar; MCA = MacConkey agar; EMB = Eosin methylene blue agar; SSA = Salmonella – Shigella agar; CLED= Cystine Lactose electrolyte deficient agar; TSI = Triple sugar iron agar; TCBS = Thiosulphate citrate bile salt agar; + = positive reaction or growth; - = Negative reaction or no growth; ± = Present or absent; A = Acid production; AG = Acid/gas production.

Tables 3: Comparative Antibacterial Activity and Potency of Nigeria Honey Assessed by AWD and ADD Techniques

Bacterial isolates	Antibacterial Activity/ Inhibition Zone Diameter (mm)					
	AWD			ADD		
	Ym	Yi	Standard (Gentamicin)	Ym	Yi	Standard (Gentamicin)
SA	7.0	0.50	14.0	14.0	0.78	18.0
SE	9.0	0.64	14.0	13.0	0.59	22.0
SS	8.0	0.50	16.0	13.0	0.65	20.0
SP	10.0	1.0	10.0	14.0	0.78	18.0
EC	10.0	0.67	15.0	12.0	0.67	18.0
PA	9.0	0.56	16.0	13.0	0.76	17.0
ST	9.0	0.75	12.8	11.0	0.69	16.0
PM	10.0	0.67	15.0	12.0	0.75	16.0
SD	12.0	0.75	16.0	15.0	0.83	18.0
VC	13.0	0.81	16.0	16.0	0.89	18.0

Ym = Mean of duplicate measurement; Yi = potency by activity index

Table 4: Effect of honey concentration on antibacterial activity

Bact-erial Isolate	Honey concentration ($\%^{v}/_{v}$) / Inhibition zone diameter (mm).											
	AWD						ADD					
	50	40	30	20	10	mean (SEM)	50	40	30	20	10	Mean* (SEM)
SA	7.0	6.0	5.0	5.0	5.0	5.6 ± 0.4	8.0	6.0	5.0	5.0	5.0	5.8 ±0.5
SE	8.0	7.0	6.0	6.0	5.0	6.4 ±0.5	10.0	8.0	7.0	5.0	5.0	7.0 ±0.8
SS	8.0	6.0	5.0	5.0	5.0	6.0 ± 0.5	8.0	7.0	6.0	5.0	5.0	6.5 ± 0.5
SP	7.0	6.0	6.0	5.0	5.0	5.8 ± 0.3	8.0	7.0	6.0	6.0	5.0	6.2 ±0.5
EC	8.0	6.0	5.0	5.0	5.0	5.8 ± 0.5	10.0	8.0	6.0	5.0	5.0	6.8 ± 0.9
PA	8.0	7.0	6.0	5.0	5.0	6.2 ± 0.5	11.0	6.0	6.0	6.0	6.0	7.0 ±0.9
ST	7.0	5.0	5.0	5.0	5.0	5.4 ± 0.4	8.0	6.0	5.0	5.0	5.0	5.8 ±0.5
PM	8.0	7.0	7.0	6.0	5.0	6.6 ± 0.5	9.0	8.0	7.0	5.0	6.0	7.0 ±0.6
SD	9.0	8.0	7.0	6.0	6.0	7.2 ± 0.5	11.0	10.0	8.0	7.0	7.0	8.4 ±0.8
VC	9.0	8.0	8.0	6.0	6.0	7.4 ± 0.5	12.0	10.0	9.0	7.0	8.7	9.0 ±0.8

* = significant at ($p< 0.05$).

Table 5: MIC and MBC of Honey against Clinical Bacterial Isolates

Clinical bacterial isolates	MIC	MBC	MIC/MBC index
SA	12.5	25.0	+
SE	12.5	25.0	+
SS	6.25	12.5	+
SP	12.5	25.0	+
EC	6.25	25.0	+
PA	25.0	50.0	+
ST	25.0	50.0	+
PM	12.5	25.0	+
SD	6.25	12.5	+
VC	6.25	12.5	+

+ = presence of growth (static activity)

The bacteriostatic activity of the natural honey seemed to increase with the concentration used. This property possibly adds to the earlier assertion and confirmed the natural honey used in this study to be concentration-dependent.

CONCLUSION AND RECOMMENDATION

This study has shown that the natural honey from South-Eastern Nigeria, exhibited concentration and pH dependent bacteriostatic antibacterial activity against the bacterial isolates. This was significantly higher in the assessment by ADD than AWD techniques. The antibacterial activity may be associated with the presence of phytochemicals like; alkaloids, saponins, flavonoids, cardiac-glycosides and terpenes; as well as high-acidity and low water activity. These findings have revealed the efficacy and precision of the ADD as precise analytical technique in antimicrobic- culture sensitivity evaluation. It has also revealed the potential and efficacy of natural honey in the formulation and production of potent pharmaceuticals to combat the myriads of bacterial infections.

REFERENCES

Al- Waili, N, S. (2004). Investigation of the antimicrobial activity of natural honey and its effects on the pathogenic bacteria infections of surgical wounds and conjuntivitis. *Journal of Medicine and Food*, 10(5):112-117.

Ali, A. T. (1995). Natural honey accelerates healing of indomethacin-induced arthral ulcers in rats. *Saudi-Arabia Medical Journal*, 16 (2): 161 – 166.

Allen, K. L., Molan, P. C. and Reid, G. M. (1991). A survey of the antibacterial activity of some New- Zealand honey. *Journal of Pharmacy and Pharmacology* 43: 812 – 822.

Allen, K. L., Radwan, S., and Reid, G. M. (2000). Antimicrobial potential of honey on some microbial isolates. *Medical and Pharmaceutical Sciences*, 2:75- 79.

Armon, P. J. (1980). The use of honey in the treatment of infected wounds. *Tropical Doctors*, 10: 91 – 93.

Baron, J. E. and Finegold, S. M. (1990). Methods for Testing Antimicrobial Effectiveness . *In*: C. V. Mosby (eds). *Bailey Scotts Diagnostic Microbiology* (8ed). Missouri, USA. 171 – 174

Batisa, M., De Almeida, A., De Pietro-Magri, L., Toma, W., Calvo, T., Villegas, W, and Souza, A. (2004). Gastric and anti-ulcer activity of *Syngonanthus arthroticus* (Bill). *Pharmaceutical Bulletin* 27 (3):328 -332.

Belts, J. A. and Molan, P. (2001). A pilot trial of honey as a wound dressing. *A conference of the European Wound Management Association*, Dublin Ireland.

Burgget, O. (1995). Osmotic pressure *In: The Illustrated Encycolpaedia of Bee Keeping,* Hooper T. Publishers Inc., New York, pp 11 – 15.

Collins, C. H. and Lyne, A. (1979). *Microbiological Methods* (4ed). Butterworth, London.

Cooper, R. A., Molan, P. C., Halas, E., Davies, R. and Harding, K. G. (2000). The inhibition of Gram-positive cocci of clinical importance by Honey. *1st World wound Healing Congress*, Melbourne, Australia.

Crane, B. (1980). *A Book of Honey*. Oxford University Press, London. PP. 198 – 216.

Dunford, C., Copper, R. and Molan, P. (2000). Using honey as a dressing for infected skin lessons. *Nursing Times,* 1: 92 – 96.

Dustmann, J. A. (1979). *Antibacterial effect of honey*. Apiacta, 14 (1): 7 – 11.

Efen, S. E. (1988). Clinical observations on wounds healing properties of honey. *British Journal of Surgey,* 75: 679 – 681.

Ekong, U. S., Mgbor, N. C., Moneke, A. N. and Obi, S. K. C. (2004). Evaluation of the antimicrobial and some pharmacokinetic properties of an antibiotic substance produced by an environmental *Aspergillus* species SK2 isolated from soil. *Nigerian Journal of Microbiology*, 18: (1.2); 199 – 206.

Green, A. E. (1988). Wound healing properties of honey. *British Journal of Surgery,* 75 (12): 1278 – 1283.

Greenwood, D. (1993): Honey for superficial wounds and ulcers. *Lancet*, 88 (37): 90 – 100.

Harbone, J. (1973). *Phytochemical Methods*. Chapmall and Hall Publishers, London, pp. 41 – 78.

Isaac, B. (2006). *Definition of Honey and Honey Products*. Chapman and Hall Publisher, New-York. Pp. 21-33.

Jeddar, A., Kharsany, U., Bhanjee, A, and Moosa, A. (1985). The antibacterial action of honey. *South Africa Medical Journal, 67: 257 – 258.*

Kucuk, M., Kolaylis, C., Karaoglu, S., Ulusoy, E. and Baltaic, C. (2007). Biological activities and chemical composition of three honeys of different types from Anatolia. *Food Chemistry,* 100: 526 – 534.

Lee, C. (1996). Substitution of honey for sulphur-dioxide in grape juice processing. *American Bee Journal,* 136: 872 – 873.

Molan, P. (1992). The antibacterial properties of honey. *Bee world,* 73: 59 – 76.

Molan, P. and Russed, K. (1998). Non-peroxide antibacterial activity in some New Zealand Honeys. *Journal of Apiculture Research,* 27: 62 – 67.

Sofowora, E.(1993). Research on medicinal plants in Africa. *Journal of Alternative Complementary Medicine,* 2 (3) 365 – 372.

Standifier, L. (2007). *Honey Bee Nutrition and Supplementary Feeding.* Oxford University press, New-York, USA, pp.37-59.

Subrahamanyam, M., Shahapure, A. G. and Nagame, N. S. (2003). Free radicals control the mechanism of the action of honey in burns. *Annals of Burns and Fire Disasters,* 16: 135 – 137.

Tilton, R. C. and Howard, B. J. (1987). Antimicrobial Susceptibility Testing. *In*: B. J. Howard *et. al* (eds), *Clinical and Pathogenic Microbiology,* C. V. Mosby, Missouri, USA.

Trease, G. and Evans, W. (1989). *Pharmacognosy (4ed).* Macmillan Publisher, Nigeria, pp. 25 – 35.

Udoidem, I. U., Ekong, U.S., Udofia, U.S. and Antai, C. O. (2012). Phytochemical screening and preservative potential of natural honey. *Journal of Agriculture, Biotechnology, and Ecology,* 5(2): 91 -99

Willix, D. J., Molan, P. C. and Harfoot, G. G. (2000), A comparison of the sensitivity of wound –infecting species of bacteria to the antibacterial activity of Manuka honey and other honeys. *New Zealand Medical Journal,* 12: 201 – 206.

Wood, B., Rade-Maker, M. and Molan, P. (1997). Manuka honey: a low cost regular Wound dressing. *New Zealand Medical Journal,* 10 (46): 107 – 110.