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ANTIMICROBIAL ACTIVITIES AND PHYSICO-CHEMICAL ANALYSES
OF HONEYS FROM Hypotrigona sp., Melipona sp. and Apis mellifera (BEE HONEY)

BY

NWEZE, JUSTUS AMUCHE
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DEPARTMENT OF MICROBIOLOGY
FACULTY OF BIOLOGICAL SCIENCES
UNIVERSITY OF NIGERIA, NSUKKA

JUNE, 2016
ANTIMICROBIAL ACTIVITIES AND PHYSICO-CHEMICAL ANALYSES
OF HONEYS FROM Hypotrigona sp., Melipona sp. and Apis mellifera (BEE
HONEY)

BY

NWEZE, JUSTUS AMUCHE

PG/MSC/13/64893

A THESIS SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY,
FACULTY OF BIOLOGICAL SCIENCES, AS PART OF THE
REQUIREMENTS FOR THE AWARD OF MASTERS OF SCIENCE
(MEDICAL MICROBIOLOGY) DEGREE OF UNIVERSITY OF NIGERIA.

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DEPARTMENT OF MICROBIOLOGY

UNIVERSITY OF NIGERIA, NSUKKA
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Sherlock, O., Dolan, A., Athman, R., Power, A., Gethin, G., Cowman, S., et al. (2010). Comparison of the antimicrobial activity of Ulmo honey from Chile and
Manuka honey against methicillin-resistant *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. *BMC Complementary and Alternative Medicine*, 10, 47.


CERTIFICATION

This is to certify that Nweze Justus Amuche (PG/MSC/13/64893) a postgraduate student in the Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, has satisfactorily completed the requirements for the award of degree of Masters of Science (M.Sc) in Medical Microbiology. The work embodied in this thesis is original and has not been submitted in part or full for any other diploma or degree of this or any other University.

----------------------------------------      ----------------------------------------
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                                                Nsukka
DEDICATION

This project is lovingly dedicated to Almighty God
ACKNOWLEDGEMENTS

My immense gratitude to God Almighty for the knowledge, wisdom, and guidance and above all, love bestowed upon me during this period of my life. If not of Him I wouldn’t be where I am because He is my strength and at any point in time that I thought I was down to nothing God was up to something.

I feel great honour and indebtedness in expressing my incessant gratitude to my eminent supervisor Professor (Mrs) J. I. Okafor, for her benevolence, constant guidance and keen interest throughout my study period, which enabled me to accomplish the assigned research task. I also wish to express my special and sincere thanks to Dr. I. E. Nweze and Dr. E.A. Eze for their help, guidance and valuable suggestions during the execution of this project.

My special thanks and gratitude the Head of Department, Professor A. N. Moneke, lecturers and staff in the Department of Microbiology, University of Nigeria, Nsukka for their valuable suggestions in designing this work.

I would like to thank Dr. Ike, Department of Zoology, University of Nigeria, Nsukka for his help in identification of the honey producing insects, *Hypotrigona* and *Melipona* species. I earnestly believe that the success of my present research assignment has become possible only due to the cordial, helpful and deep interest of my colleagues, Mr. Chidebelu Paul, Mr. Onoyima Nnaemeka, Mr. Akpi Uchenna, Miss Roda and Mr. Obi J. Okechukwu, who provided me with some of the test organisms.

I would like to express my appreciation to my family who support and encourage me to do my best in this postgraduate studies. Special thanks to my twin brother, Mr. Nweze Julius for his assistance and encouragement.
ABSTRACT

Honey has been used traditionally for ages to treat infectious diseases. Antimicrobial activity of honey is complex due to the involvement of multiple bioactive compounds. The physico-chemical and antimicrobial properties of honey varieties from *Apis mellifera* and stingless bees, *Hypotrigona* sp. (Okotobo - Igbo) and *Melipona* sp. (Iffufu - Igbo) were studied using International Honey Commission protocols and microbiological methods (agar-well diffusion and broth microdilution) respectively. A total of nine honey samples (3 from each) were used. The physico-chemical analyses of the honey varieties showed that the honeys had mean pH range of 3.73±0.08 - 4.24±0.20. Honey samples from *Hypotrigona* sp. had the highest mean moisture (17.50 ± 0.80 %), total dissolved solids (370.01 ± 22.51 ppm), hydromethylfurfural (16.58 ± 0.37 mg/kg), total acidity (35.57 ± 0.42meq/kg), protein content (16.58 ± 0.37 g/kg)and phenol content (527.41 ± 3.60 mg/kg). *Melipona* sp. honey had the highest average flavonoids (86.39 ± 4.69 mg/kg), total sugar (80.71 ± 1.37 % (g/100g) and reducing sugar (75.64 ± 1.99 % (g/100g) contents. There were no statistically significant differences between the mean pH, electrical conductivity and protein contents of *A. mellifera* and *Melipona* sp. honeys (p< 0.05). Several strong correlations were observed among some of the physicochemical properties of these honey varieties. In the initial antimicrobial activity testing, *Hypotrigona* sp. honey samples had statistically the highest mean inhibition zones diameter (mm) against MDR *Staphylococcus aureus* (7.14 ± 4.11), *Klebsiella pneumonia* (7.92 ± 3.96), *Pseudomonas aeruginosa* ATCC 25783 (9.77 ±4.58), MDR *S. enterica* (6.96 ± 4.03), and *Aspergillus niger* (10.12 ± 5.51).The minimum inhibitory concentrations (MICs) of the honey varieties from *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. ranged from 6.3 – 25.0%, 3.1 – 12.5% and 6.3 – 25.0% (v/v) respectively. There were no statistically significant differences between the mean MICs of *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp.honeys against *P. aeruginosa* ATCC 25783 (7.64 ± 2.76, 7.28 ± 4.14 and 8.33 ± 3.31 % v/v respectively).*Hypotrigona* sp. honey had the least mean MICs (4.15 ± 1.58 – 11.11 ± 2.76 % v/v) against most of the test organisms. The minimum biocidal concentration (MBC) of the honey varieties from *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. against the test organismsvaried from 6.3 – 50%, 3.1 – 25% and 12 – 50% (v/v) respectively. There were no statistically significant differences between the mean MBCs of the honey varieties against *Klebsiella pneumonia*(p = 0.669), *P. aeruginosa* ATCC 25783 (p = 0.977), *A. niger*(p = 0.688) and *C. albicans* (p = 0.168).The honey varieties had exceptional levels of hydrogen peroxide-dependent activity, and non-peroxide activity against the test organisms. This research has also shown that the honey varieties varied significantly in their physicochemical and antimicrobial properties. ‘Okotobo’ and ‘ифufu’ honeys that are both not consumed as widely as regular bee honeyhave shown to contain bioactive compounds and have antimicrobial properties similar to those of regular bee honey.
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<th>Description</th>
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<tr>
<td>%</td>
<td>Percentage</td>
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<tr>
<td>A. niger</td>
<td>Aspergillus niger</td>
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<tr>
<td>ABS&lt;sub&gt;450&lt;/sub&gt;</td>
<td>Colour Intensity</td>
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<tr>
<td>AM</td>
<td>Apis mellifera</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>B. cereus</td>
<td>Bacillus Cereus</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
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<tr>
<td>C. tropicalis</td>
<td>Candida tropicalis</td>
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<tr>
<td>CEQ</td>
<td>Catechin equivalents</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
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<tr>
<td>DNSA</td>
<td>3,5-dinitrosalicylic acid</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>EC</td>
<td>Electrical conductivity</td>
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<tr>
<td>GAEs</td>
<td>Gallic acid equivalents</td>
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<tr>
<td>HMF</td>
<td>Hydroxymethylfurfural</td>
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<tr>
<td>HY/HYP</td>
<td>Hypotrigona sp.</td>
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<tr>
<td>IHC</td>
<td>International Honey Commission</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>Klebsiella pneumonia</td>
</tr>
<tr>
<td>KW</td>
<td>Kruskal Wallis</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum Biocidal Concentration</td>
</tr>
<tr>
<td>MBCC</td>
<td>Minimum Inhibitory Concentration for catalase treated honey</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi Drug Resistance</td>
</tr>
<tr>
<td>MEP</td>
<td>Melipona sp.</td>
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<tr>
<td>Meq</td>
<td>Miliequivalent</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHB</td>
<td>Mueller Hinton Agar</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<tr>
<td>MICC</td>
<td>Minimum Inhibitory Concentration for catalase treated honey</td>
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mm  Millimeter
NZI  No zones of inhibition
P. aeruginosa  Pseudomonas aeruginosa
S. aureus  Staphylococcus aureus
S. enterica  Salmonella enterica
SD  Standard Deviation
SDB  Sabouraud Dextrose Broth
SPSS  Statistical Package for Social Sciences
TDS  Total dissolved solids
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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Traditional medicine has been used to treat infections since the origin of mankind and honey is one of the oldest medicines considered as a remedy for microbial infections (Cooper et al., 2009). It was not until late 19th century that researchers discovered that honey has natural antimicrobial qualities (Zumla and Lulat, 1989). Resistance to antibiotics continues to rise and few new therapies are on the horizon, there is further increased interest in the antimicrobial potency of honey (Fahim et al., 2014). Previous studies showed that honey had remarkable antimicrobial activity against fungi, bacteria, viruses and protozoa (Molan, 1992; Sherlock et al., 2010; Mohapatra et al., 2011; Fahim et al., 2014).

Honey is a natural sweet mixture produced by honey insects from the nectar of flowers or from living parts of plants. The insect transform the nectar into honey by combining this mixture with substances of their own. The mixture is then regurgitated, dehydrated and stored in the waxy honeycomb inside the hive to ripen and mature for further use (Iurlina and Fritz, 2005). Honey is composed mainly of carbohydrates, smaller amount of water and a great number of minor components. Sugars are the main constituents of honey, constituting of about 95%. Honey characterization is based on the determination of its chemical, physical or biological properties (Gomes et al., 2010).

Even though honey is produced worldwide, its composition and antimicrobial activity can be variable, and are dependent primarily on their botanical origin, geographical and entomological source (Maryann, 2000). Other certain external factors, such as harvesting season, environmental factors, processing and storage condition, also play important roles (Gheldof and Engeseth, 2002). Entomologically, the honey variety produced by honey bees (the genus *Apis*) is one most commonly referred to, as it is the type of honey collected by most beekeepers and consumed by most people in Nigeria. Honeys produced by other insects (stingless insects) have different properties (Sherlock et al., 2010).

Antimicrobial activity of honey is highly complex due to the involvement of multiple compounds and also due to large variations in the concentrations of these compounds among honeys. It depends on osmotic effect (sugar concentration), hydrogen peroxide, and low pH, as well as more recently identified compounds, methyl glyoxal and antimicrobial peptide, bee defensin-1 (Fahim et al., 2014).
contains significant antioxidant contents including glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids and proteins. The biological properties that make it ideal as a medicine are: antibacterial, bacteriostatic, anti-inflammatory, wound and sunburn healing effects, antioxidant activity, radical scavenging activity and antimicrobial activity (Al-Mamary et al., 2002; Gheldof and Engeseth, 2002; Beretta et al., 2005; Aljadi and Kamaruddin, 2004; Fahim et al., 2014).

Generally, the darker the honey is the higher its phenolic content and its antioxidant power. Antioxidant activity depends on the botanical origin of honey and shows variations in different honeys acquired from different sources (Küçük et al., 2007). Furthermore, antimicrobial activity of honey is linked to hydrogen peroxide which is produced by glucose oxidase especially when honey is diluted. In the diluted form of honey, produced hydrogen peroxide is not tissue damaging and is an important stimulant of the growth of tissues and has the potential for wound healing (Malik et al., 2010). The hydrogen peroxide activity of most of the honeys can be destroyed by heat or by the presence of catalase. However, some honeys retain their antimicrobial activity even in the presence of catalase which are known as “non-peroxidase honeys”. This activity is important especially in the context of topical antimicrobial and wound dressing fluids (Bang et al., 2003; Malik et al., 2010).

Honey is considered among the possible alternatives, which is natural, non-toxic and with broad spectrum of action. This could be a promising substitute or supplement to antimicrobial agents, but some factors limits its use. Clinical applicability of honey has been hindered by incomplete knowledge of the antimicrobial activity and lack of precise mechanisms for determining the type of activity of honey, variations of honey and its cost in some countries (Malik et al., 2010; Mandal and Mandal, 2011).

In Nigeria, honey is considered to be important in traditional treatment of respiratory ailments, surface infections, diarrhoea and other diseases as reported by Eleazu et al., 2013. There are enormous reports on the physico-chemical, antimicrobial, microbiological and medicinal properties of honey from different parts of the world, including North America, Europe, Asia, Australia, and South Africa (Cooper et al., 2009; Gomes et al., 2010; Mandal and Mandal, 2011; Fahim et al., 2014). There is paucity of information on Nigerian honey, although some researchers (Adesunkanmi and Oyelami, 1994; Adebiyi et al., 2004; Omafuvbe and Akanbi,
2009, Anyanwu, 2012; Eleazu et al., 2013; Buba et al., 2013) have reported antimicrobial activity and some chemical and physical properties of honey collected from Nigeria. The research on antifungal activity of Nigerian honey is also scarce (Anyanwu, 2012). All the reports so far have shown that Nigerian honeys have bacteriostatic and bactericidal activities against wide range of bacteria and fungi (Akujobi and Njoku, 2010; Anyanwu, 2012; Eleazu et al., 2013; Buba et al., 2013).

However, most of the antimicrobial studies against pathogenic bacteria, viruses and fungi were carried out using honey produced by the honey bee (Eg. *Apis mellifera*), while it appears that no research has been done on honey produced by other insects. To our understanding, nothing has been done on the antimicrobial potential of these honeys indigenous to Nsukka produced by other insects (not bees) like *Hypotrigona* spp. and *Melipona* spp. This study will focus on the analysis of these different honey samples. Prior to antimicrobial activity testing, the physico-chemical properties (such as pH, colour, ash, protein, and moisture contents, hydroxymethyl furfural contents, total sugar contents, reducing sugar and non-reducing sugar contents), antimicrobial properties (non-peroxidase/peroxidase activity) and antioxidative properties (such as phenol and flavonoid contents) of various ‘none bee’ honey samples were analyzed to assess their quality.

1.2. STATEMENT OF PROBLEM

Antimicrobial agents (such as newly synthesized antimicrobial drugs) are essentially important in reducing the global burden of infectious diseases. However, the rapid increase in multiple drug resistant microorganisms associated with conventional agents has necessitated the search for new antimicrobial agents that are safer and less expensive. Therefore, a re-evaluation of the therapeutic use of ancient remedies, such as honey is clearly needed.

1.3. AIM OF THE STUDY

To evaluate the *in vitro* antimicrobial activities of honeys from *Apis mellifera* and stingless bees, *Hypotrigona* spp. and *Melipona* spp. against some bacterial and fungal species.

1.4. OBJECTIVES OF THE STUDY

- To evaluate and compare the physicochemical properties of the honey varieties.
- To correlate some physico-chemical properties of the honey varieties.
- To evaluate the antimicrobial activities of the honey varieties using agar well diffusion methods.
To determine and compare the minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) of the honey varieties.

To determine non-peroxidase antimicrobial activities of the honey varieties.

1.5. LITERATURE REVIEW

1.5.1. Ancient Use of Honey as a Medicine

Honey is known as one of the oldest medicines and has been used by almost all societies on earth, dating back to 210 B.C. It has been used in different places such as Spain, India, Africa and Australia (Molan, 1998; Joshi et al., 2008). It has been not only been used as food, but also it has been used to treat many clinical conditions such as gastrointestinal tract diseases, ulcers and ophthalmic problems (Khan et al., 2007).

Evidences of old pictures drawn on rocks have been found to support that the honey was gathered and used ages before even written history. Honey was mentioned in very old civilisations like the Sumerians and Babylonians (Ransome, 2004). Indeed, the medical use of honey was stated in Ancient Egyptian records as well as in that of the Ancient Greeks (Simon et al., 2009). The use of honey by Ancient Egyptians was recorded in the Edwin's Smith surgical papyrus (Jones et al., 2000). This papyrus is believed to be written before 1700 B.C. (Ransome, 2004; Chattopadhyay et al., 2009). Ancient Greeks were probably, the first civilisation to keep hives for honey production (Ransome, 2004; Chattopadhyay et al., 2009). They believed that honey is an essential healthy food (Crane, 1999; Ransome, 2004; Chattopadhyay et al., 2009).

Also, more than 15,000 years ago, paintings in caves near Valencia in Spain showed people collecting honey (Joshi et al., 2008). In addition, almost all religions and folk beliefs have mentioned honey as a good food for people's health. For example, when the Children of Israel were in Egypt, the promise to them was 'land flowing with milk and honey'. In Islam more than 1,400 years ago, both the holy Quran and Hadith (Prophet Talk) referred to honey as a healer of disease. This appears in the special chapter of Quran on honey and the translation (16:68-69) is “And your Lord revealed to the bee saying: Make hives in the mountains and in the trees and in what they build. Then eat of all the fruits and walk in the ways of your Lord submissively. There comes forth from within it a beverage of many colours, in which there is healing for men; most surely there is a sign in this for a people who reflect” (Crane, 1975; Ali, 1989). Also, honey was mentioned in the Bible. Solomon in
his proverbs (24:13) has advised his son: “my son, eat thou honey for it is good” (Joshi et al., 2008; Cooper et al., 2009).

Honey was used for healing of wounds for more than 4,000 years. The use of honey to cover wounds was recorded in Ancient Egypt (Gunther, 1934). Aristotle (384-322 B.C.) is one of the famous ancient physicians who used honey for wound healing. He mentioned that pale honey was “good as a salve for sore eyes and wounds”. Dioscorides (c. 50 AD) is another famous ancient physician and he has stated that yellow honey was the best, being “good for all rotten and hollow ulcers” (Zumla and Lulat, 1989; Molan, 1998).

Chattopadhyay et al. (2009) mention that details of using of honey for wound cure practices in the Middle Ages have been documented from 1392. In recent years, many researchers have concluded that honey is a good therapy for wounds (Molan and Betts, 2004; Bell, 2007; Evans and Flavin, 2008; Robson et al., 2009).

1.5.2. Honeys as Modern Medicine

In the last 30 years interest in the use of honey as a treatment agent has increased. Most research that has been undertaken has focused on the employment of honey in wound treatment (Ahmed et al., 2003; Berguman et al., 1983; Dumronglert, 1983; Emarah, 1982; Wadi et al., 1987; Ingle et al., 2006).

Efem (1988) reported the first large clinical cohort study involving 59 patients who had a variety of wounds such as Fournier’s gangrene, burns and ulcers. The use of honey on these patients resulted in successful wound healing and the clearance of infection. In addition, Subrahmanyam (1993, 1994, 1996, 1998) and Subrahmanyam et al., 2001, 2003, 2007) reported several clinical trials on burns patients with honey compared to various different treatments.

Clinically, many researchers have studied the uses of honey in wound management and it has been reported to clear wound pathogens rapidly (Al-Waili and Saloom, 1999; Lusby et al., 2002), to stimulate immune response and to reduce inflammation (Molan and Betts, 2004; Tonks et al., 2003) and to support the debridement of wounds by autolysis (Stephen-Haynes, 2004). In addition, honey has been reported to have a deodorising property on wounds, due to the oxidation of glucose by bacteria resulting in production of lactic acid rather than malodorous compounds such as ammonia, sulphur compounds and amines produced by the breakdown of amino acids (Cooper, 2005; Molan, 2002; Stephen-Haynes, 2004). Moreover, honey has been used effectively on skin grafts (Schumacher, 2004),
diabetic foot ulcers (Eddy and Gideonsen, 2005), malignant ulcers (Simon et al., 2005) and abscesses (Okenyi et al., 2005). Some researchers have observed that honey promotes tissue regeneration through the stimulation of angiogenesis and the growth of fibroblasts and epithelial cells (Efem, 1988, 1993; Stephen-Haynes, 2004; Subrahmanyan, 1994, 1998). Fast healing can therefore minimise the need for skin grafts (Subrahmanyan, 1998).

Recently, (Gethin et al., 2008) observed that the use of manuka honey as a wound dressing reduced wound pH which in turn decreased protease activity, increased fibroblast activity and released more oxygen from haemoglobin to promote rapid wound healing. Furthermore, after honey is applied to the wound, it forms a film of liquid between the wound and the dressing that prevents the dressing from sticking to the wound, reducing pain and not damaging the newly formed cells. As honey has no adverse effects on tissue, it can be used on wounds safely and introduced into cavities and sinuses to clear infection (Molan, 2000).

1.5.3. Some Physical and Chemical Composition of Natural Honey

Honey according to Codex Alimentarius Commission (1989) has several important qualities in addition to composition and taste. Freshly extracted honey is a viscous liquid. Its viscosity depends on large variety of substances and therefore varies with its composition and particularly with its water content. Hygroscopicity is another property of honey and describes the ability of honey to absorb and hold moisture from environment. Normal honey with water content of 18.8% or less will absorb moisture from air of a relative humidity of above 60%. The surface tension of honey varies with the origin of the honey and is probably due to colloidal substances. Together with high viscosity, it is responsible for the foaming characteristics of honey (Olaitan et al., 2007).

Natural honey contains about 200 substances, including amino acids, vitamins, minerals and enzymes, but it primarily contains sugar and water. Sugar accounts for 95–99% of honey dry matter. The principal carbohydrate constituents of honey are fructose (32.56 to 38.2%) and glucose (28.54 to 31.3 %), which represents 85–95% of total sugars that are readily absorbed in the gastrointestinal tract (Ezz El-Arab et al., 2006; Moundoi et al., 2001).

Other sugars include disaccharides such as maltose, sucrose, isomaltose turanose, nigerose, meli-biose, panose, maltotriose, melezitose. A few oligosaccharides are also present. Honey contains 4 to 5% fructo-oligosaccharides,
which serve as probiotic agents (Chow, 2002; Ezz El-Arab et al., 2006). Water is the second most important component of honey. Organic acids constitute 0.57% of honey and include gluconic acid which is a by-product of enzymatic digestion of glucose. The organic acids are responsible for the acidity of honey and contribute largely to its characteristic taste (Olaitan et al., 2007). The concentration of mineral compounds ranges from 0.1% to 1.0%. Potassium is the major metal, followed by calcium, magnesium, sodium, sulphur and phosphorus. Trace elements include iron, copper, zinc and manganese (Sampath et al., 2010; Lachman et al., 2007; Rashed and Soltan, 2004).

Nitrogenous compounds, vitamins C, B1 (thiamine) and B2 complex vitamins like riboflavin, nicotinic acid, B6 and panthothenic acid are also found (Jagdish and Joseph, 2004). Honey contains proteins only in minute quantities, 0.1–0.5 percent (Lee et al., 1998). According to a recent report, specific protein quantities differ according to the honeybee origin (Won et al., 2009).

A variety of enzymes such as oxidase, invertase, amylase, catalase, etc. are present in honey. However, the main enzymes in honey are invertase (saccharase), diastase (amylase) and glucose oxidase. They have an important role in the formation of honey (Olaitan et al., 2007). The enzyme glucose oxidase produces hydrogen peroxide (which provides antimicrobial properties) along with gluconic acid from glucose which helps in calcium absorption. Invertase converts sucrose to fructose and glucose. Dextrin and maltose are produced from long starch chains by the activity of amylase enzyme. Catalase helps in producing oxygen and water from hydrogen peroxide (Bansal et al., 2005).

**Colour Characteristics**

The colour in liquid honey varies from clear and colourless (like water) to dark amber or black. The various honey colours are basically all shades of yellow and amber. Colour varies with botanical origin, age, and storage conditions, but transparency or clarity depends on the amount of suspended particles such as pollen (Olaitan et al., 2007). Less common honey colours are bright yellow (sunflower), reddish undertones (chest nut), grayish (eucalyptus) and greenish (honeydew). Once crystallized, honey turns lighter in colour because the glucose crystals are white. Honey crystallization results from the formation of monohydrate glucose crystals, which vary in number, shape, dimension, and quality with the honey composition and
storage conditions. The lower the water and the higher the glucose content of honey, the faster the crystallization (Olaitan et al., 2007).

In all over the world, dark honeys are especially appreciated. The most commonly used methods are based on optical comparison, using simple colour grading after Pfund (Fell, 1978) or Lovibond (Aubert and Gonnet, 1983). The values of these comparators give a measure of colour intensity, but only along the normal amber tone of honey. The Lovibond comparators are easier to handle than the Pfund graders, but honey is generally marketed according to the Pfund colour scale. That is why at present Lovibond graders with a Pfund scale are marketed.

**Electrical conductivity**

The measurement of electrical conductivity (EC) was introduced a long time ago (Vorwohl, 1964). At present it is the most useful quality parameter for the classification of unifloral honeys, which can be determined by relatively inexpensive instrumentation. This has been confirmed by the data, published in this issue (Persano-Oddo and Piro, 2004). On the basis of an extensive survey of EC values on honeys originating from different parts of the world (Bogdanov et al., 2002), this parameter was included recently in the new international standards for honey (Codex Alimentarius, 2001; European Commission, 2002), replacing the determination of ash content. Indeed, EC correlates well with the mineral content of honey, (Accorti et al., 1987). In these standards maximal EC values for blossom honeys (except chestnut honey) are introduced for differentiation between honeydew and blossom honeys.

The method for the determination of electrical conductivity is described in Bogdanov et al. (2002). Honey EC values are expressed in milli Siemens/cm at 20 °C, while nowadays the international reference measurements should be carried out at 25 °C. This contradiction needs to be resolved.

**pH and acidity**

All honeys are acidic with a pH-value generally lying between 3.5 and 5.5, due to the presence of organic acids that contribute to honey flavour and stability against microbial spoilage. In honey the main acid is gluconic acid, which is found together with the respective glucono-lactone in a variable equilibrium (White et al., 1958). Free acidity, total acidity and pH have some classification power for the discrimination between unifloral honeys, while lactones, due to their strong variability, do not provide useful information (Persano-Oddo and Piro, 2004). The methods for the determination of free acidity by titration to pH 8.3 or to an
equivalence point have a poor reproducibility (Bogdanov et al., 2002), due to lactone hydrolysis during titration. The reproducibility of the measurement of total activity (free acidity + lactones) is slightly better (Conte et al., 2002).

**Acids**

According to Codex Alimentarius (2001) and European Commission (2002), the flavour of honey results from the blending of many “notes,” not the least being a slight tartness or acidity. The acids of honey account for less than 0.5 percent of the solids, but this level contributes not only to the flavour, but is in part responsible for the excellent stability of honey against microorganisms. Several acids have been found in honey, gluconic acid being the major one. It arises from dextrose through the action of an enzyme called glucose oxidase. Other acids in honey are formic, acetic, butyric, lactic, oxalic, succinic, tartaric, maleic, pyruvic, pyroglutamic, α-ketoglutaric, glycollic, citric, malic, 2- or 3-phosphoglyceric acid, α- or β-glycerophosphate, and glucose 6-phosphate.

**Water content**

The water content is a quality parameter, important above all for honey shelf life. It has a minor importance for the characterisation of unifloral honeys. However, depending on the production season and the climate, unifloral honeys show some typical differences in water content, which affect the physical properties of honey (viscosity, crystallisation) and also influence the value of the glucose/water ratio (Persano-Oddo and Piro, 2004). However, water content can be artificially altered during honey processing.

Moisture is routinely determined by refractometry by an Abbé analogue refractometer. Digital refractometers can also be used for the determination of water content, as the results achieved are not significantly different from those obtained with the analogue ones. The values, determined by refractometry are somewhat lower than the true water content, which can be measured only by Karl Fischer titration (Bogdanov, 1999).

**Enzymes: diastase and invertase**

One of the characteristics that sets honey apart from all other sweetening agents is the presence of enzymes. These conceivably arise from the bee, pollen, nectar, or even yeasts or micro-organisms in the honey. Those most prominent are added by the bee during the conversion of nectar to honey. Enzymes are complex protein materials that under mild conditions bring about chemical changes, which may
be very difficult to accomplish in a chemical laboratory without their aid. The changes that enzymes bring about throughout nature are essential to life. Some of the most important honey enzymes are invertase, diastase, and glucose oxidase (Bogdanov et al., 2008).

Invertase, also known as sucrase or saccharase splits sucrose into its constituent simple sugars, dextrose, and levulose. Other more complex sugars have been found recently to form in small amounts during this action and in part explain the complexity of the minor sugars of honey. Although the work of invertase is completed when honey is ripened, the enzyme remains in the honey and retains its activity for some time. Even so, the sucrose content of honey never reaches zero. Since the enzyme also synthesizes sucrose, perhaps the final low value for the sucrose content of honey represents an equilibrium between splitting and forming sucrose (Ortiz-Vázquez et al., 2013).

Diastase (amylase) digests starch to simpler compounds but no starch is found in nectar. What its function is in honey is not clear. Diastase appears to be present in varying amounts in nearly all honey and it can be measured. It has probably had the greatest attention in the past, because it has been used as a measure of honey quality in several countries (Bogdanov et al., 2008).

Glucose oxidase converts dextrose to a related material, a gulconolactone, which in turn forms gluconic acid, the principal acid in honey. Since this enzyme previously was shown to be in the pharyngeal gland of the honey bee, this is probably the source. Here, as with other enzymes, the amount varies in different honeys. In addition to gulconolactone, glucose oxidase forms hydrogen peroxide during its action on dextrose, which has been shown to be the basis of the heat-sensitive antibacterial activity of honey. Other enzymes are reported to be present in honey, including catalase and an acid phosphatase. All the honey enzymes can be destroyed or weakened by heat (DebMandal and Mandal, 2011).

The methods for the determination of diastase and invertase activity are described (Bogdanov et al., 1997). Later, another formula was found for the diastase determination with the Phadebas method in honeys with low enzyme content (Persano-Oddo and Pulcini, 2004). For the expression of invertase results, international units (U/kg) were proposed instead of Hadorn numbers (von der Ohe et al., 1999). These changes were included in the online IHC methods.

**Hydroxymethylfurfural**
Fresh honey does not contain hydroxymethylfurfural (HMF). Thus, HMF is not a criterion for the botanical classification of honey. However, before determining storage-dependent parameters like enzyme activity and colour, one should ensure that honeys are fresh and unheated. Before testing these parameters, it should be checked that the HMF content is below 15 mg/kg (European Commission, 2002).

Three methods for the determination of HMF are described and validated by the IHC (Bogdanov et al., 1997). Only two of them are recommended for use: the HPLC and the White method. The Winkler method should not be used because one of the reagents (p-toluidine) is carcinogenic. Since the publication of the IHC methods there is a change in the procedure of the HMF determination by HPLC: a Carrez treatment of the honey solution is necessary in order to prevent HMF break-down (DebMandal and Mandal, 2011).

**Phenolic acids and polyphenols**

Phenolic acids and polyphenols are plant derived secondary metabolites. These compounds have been used as chemotaxonomic markers in plant systematics. They have been suggested as possible markers for the determination of botanical origin of honey. Considerable differences in composition and content of phenolic compounds between different unifloral honeys were found. Dark coloured honeys are reported to contain more phenolic acid derivatives but less flavonoids than light coloured ones (Alvarez-Suarez et al., 2013, 2010, 2009).

**Proteins and Amino Acids**

The amount of nitrogen in honey is low, 0.04 percent on the average, though it may range to 0.1 percent. Recent work has shown that only 40 to 65 percent of the total nitrogen in honey is in protein, and some nitrogen resides in substances other than proteins, namely the amino acids. Of the 8 to 11 proteins found in various honeys, 4 are common to all, and appear to originate in the bee, rather than the nectar. Little is known of many proteins in honey, except that the enzymes fall into this class (European Commission, 2002; Alvarez-Suarez et al., 2013).

The presence of proteins causes honey to have a lower surface tension than it would have otherwise, which produces a marked tendency to foam and form scum and encourages formation of fine air bubbles. Beekeepers familiar with buckwheat honey know how readily it tends to foam and produce surface scum, which is largely due to its relatively high protein content (European Commission, 2002).
The amino acids are simple compounds obtained when proteins are broken down by chemical or digestive processes. They are the “building blocks” of the proteins. Several of them are essential to life and must be obtained in the diet. The quantity of free amino acids in honey is small and of no nutritional significance. Breakthroughs in the separation and analysis of minute quantities of material (chromatography) have revealed that various honeys contain 11 to 21 free amino acids. Proline, glutamic acid, alanine, phenylalanine, tyrosine, leucine, and isoleucine are the most common, with proline predominating (DebMandal and Mandal, 2011).

Amino acids are known to react slowly, or more rapidly by heating, with sugars to produce yellow or brown materials. Part of the darkening of honey with age or heating may be due to this. Proline, the main amino acid of honey, added to honey by the bee, is a criterion of honey ripeness (von der Ohe et al., 1991). This parameter shows characteristic values in different unifloral honeys (Sabatini and Grillenzoni, 2002; Persano-Oddo and Piro, 2004), roughly correlated with the enzyme activity (Sabatini and Grillenzoni, 2002).

However, the variation of this parameter in different unifloral honeys is quite high and it is not possible to classify unifloral honey on the basis of proline content only (Sanchez et al., 2001; Sabatini and Grillenzoni, 2002; Persano-Oddo and Piro, 2004). The proline content is easily determined by photometry (Bogdanov et al., 1997).

**Sugars**

Honey is above all a carbohydrate material according to Codex Alimentarius (2001) and European Commission (2002), with 95 to 99.9 percent of the solids being sugars, and the identity of these sugars has been studied for many years. Sugars are classified according to their size or the complexity of the molecules of which they are made. Dextrose (glucose) and levulose (fructose), the main sugars in honey, are simple sugars, or monosaccharides, and are the building blocks for the more complex honey sugars. Dextrose and levulose account for about 85 percent of the solids in honey.

Until the middle of this century, the sugars of honey were thought to be a simple mixture of dextrose, levulose, sucrose (table sugar), and an ill-defined carbohydrate material called “honey dextrin.” With the advent of new methods for separating and analyzing sugars, workers in Europe, the United States, and Japan have identified many sugars in honey after separating them from the complex honey.
mixture. This task has been accomplished using a variety of physical and chemical methods (European Commission, 2002).

Dextrose and levulose are still by far the major sugars in honey, but 22 others have been found. All of these sugars are more complex than the monosaccharides, dextrose and levulose. Ten disaccharides have been identified: sucrose, maltose, isomaltose, maltulose, nigerose, turanose, kojibiose, laminaribiose, $a$, $B$-trehalose, and gentiobiase. Ten trisaccharides are present: melezitose, 3-$a$-isomaltosylglucose, maltotriose, 1-kestose, panose, isomaltotriose, erlose, theanderose, centose, and isopanose. Two more complex sugars, isomaltotetroase and isomaltopentaose, have been identified. Most of these sugars are present in quite small quantities (Codex Alimentarius, 2001; European Commission, 2002).

Most of these sugars do not occur in nectar, but are formed either as a result of enzymes added by the honeybee during the ripening of honey or by chemical action in the concentrated, somewhat acid sugar mixture we know as honey (Bogdanov et al., 2008).

1.5.4. Factors contributing to Antimicrobial Properties of Honey

Until 1963, it was thought that the antimicrobial properties of honey were mainly because of hydrogen peroxide, but further studies have indicated that other physical factors like acidity, osmolarity (Molan, 1992) and electrical conductivity, and chemical factors including volatile compounds (Yao et al., 2003), antioxidant (Gheldoff et al., 2002; Henriques et al., 2006), beeswax, propolis and pollen (Viuda-Martos et al., 2008) play a considerable role in antimicrobial activity.

1.5.4.1. Osmotic Effect

Honey is a super saturated solution of sugar (80%) and water (17%). The osmolarity of honey inhibits microbial growth because of the strong interaction of sugar molecules with water molecules thus; insufficient water molecules are available to support microbial growth. This availability is known as water activity (aw). Water involved in many metabolic processes in many organisms. Depending on the permeability of cell membrane in each organism the water activity (aw) of many bacterial species vary between 0.94-0.99. The water activity of honey is 0.6 because of high sugar molecules and low water thus many species cannot grow in that environment. Fungi can tolerate a lower aw than bacteria, so reports of antifungal activity with diluted honey reveal that there are more factors involved than only the sugar content of honey. Also, Staph aureus has a high tolerance of low aw (0.86) ie.
can tolerate high NaCl level but not high sugar therefore, it is considered as one of the most susceptible species to the antibacterial activity of honey (Molan, 1992).

1.5.4.2. Acidity

Honey is quite acidic; normally, it has an average pH of 3.9 (with a typical range of 3.2 to 4.5). It has been known that this acidity is a result of the conversion of glucose to gluconic acid with help of glucose oxidase enzyme (Molan, 2001). The optimum pH for growth of many bacterial species is 7.2 – 7.4. However, the lowest pH value for growth of some wound pathogens is 4.3 for \textit{E. coli} and 4.4 for \textit{P. aeruginosa}. The low pH of honey is therefore important to slow down or inhibit bacterial growth (Bogdanov, 2009; Molan, 2000). Since 2001, the osmotic effect was thought to be the main factor for antimicrobial activity (Molan, 2001). However, in 2005 a study compared honey and sugar solution of same osmotic effect on coagulase negative staphylococci. The study confirmed that antimicrobial properties are not exclusively due to osmotic effect (French \textit{et al.}, 2005). It has been noted that the pH of honey also generates and maintains good environment for fibroblast activity (Lusby \textit{et al.}, 2002).

1.5.4.3. Hydrogen Peroxide Production

In 1919, Sackett reported that in diluted honey the antibacterial properties of honey were increased. This is because when honey is diluted, hydrogen peroxide is released with the help of an enzyme (glucose oxidase) that is found in honey (Molan, 1992). This enzyme is secreted by the hypopharyngeal gland of bees and added to nectar during honey formation (Alvarez-Suarez \textit{et al.}, 2013).

\begin{equation}
\text{Glucose} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{glucose oxidase} \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2
\end{equation}

Hydrogen peroxide (H$_2$O$_2$) is considered to be one of the main factors in antibacterial activity of honey. It is involved in cell multiplication in different cell types in the body as certain concentration of H$_2$O$_2$ can support epithelial cells and fibroblast growth to repair damage or injury (Burdon, 1995). It also promotes wound healing by regeneration of new capillaries (Tur \textit{et al.}, 1995). The enzyme (glucose oxidase) is inactive in full strength honey due to the low pH, so the diluting action of fluids produced by the wound is thought to activate glucose oxidase to produce hydrogen peroxide. In addition, it stays in the honey during storage without losing activity. Hydrogen peroxide was used for long time to disinfect wounds in hospitals. This chemical causes damage to the tissues and inflammation due to free radical that is produced. The levels of H$_2$O$_2$ in honey are around 1000 times lower than those
applied as antiseptic on wounds (Molan, 2001). As a result it does not inflame a wound or damage the tissue (Bang et al., 2003). Weston (2000) suggested that the level of \( \text{H}_2\text{O}_2 \) was related to floral source, and that it depended on the balance between the production and destruction rate of \( \text{H}_2\text{O}_2 \). Destruction of \( \text{H}_2\text{O}_2 \) is due to catalase which derives from both the pollen and the nectar of plants, and the amount of catalase in different sources is variable. In addition, Brudzynski (2006) studied the effect of \( \text{H}_2\text{O}_2 \) on the antibacterial activity of 42 honey samples from Canada. She found that the antibacterial activity was correlated with production of \( \text{H}_2\text{O}_2 \) in honey.

### 1.5.4.4. Non-Peroxide Components

Several efforts were made to identify the non-peroxide antibacterial components present in the honey (Allen et al., 1991). Weston et al., (1999) separated the antibacterial phenolic fraction (APF) from the honey which consisted of benzonic acids, cinnamic acids and flavonoids. It was determined that APF plays a small role in manuka honey as non-peroxide antibacterial component, therefore, there are other factors which were need to be identified. Honey contains a variety of polyphenolic compounds that may be capable of chelating metal ions and decreasing oxidation (Gheldof et al., 2002). Two important classes of phenolic compounds are flavonoids and phenolic acid which are known as natural antioxidants (Molan, 1992; Pyrzsnska and Biesaga, 2009; Yao et al., 2003). In a study performed by Wahdan (1998), two phenolic acids were extracted for the first time; these were caffeic acid and ferulic acid. Flavonoids had shown a range of biochemical and pharmacological actions, which affect the inflammatory cells and the generation of inflammatory processes (Viuda-Martos et al., 2008). The use of flavonoids in medicine is increasing due to their ability to trap free radicals, to stimulate hormones and neurotransmitters, and to inhibit specific enzymes which produce superoxide anions (Pyrzsnska and Biesaga, 2009).

However, it has been identified that several organic components in the ether extract of honey possess antibacterial activity; these include 3,5-dimethoxy-4-hydroxy benzoic acid (syringic acid), and methyl 3,5-dimethoxy-4- hydroxy benzoate (methyl syringate) (Alvarez-Suarez et al., 2013). By using high performance liquid chromatography (HPLC), some other flavonoids and phenolic acids have also been identified in different honeys, for example, pinocembrin, pinobanksin and chrysin.
(Bogdanove et al., 1989), gallic acid and abscisic acid (Yao et al., 2003) caffeic acid and ferulic acid (Wahdan, 1998), and vanillic acid, cinnamic acid, and benzoic acid (Weston et al., 1999; Weston et al., 2000).

1.5.4.5. Antioxidant Activity

Antioxidants are substances that protect wound tissues from being damaged by oxygen radicals. The free radicals may be produced by hydrogen peroxide and cause cellular damage. Free radicals are involved in cell toxicity and can alter cell biomolecules such as proteins, carbohydrates, lipids and nucleic acids causing cell death (Alvarez-Suarez et al., 2013).

Gheldof et al., (2002) analysed the antioxidant activity in different honey fractions and determined that most of the antioxidant components were found in the water-soluble fraction. These include gluconic acid, protein, ascorbic acid, hydroxymethylfuraldehyde, and the combined activities of the enzymes glucose oxidase, catalase and peroxidase. The same study also showed that the phenolic compounds in honey contributed very significantly to its antioxidant capacity.

When honey is diluted the release of high levels of hydrogen peroxide may lead to tissue damage by formation of free radicals such as hydroxyl and superoxide. Many honeys including manuka honey have the ability to quench free radicals. This property may play a role in reducing inflammation and chronic wound infection (Henriques et al., 2006)

A recent study was completed by Van de Berg et al., (2008) with regard to the antioxidant level in buckweat honey showing that this type of honey reduced the level of reactive oxygen species (ROS) which affect the wound healing process. Also, beside the low pH and high acidity buckwheat honey was shown to contain high amounts of phenolic components that aid the antimicrobial mechanisms and block the oxidative reaction system (Alvarez-Suarez et al., 2013). In addition, several reports demonstrated the relationship between the antioxidant and the colour of honey, where darker honey exhibited higher antioxidant content (Bogdanov et al., 2004; Estevinho et al., 2008; Turkmen et al., 2006). It has been thought that non-hydrogen peroxide activity in manuka honey may be due to plant derived components such as flavonoids and phenolic compounds. Recently, two research groups have reported that the activity of Leptospermum honeys correlates with the presence of methyglyoxal (MG), an alpha-oxoaldehyde that reacts with macromolecules such as DNA, RNA and proteins (Adams et al., 2008). High amount of MG was present in some manuka
honey which is equivalent to the non-peroxide activity. MG was, therefore, known as a bioactive complex responsible for the antibacterial activity in manuka honey (Adams et al., 2008).

Recently Atrott and Henle (2009) studied the presence of methylglyoxal in 61 samples of manuka honey. They found that the antibacterial activity ranged between 12.4% to 30.9% equivalent to phenol concentration.

More recently Kwakman et al., (2010) discovered an antibacterial bee peptide called bee defensin-1 in honey. To date this peptide has been isolated only from a honey used in the production of revamil and it was confirmed that this protein exhibits most of the antibacterial activity. The exact mechanism of bee defensin-1 on bacteria is not yet known.

1.5.7. Therapeutic Properties of Honey

Researchers have reported that honey is becoming acceptable as a reputable and effective therapeutic agent by Practitioners of conventional medicine and by the general public. Its beneficial role has been endorsed to its antimicrobial, anti-inflammatory and anti-oxidant activities as well as boosting of the immune system (Mohapatra et al., 2011; Sherlock et al., 2010; Buba et al., 2013; Fahim et al., 2014).

1.5.5.1. Antimicrobial Activity

The antimicrobial activity is very important therapeutically, especially in situation where the body’s immune response is insufficient to clear infection. In other words, it has shown powerful antimicrobial effects against pathogenic and non-pathogenic micro-organisms (yeasts and fungi) even against those that developed resistance to many antibiotics (Fahim et al., 2014). The antimicrobial effects could be bacteriostatic or bactericidal depending on the concentration that is used. However, such activity has been attributed to certain factors like high osmolarity (low water activity), acidity (low pH), and hydrogen peroxide and nonperoxide components (Sherlock et al., 2010).

Furthermore, honey is a supersaturated sugar solution; these sugars have high affinity for water molecules leaving little or no water to support the growth of micro-organisms (bacteria and yeast). Consequently, the micro-organisms become dehydrated and eventually die (Mohapatra et al., 2011). In addition, the natural acidity of honey will inhibit many pathogens.

According to Fahim et al. (2014), the usual pH range of most of the pathogens is around 4.0-4.5. However, the major antimicrobial activity has been found to be due
to hydrogen peroxide, produced by the oxidation of glucose by the enzyme glucose oxidase, when honey is diluted. As hydrogen peroxide decomposes, it generates highly reactive free radicals that react and kill the bacteria. In most cases, the peroxide activity in honey can be destroyed easily by heat or the presence of catalase.

Notwithstanding, some honeys have antibacterial action separate to the peroxide effect, resulting in a much more persistent and stable antibacterial action (non-peroxide activity). They are however called “non-peroxide honeys. Manuka honey (*Leptospermum scoparium*) from New Zealand and jelly bush (*Leptospermum polygalifolium*) from Australia are non-peroxide honeys which are postulated to possess unidentified active components in addition to the production of hydrogen peroxide. They retain their antimicrobial activity even in the presence of catalase (Buba *et al.*, 2013).

Weston (2000) suggested that the main part of this activity might be of honeybee origin, while part may be of plant origin. The compounds exhibiting this activity can be extracted with organic solvents (e.g. n-hexane, diethyl ether, chloroform, ethyl acetate) by liquid-liquid (Manyi-Loh *et al.*, 2010) or solid phase extraction methods (Aljadi and Yusoff, 2003). The extracted compounds have been reported to include flavonoids, phenolic acids, volatile compounds (ascorbic acid, carotenoid-like substances, organic acids, neutral lipids, and Maillard reaction products), amino acids and proteins (Vela *et al.*, 2007).

Other important effects of honey have been linked to its oligosaccharides. They have prebiotic effects, similar to that of fructo-oligosaccharides (Sanz *et al.*, 2005). The oligosaccharides have been reported to cause an increase in population of bifidobacteria and lactobacilli, which are responsible for maintaining a healthy intestinal microflora in humans. As a matter of fact, *Lactobacillus* spp. protect the body against infections like salmonellosis; and *Bifidobacterium* spp restrict the overgrowth of the gut walls by yeasts or bacterial pathogens and, perhaps reduce the risk of colon cancer by out-competing putrefactive bacteria capable of liberating carcinogens (Kleerebezem and Vaughan, 2009).

The use of honey as a traditional remedy for microbial infections dates back to ancient times (Lusby *et al.*, 2005). Research has been conducted on manuka (*L. scoparium*) honey (Visavadia *et al.*, 2006), which has been demonstrated to be effective against several human pathogens, including *Escherichia coli* (*E. coli*), *Enterobacter aerogenes*, *Salmonella typhimurium*, *S. aureus* (Tan *et al.*, 2009).
Laboratory studies have revealed that the honey is effective against methicillin-resistant *S. aureus* (MRSA), streptococci and vancomycin resistant Enterococci (VRE) (Rajeswari et al., 2010).

However, the newly identified honeys may have advantages over or similarities with manuka honey due to enhanced antimicrobial activity, local production (thus availability), and greater selectivity against medically important organisms (Tan et al., 2009). The coagulase negative staphylococci are very similar to *S. aureus* (Rajeswari et al., 2010) in their susceptibility to honey of similar antibacterial potency and more susceptible than *Pseudomonas aeruginosa* and *Enterococcus* species (Fahim et al., 2014). The disc diffusion method is mainly a qualitative test for detecting the susceptibility of bacteria to antimicrobial substances; however, the minimum inhibitory concentration (MIC) reflects the quantity needed for bacterial inhibition. Following the in vitro methods, several bacteria (mostly multidrug resistant; MDR) causing human infections that were found susceptible to honeys (Mohapatra et al., 2011; Fahim et al., 2014).

**Zone diameter of inhibition**

The zone diameter of inhibition (ZDI) of different honey samples (5%-20%) has been determined against *E. coli* O157: H7 (12 mm -24 mm) and *S. typhimurium* (0 mm -20 mm) (Badawy et al., 2004). The ZDIs of Nigerian honeys were found to be (20-21) mm, (15-16) mm and (13-14) mm for *S. aureus*, *P. aeruginosa* and *E. coli*, respectively (Agbagwa and Frank- Peterside, 2010). Agbagwa and Frank- Peterside (2010) and Anyanwu (2012) examined different honey samples: Western Nigerian honey, Southern Nigerian honey, Eastern Nigerian honey and Northern Nigerian honey, and compared their abilities to inhibit the growth of *S. aureus*, *P. aeruginosa*, *E. coli* and *Proteus mirabilis* (*P. mirabilis*) with an average of ZDIs (5.3-11.6) mm, (1.4-15.4) mm, (4.4-13.5) mm and (9.1-17) mm, respectively, and with honey concentrations of 80%-100%. The extracts of raw and processed honey showed ZDI (6.94-37.94) mm, against gram-positive bacteria viz., *S. aureus*, *Bacillus subtilis*, *Bacillus cereus*, as well as gram negative bacteria like *E. coli*, *P. aeruginosa* and *S. enterica* serovar Typhi (Chauhan et al., 2010).

**Minimum inhibitory concentration**

The minimum inhibitory concentration(MIC) assay showed that a lower MIC was observed with ulmo (*Eucryphia cordifolia*) honey (3.1% - 6.3% v/v) than with manuka honey (12.5% v/v) for MRSA isolates; for the *E. coli* and *Pseudomonas*
strains equivalent MICs were observed (12.5% v/v) (Sherlock et al., 2010). The MICs for Tualang honey ranged 8.75% - 25%, while those for manuka honey ranged 8.75% - 20% against many pathogenic gram-positive and gram negative bacteria (Tan et al., 2010). The MICs of manuka, heather, khadikraft and local honeys against clinical and environmental isolates of *P. aeruginosa* were recorded as 10% - 20%, 10% - 20%, 11% and 10% - 20%, respectively (Mullai and Menon, 2007). The MICs of *A. mellifera* honey ranged (126.23 - 185.70) mg/ml and of *Tetragonisca angustula* honey (142.87 - 214.33) mg/ml against *S. aureus* (Miorin et al., 2003). The Egyptian clover honey MIC was 100 mg/ml for *S. typhimurium* and *E. coli* O157:H7 (Badawy et al., 2004). The Nilgiri honey MICs were 25%, 35% and 40% for *S. aureus*, *P. aeruginosa* and *E. coli*, respectively (Rajeswari et al., 2010). MIC values of honey extracts were found in the range of (0.625-5.000) mg/ml, for *S. aureus*, *B. subtilis*, *B. cereus*, and gram-negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhi* (Chauhan et al., 2010).

By visual inspection, the MICs of Tualang honey ranged 8.75% - 25% compared with those of manuka honey (8.75% - 20%) against wound and enteric microorganisms: *Streptococcus pyogenes*, coagulase-negative *Staphylococci*, MRSA, *Streptococcus agalactiae*, *S. aureus*, *Stenotrophomonas maltophilia* (*S. maltophilia*), *Acinetobacter baumannii*, *S. typhi*, *P. aeruginosa*, *Proteus mirabilis*, *Shigella flexneri*, *E. coli*, *Enterobacter cloacae* (Tan et al., 2010). Six bacterial strains from burn-wound patients, namely, *Aeromonas schubertii*, *Haemophilus paraphrophilus*, *Micrococcus luteus*, *Cellulosimicrobium cellulans*, *Listonella anguillarum* and *A. baumannii* had MICs of 35%-40%, 35%-40%, 35%-40%, 25%-30%, respectively, as has been reported by Hassanein et al. (2010). The honeys were inhibitory at dilutions down to 3.6% - 0.7 % (v/v), for the pasture honey, 3.4% - 0.5% (v/v), and for the manuka honey, against coagulase-negative *Staphylococci* (Fahim et al., 2014).

**Time-kill study**

The kill kinetics provides more accurate description of antimicrobial activity of antimicrobial agents than does the MIC (Tan et al., 2010). In our earlier study, we explored the time-kill activity of autoclaved honey against *E. coli*, *P. aeruginosa* and *S. Typhi* in order to establish the potential efficacy of such local honey (not studied before) collected from villages (Mandal and Mandal, 2011). Antibiotic susceptible and resistant isolates of *S. aureus*, *S. epidermidis*, *Enterococcus faecium*, *E. coli*, *P. aeruginosa*, *E. cloacae*, and *Klebsiella oxytoca*, etc, were killed within 24 h by 10%-40% (v/v) honey (Kwakman et al., 2011). Thus, more studies are required to establish
various local honeys based upon kill kinetics and their effective in vivo application against MDR infections.

1.5.5.2. Anti-Inflammatory Activity

Although inflammation is a vital part of the normal response to infection or injury, when it is excessive or prolonged it can prevent healing or even cause further damage. The most serious consequence of excessive inflammation is the production of free radicals in the tissue. These free radicals are initiated by certain leucocytes that are stimulated as part of the inflammatory process (van den Berg et al., 2008), as inflammation is what triggers the cascade of cellular events that give rise to the production of growth factors which control angiogenesis and proliferation of fibroblasts and epithelial cells (Simon et al., 2009).

They can be extremely damaging and break down lipid, proteins and nucleic acids that are the essential components of the functioning of all cells. However, the anti-inflammatory properties of honey have been well established in a clinical setting (Subrahmanyam et al., 2003) and its action is free from adverse side effects.

1.5.5.3. Anti-Oxidant Activity

Antioxidant capacity is the ability of honey to reduce oxidative reactions within the human body. It has been found to have a significant antioxidant content measured as its capacity to scavenge free radicals (Gheldof et al., 2002). This antioxidant activity may be at least partly what is responsible for its anti-inflammatory action because oxygen free radicals are involved in various aspects of inflammation (Henriques et al., 2006). Even when the antioxidants in honey do not directly suppress the inflammatory process, they can be expected to scavenge free radicals in order to reduce the amount of damage that would otherwise have resulted.

Honey exerts its anti-oxidant action by inhibiting the formation of free radicals, catalyzed by metal ions such as iron and copper. Flavonoids and other polyphenols, common constituents of honey have the potential to impound these metal ions in complexes, preventing the formation of free radicals in the first place (Makawi et al., 2009).

Honey contains a variety of phytochemicals (as well as other substances such as organic acids, vitamins, and enzymes) that may serve as sources of dietary antioxidants. The amount and type of these antioxidant compounds depends largely upon the floral source/variety of the honey. In general, darker honeys have been shown to be higher in antioxidant content than lighter honeys (Gheldof et al. 2002).
Researchers at the University of Illinois Champaign/Urbana examined the antioxidant content (using an assessment technique known as Oxygen Radical Absorbance Capacity or ORAC) of 14 uni-floral honeys compared to a sugar analogue. ORAC values for the honeys ranged from 3.0 μmol TE/g for acacia honey to 17.0 μmol TE/g for Illinois buckwheat honey. The sugar analogue displayed no antioxidant activity (Alvarez-Suarez et al., 2013). Free radicals and reactive oxygen species (ROS) have been implicated in contributing to the processes of aging and disease. Humans protect themselves from these damaging compounds, in part, by absorbing antioxidants from high-antioxidant foods (Makawi et al., 2009). This report describes the effects of consuming 1.5 g/kg body weight of corn syrup or buckwheat honey on the antioxidant and reducing capacities of plasma in healthy human adults. It can be speculated that these compounds may augment defences against oxidative stress and that they might be able to protect humans from oxidative stress. Given that the average sweetener intake by humans is estimated to be in excess of 70 kg per year, the substitution of honey in some foods for traditional sweeteners could result in an enhanced antioxidant defense system in healthy adults (Henriques et al., 2006).

Antioxidant properties shown by volatile oil of propolis (VOP) from India were investigated by spectrophotometric methods and a photochemiluminescence method and it was found that from IC50 values it could be concluded that the efficiency of scavenging ABTS radicals by the VOP was more pronounced as compared to scavenging other radicals (Kwakman et al., 2010).

1.5.5.4. Boosting of Immune System

As well as having a direct antibacterial action, honey may clear infection through stimulating the body’s immune system to fight infections. It has been reported that honey stimulates B60 lymphocytes and T-lymphocytes in cell culture to multiply, and activate neutrophils (Tonks et al., 2003). Furthermore, Jones et al. (2000) in their study reported the stimulation of monocytes in cell cultures to release the cytokines TNF-alpha, IL-1 and IL-6, the cell “messengers” that activate the many facets of the immune response to infection. Recently, Tonks et al. (2007) discovered a 5.8k DA component of Manuka honey which stimulates the production of TNF-α in macrophages via Toll-like receptor. In addition, honey provides a supply of glucose, which is essential for the “respiratory burst” in macrophages that produce hydrogen peroxide, the dominant component of their bacteria-destroying activity (Molan, 2001).
Moreover, it provides substrates for glycolysis, the major mechanism for energy production in the macrophages, and thus allows them to function in damaged tissue and exudates where the oxygen supply is often poor. The acidity of honey may also assist in the bacteria destroying action of macrophages, as an acid pH inside the phagocytic vacuole is involved in killing ingested bacteria (Molan, 2001).

1.5.6. Clinical Conditions for Treatment with Honey

1.5.6.1. As Remedy for Diarrhoea

Infections of the intestinal tract are common throughout the world, affecting people of all ages (Badawy et al., 2004). The infectious diarrhoea exacerbates nutritional deficiencies in various ways, but as in any infection, the calorific demand is increased. Pure honey has bactericidal activity against many enteropathogenic organisms, including those of the Salmonella and Shigella species, and enteropathogenic E. coli (Badawy et al., 2004; Agbagwa et al., 2010).

In vitro studies of Helicobacter pylori isolates which cause gastritis have been shown to be inhibited by a 20% solution of honey. Even isolates that exhibited a resistance to other antimicrobial agents were susceptible (Chattopadhyay et al., 2009). In a clinical study, the administration of a bland diet and 30 mL of honey three times a day was found to be an effective remedy in 66% of patients and offered relief to a further 17%, while anaemia was corrected in more than 50% of the patients (Ndip et al., 2007).

1.5.6.2. As Medicine for Gastric Ulcers

Gastric ulcers have been successfully treated by the use of honey as a dietary supplement (Ali, 1991). Honey administered subcutaneously or orally before oral administration of ethanol affords protection against gastric damage and reverses changes in pH induced by ethanol (French et al., 2005).

Honey consumption delayed the postprandial ghrelin response (p = 0.037), enhanced the total PYY (p=0.007) response, and blunted the glucose response (p=0.039) compared with consumption of the sucrose-containing meal (Larson-Meyer et al., 2010).

1.5.6.3. As Medicine for Canine Recurrent Dermatitis

The antibacterial activity of honeymade honey and propolis was evaluated in vitro against Staphylococcus aureus strains isolated from canine patients with dermatitis and found that the honey showed bactericidal effects against the bacterial
tested *S. aureus* stains, but was less efficient than the propolis at certain concentrations (Iulia *et al.*, 2007; Moussa *et al.*, 2012).

1.5.6.4. As Immune Inducer

*In vivo* antibacterial activity of honey resulted in the induction of increased lymphocyte and phagocytic activity. Recent studies showed that the proliferation of peripheral blood B-lymphocytes and T-lymphocytes in cell culture is stimulated by honey at concentrations as low as 0.1% and phagocytes are activated by honey at concentrations as low as 0.1% (Halawani and Shohayeb, 2011). Honey at a concentration of 1% also stimulates monocytes in cell culture to release cytokines, tumor necrosis factor (TNF)-alpha, interleukin (IL)-1 and IL-6, which activate the immune response to infection (Alvarez-Suarez *et al.*, 2010; Tonks *et al.*, 2001; Tonks *et al.*, 2003).

It has been reported that Manuka honey increased IL-1β, IL-6, and TNF-α production from Mono Mac6 cells or human monocytes, and the active component was 5.8 kDa, which increased production of these cytokines via TLR4. In addition, it was reported that oral intake of honey augmented antibody productions in primary and secondary immune responses against thymus-dependent and thymus-independent antigens (Alvarez-Suarez *et al.*, 2010).

1.5.6.5. As Anti-diabetic Agent

In the past, people with diabetes were advised to avoid “simple sugars” including honey. It was thought that consuming simple sugars would cause a sharp and rapid elevation in blood glucose levels and an overwhelming insulin demand. Some even speculated that eating simple sugars could cause diabetes, a notion that has not been supported by scientific research (Al-Waili and Haq, 2004).

In fact, research has shown that some complex carbohydrates raise blood glucose levels more significantly than certain simple sugars. Both honey and sucrose have been shown to produce a lower glucose response than starchy foods such as white bread. Moreover, it has been shown that the total amount of carbohydrate consumed is probably more important than the type of carbohydrate when it comes to blood sugar levels. Thus, experts agree that diabetics may include moderate amounts of “simple sugars” in a balanced diet. Honey compared with dextrose caused a significantly lower rise in plasma glucose levels in diabetic subjects. It also caused reduction of blood lipids, homocysteine levels and CRP (C reactive protein) levels in normal and hyperlipidemic subjects (Tonks *et al.*, 2007).
1.5.6.6. Antimutagenic and Antitumor Activity

Mutagenic substances act directly or indirectly by promoting mutations of the genetic structure. During the roasting and frying of food heterocyclic amines are formed, e.g. Trp-p-1 (3-Amino-1,4-dimethyl-5H-pyridol [4,3-b] indole). The antimutagenic activity of honeys from seven different floral sources (acacia, buckwheat, fireweed, soybean, tupelo and Christmas berry) against Trp-p-1 was tested by the Ames assay and compared to a sugar analogue as well as to individually tested simple sugars (Wang et al., 2002). All honeys exhibited a significant inhibition of Trp-p-1 mutagenicity. Glucose and fructose were found to have a similar antimutagenic activity as honey. Nigerose, another sugar, present in honey has an immunoprotective activity (Murosaki et al., 2002). The antimetastatic effect of honey and its possible mode of anti-tumor action was studied by the application of honey in spontaneous mammary carcinoma in methylcholanthrene-induced fibrosarcoma of CBA mice and in anaplastic colon adenocarcinoma of Y59 rats (Orsolic and Basic, 2004). A statistically significant anti-metastatic effect was achieved by oral application of honey (Orsolic et al., 2003). These findings indicate that honey activates the immune system and honey ingestion may be advantageous with respect to cancer and metastasis prevention.

In addition, it is postulated that honey given orally before tumour cell inoculation may have a decreased effect on tumour spreading. In another study of the same group the effect of honey on tumour growth, metastasising activity and induction of apoptosis and necrosis in murine tumour models (mammary and colon carcinoma) was investigated. A pronounced antimetastatic effect was observed when honey was applied before tumour-cell inoculation (per oral 2 g kg⁻¹ for mice or 1 g kg⁻¹ for rats, once a day for 10 consecutive days) (Swellam et al., 2003).

In another study the anti-tumour effect of honey against bladder cancer was examined in vitro and in vivo in mice. According to these results honey is an effective agent for inhibiting the growth of different bladder cancer cell lines (T24, RT4, 253J and MBT-2) in vitro. It is also effective when administered intralesionally or orally in the MBT-2 bladder cancer implantation mice models (Swellam et al., 2003).

1.5.6.7. As Treatment for Arthritis

Apparently also boron stimulates in a positive way, hormonal factors for both men and women, resulting in healthy bones. If this hormonal balance is disturbed, it
will lead to osteoarthritis and as honey contains boron, it’s routinely consumption can avoid such problems (Bingham et al., 2010).

1.5.6.8. As Skin Disinfectant

Typical honeys are about eight times more potent against coagulase-negative staphylococci than if bacterial inhibition were due to their osmolarity alone. Therefore, honey applied to skin at the insertion points of medical devices may have a role in the treatment or prevention of infections by coagulase-negative staphylococci (French et al., 2005).

1.5.6.9. The Action of Honey in Wound Healing

Honey is an effective treatment of wounds because it is non-irritating, non-toxic, self-sterile, bactericidal, nutritive, easily applied and more comfortable than other dressings (Singh et al., 2012). The treatment of wounds with honey has rendered them bacteriologically sterile within 7-10 days of the start of the treatment and promoted healthy granulation of tissue according to Tan et al. (2009). Anti-fungal activity of honey has been also tested on Candida albicans, C. pseudotropicalis, C. stellatoidea and C. tropicalis and all were found to be susceptible (Akujobi and Njoku, 2010; Anyanwu, 2012). Honey was also found to be more effective as an antibacterial agent against several Pseudomonas and Staphylococcus strains than the antibiotic, gentamicin (Tan et al., 2009).

In a clinical study involving 59 patients with wounds and ulcers, most of which had failed to respond to conventional treatments, 15-30 ml fresh honey was applied daily. The bacteria isolated from 58 of these wounds (E. coli, S. aureus, P. mirabilis, mixed coliforms, Klebsiella species, and S. faecalis) were all susceptible to honey in vitro according to Tan et al. (2009). One other bacteria, Ps. pyocyanea did not undergo complete lysis in vitro tests but it was completely sterilised in vivo. In one case in which the patient had a buruli ulcer infected with Mycobacterium ulcerans, honey treatment was ineffective an in vitro tests showed the mycobacteria to be resistant to honey (Subrahmanyam, 2007).

Honey has cleansing action of wounds, stimulates tissue regeneration, reduces inflammation and honey impregnated pads act as non-adhesive tissue dressing (Singh et al., 2012).

1.5.7. Factors that Affect Antimicrobial Activity of Honey

The instability of honey inhibine was first recognized by Dold, who found that it was destroyed by heating and by exposure to light. These observations have since
been confirmed by numerous other researchers, but there have been differences in the degree of instability reported (Moussa et al., 2012; Chen et al., 2012).

a. Sensitivity to heat

According to Chen et al. (2012) and Hassan (2013), the report on the loss of antibacterial activity on exposure of honey to heat was of complete loss of inhibition by 17% honey after exposure of 50% honey to 100°C for 5 min, 80°C for 10 min, or 56°C for 30 min. However, this did not mean that antibacterial activity was lost completely: if the unheated honey had been of just high enough activity to inhibit growth when at 17%, not much activity would have to be lost on heating for inhibition no longer to be seen. This also applies to the similar finding of Pothmane that exposure of honey to 100°C for 5 min or 56°C for 1 h caused complete loss of inhibition by 17% honey. In later reports the researchers used a dilution series for the assay of activity. Although complete loss of inhibition in their studies still did not mean that antibacterial activity was lost completely, its reduction to a level below detestability would generally represent a loss of 80% or more, if not a complete loss. In these reports 'complete loss' was found to result from exposure of honey to: 100°C for 30 minutes; 100°C for 15 min (Schade et al., 1958; Hassan, 2013); 90°C for 8 minutes (Gryuner and Arinkia, 1970); 100°C for 5 min, 90°C for 15 min, 70-80°C for 20-30 min, and 56°C for 60 min (Franco and Sartori, 1940); 80°C for 15 min (Stomso-Stitz and Kominos, 1960); 80°C for 30 min (Ialomiteanu and Daghie, 1973); 60°C for 15 min and from use of 'heated honey' (Dustmann, 1987). An almost complete loss was found on heating honey for 100°C for 10 min (Chambonnaud, 1966). In another report the activity was not lost completely after exposure of honey to 100°C for 15 min, but was reduced to the same level as that of artificial honey, indicating that all activity other than that due to osmolarity had been destroyed (Christov and Mladenov, 1961). A similar finding was made with honey boiled for 10 min (Plachy, 1944). Others also have found that only part of the antibacterial activity is destroyed by heating honey. Exposure of honey to 100°C for 10 min caused complete loss of activity against seven species of bacteria, but only partial loss of activity against Bacillus pumilus and a strain of Streptomyces, and no loss of activity against Bacillus subtilis and Sarcina lutea. Another report about half of the activity against B. subtilis was found to be heat-stable (Gonnet and Lavie, 1960). Heating honey at 56°C for 30 min caused a loss of activity that was greater against some species than against others'. The presence of both heat-stable and heat-sensitive
factors has been reported by others also (Chambonnaud, 1968; Gryuner and Arinkia, 1970; Daghie et al., 1971; Bogdanov, 1983). The retention of part of the activity reported in instances where honey has been subjected to lesser degrees of heating probably results from there being only partial destruction of the heat-sensitive factor, rather than a heat-stable factor being responsible (Moussa et al., 2012).

According to Chen et al. (2012), the minimum inhibitory concentration of honey was found to increase from 4% to 8% after exposure of honey to 46°C for 8 h, to 12% after exposure to 52°C for 8 h, and to 16% after exposure of honey to 55°C for 8 h. Also reported was complete loss of activity after exposure to more than 65°C for less than 4 h, a heavy but not complete loss after exposure to 56°C for 24 h, but no loss after exposure to 40°C for 96 h (Moussa et al., 2012).

The stability of the antibacterial activity in heated honey has been found to depend on the pH, activity being more rapidly lost at low pH according to Moussa et al. (2012). According to Singh et al. (2012), there are some large differences in the findings on the stability of the antibacterial activity of honey at lower temperatures, but generally the conclusion has been that it is stable below 40°C. No decrease in antibacterial activity was seen in honeys held at 40°C for 96 h, as in the case mentioned above, nor in honey held at 37°C for 24 h. This is to be expected when it is borne in mind that the temperature in the beehive where honey can spend quite a long time is around 34°C. It may not be as stable at this temperature when diluted: the rate of production of hydrogen peroxide drops off with time, and the amount of hydrogen peroxide present after 16 h was found to be much lower than that present after the first hour (Moussa et al., 2012). Others have also reported that honey is less stable when diluted. This could be a consequence of the build-up of gluconic acid, or of damage to the glucose oxidase from free radicals generated from hydrogen peroxide as discussed earlier (Hasan, 2013). The latter suggestion is supported by the finding with the isolated enzyme that addition of a high level of hydrogen peroxide inactivated it after about 30 min. However, it has been reported that 50% honey held at room temperature for 100 h does not lose its antibacterial activity (Molen, 2007). There are several indications of the antibacterial activity of honey being very stable at room temperature.

b. Sensitivity to light

It has been known since some of the earliest work on the antibacterial properties of honey that the activity is unstable in light. Chen et al. (2012) reported
that honey lost its ability to inhibit bacterial growth (tested in a 17% solution) after exposing a thin film of it to sunlight. Others have since confirmed this observation according to Chen et al. (2012). Exposure of honey in a layer 1-2 mm thick to sunlight for 15 min was found to result in complete loss of non-osmotic activity. When not spread out in a thin layer it has not been found to be so sensitive: almost complete loss of activity after 18 days in direct sunlight, gradual disappearance of activity when exposed to direct sunlight but not with diffuse daylight, and a significant reduction in activity in honey samples stored for 3-6 months on open shelves (more than twice that lost in the same samples stored in a dark cupboard) have been reported (Tan et al., 2009). No loss of activity was found, however, when a thin film of honey was exposed for 1 h to an ultraviolet (UV) lamp (254 nm) (Molen, 2002).

A large loss of activity was found in honey left for 8 months on a window-sill on the sunny side of the building if stored in 1 or 2.5 litre jars made from clear polystyrene, but not if stored in jars made of white or ivory polyethylene with low transmission of light of wavelength below 400 nm. Glass jars coated with a film to absorb UV light were only partially successful in this study in preventing the loss of activity, indicating the necessity to protect from light of wavelengths up to 400 nm (Mandel and Mandel, 2011). This protection by absorption of light can occur within the honey itself, as is seen with the greater stability of bulk quantities compared with thin films. Dark-coloured honey was found to be more light-stable than light-coloured honey, presumably because it is less light into the bulk of the honey (Alvarez-Suarez et al., 2013). However, the sensitivity to light has been observed to depend on the floral source of the honey: in a 500 g jar kept in sunlight, some floral types of honey were found to lose their activity completely in only 48 h, and a reduction of up to 67% in the production of hydrogen peroxide (Hasan, 2013).
CHAPTER TWO: MATERIALS AND METHODS

2.0. Collection of Honey Samples

Three honey samples each from Hypotrigona spp. (Okotobo) (Appendix A, Picture 1) and Melipona spp. (Appendix A, Picture 2 (Ifufu) including Apismelliferahoney (Bee honey) (Appendix A, Picture 3) were collected from keepers at Olido, Enugu Ezike, Igbo Eze North Local Government Area of Enugu State. The matured combs, laden with honey, were harvested and aseptically collected in sterile screwed cups, and kept in a cool and dry place before transporting to the laboratory.

2.1. Extraction Procedure

The crushing method were employed for extraction of the various honey sample varieties from their different combs. Each of the honey combs, laden with honey, were tied up in a clean white cloth and squeezed to release the honey into a receptacle. While Melipona spp. were collected with sterile syringes. The released honey were stored under room temperature (27-30°C) using glass jars.

2.2. Physicochemical Analyses

The following physicochemical properties of the honey samples were determined according to harmonized methods of the International Honey Commission (Bogdanov et al., 1997) and Association of Official Analytical Chemists (AOAC, 2000): pH, electrical conductivity (mS/cm), total dissolved solid (ppm), colour characteristic, colour intensity (ABS450) (mm), moisture contents (%), free, total and lactone acidities (meq/kg), total protein content (g/kg), hydroxymethylfurfural (HMF) (mg/kg), total sugar and reducing sugar contents (%(g/100g)), total flavonoid...
(mg$_{CEQ}$/kg) and phenolic contents (mg$_{GAE}$/kg), proline (mg/kg) and ascorbic acid contents (mg/kg).

2.2.1. Determination of pH

The pH of a 10% (w/v) solution of homogenized honey prepared in boiled warm water was measured with a digital pH-meter (HI 98127, Hanna instruments, Mauritius) according to AOAC (2000). The pH meter was calibrated using standard buffers of 4.0 and 10.0 prior to measuring the pH of the samples. Sample solution was taken in the beaker and inserted. When the first reading was completed, the electrode was washed with distilled water and dried-up with tissue paper. Similarly, as a continue series, all other samples were determined accordingly.

2.2.2. Determination of Moisture Content

The moisture content was determined based on the refractometric method according to harmonized methods of International Honey Commission (Bogdanov et al., 1997). In general, the refractive index increases with the increase in the solid content. The refractive indices of honey samples were measured at ambient temperature using an Atago hand refractometer (ATAGO, N-1α, Tokyo, Japan) and the readings were further corrected for a standard temperature of 20°C by adding/subtracting the correction factor of 0.00023/°C. The percentage moisture content values corresponding to the corrected refractive index values were calculated using Wedmore’s table (AOAC, 1990).

2.2.3. Determination of Electrical Conductivity (EC) and Total Dissolved Solids (TDS)

The EC and TDS were measured using a conductivity meter HI 98311 (Hanna Instruments, Mauritius) in a 20% (w/v) solution of honey suspended in deionized water as recommended by Bogdanov (1997). The EC and TDS of each sample were analyzed and the means are expressed as mS/cm and ppm, respectively. The EC of deionized water alone was less than 10 μS/cm.

2.2.4. Determination of Colour Characteristics

The colours of the honey samples (Appendix A, Picture 1) were determined by spectrophotometric measurement (PG Instruments Ltd, UK) of the absorbance of 50% (w/v) honey solution (homogenized and centrifuged at 3200rpm for 5 minutes) at 560 nm according to the method of White (1984). The colour of honey samples was
classified according to the Pfund scale after conversion of the absorbance values in millimetre (mm).

2.2.5. **Determination of Colour Intensity (ABS$_{450}$)**

The mean absorbance of honey samples was determined using the method of Beretta et al. (2005). Briefly, honey samples were diluted to 50% (w/v) with warm (45-50°C) distilled water and the resulting solution was filtered using a 0.45µm filter to remove large particles. The absorbance was measured at 450 and 720nm using a spectrophotometer (PG Instruments Ltd, UK) and the difference in the absorbance readings was expressed as µAU.

2.2.6. **Determination of Total Sugar Contents**

Honey samples were suspended in distilled water to produce a 25% (w/v) solution. The total sugar content of each honey sample was determined using the refractometric method (Atago handheld refractometer, ATAGO, N-1α, Tokyo, Japan) and the percentage of sucrose content was measured in g/100g of honey (AOAC, 2000).

2.2.7. **Determination of Reducing and non-Reducing Sugar Contents**

The total reducing sugar content was measured using 3,5-dinitrosalicylic acid (DNSA). The reducing sugar reduces DNSA to 3-amino-5-nitrosalicylic acid, resulting in a solution with reddish-orange colouration that was measured spectrophotometrically at 540 nm (Saxena et al., 2010). The honey solution (0.1 g/mL) was diluted 100-fold with distilled water. A 1-mL aliquot of this diluted solution was mixed with equal amounts of DNSA solution and incubated in a boiling water bath for 10 min. The mixture was allowed to cool to ambient temperature for 10 min and mixed with 7.5 mL of distilled water followed by measurement of the absorbance at 540 nm using a spectrophotometer. Glucose solutions of known concentrations (100, 200, 400 and 600 µg/mL) was used as standards.

The amount of non-reducing sugars, such as sucrose content (%), was measured by subtracting the reducing sugar content from total sugar content, which was expressed by the following equation (AOAC, 2000):

\[
\text{Sucrose content} \% = \text{Total sugar content} - \text{Reducing sugar}
\]

2.2.8. **Determination of Hydroxymethyl Furfural (HMF)**

Hydroxymethylfurfural (HMF) was detected using a technique based on the method described by Winkler (1955). Five grams of honey was dissolved, without heating, in oxygen free distilled water and transfer to a 125 ml graduated flask and
diluted to volume with oxygen free distilled water. Two milliliters of honey solution was pipetted into two tubes and 5 ml of P-toluidine solution was added to each. Into one test tube, 1 ml of water was pipetted and into the other 1 ml of barbituric acid solution was added; both mixtures were then be shaken. The content of HMF is calculated as follows:

\[
\text{HMF} = \frac{192 \times A \times 10}{\text{Weight of the honey in grams}}
\]

Where: \( A = \text{Absorbance} \), \( 192 = \text{Factor for dilution and extinction coefficient} \). Express results in mg/kg to 1 decimal place (Auerbach and Borries, 1924).

2.2.9. Determination of free, total, and lactone acidities

The free acidities and lactones were determined by equivalence point titration according to International Honey Commission (IHC, 2002). 10 g sample was dissolved in 75 ml of carbon dioxide-free water (deionized) in a 250 ml beaker. Stirred with the magnetic stirrer, immersed the pH electrodes in the solution and recorded the pH. Titrated with 0.1M NaOH (rate of 5ml/minute) to pH 8.50 (a steady reading was obtained within 120 sec of starting the titration; in other words, complete the titration within 2 minutes) (free acidity). Recorded the reading to the nearest 0.2ml when using a 10ml burette and to 0.01ml if the automatic titrator has sufficient precision). Immediately, a volume of 10mL 0.05 M NaOH was added and, without delay, back titrated with 0.05 M HCl from the second burette to pH 8.30, and recorded (lactone acidity). A blank determination procedure was performed to check for possible sources of error by titrating with only 75ml of deionized water to pH of 8.5 and record. These parameters were recorded in miliequivalent.

Calculation:

**Free acidity** (F.A.) is the acidity titratable with sodium hydroxide up to the equivalence point: \[
\text{F.A}_{(\text{meq/kg})} = \frac{(\text{ml of 0.1M NaOH titrated}) \times \text{NaOH Normality F)} - (\text{ml of 0.1NaOH titrated blank}) \times \text{NaOH Normality F) \times 1000}}{\text{Sample weight}}
\]

\[
\text{F.A}_{(\text{meq/kg})} = (\text{ml of 0.1M NaOH titrated}) - (\text{ml of 0.1M NaOH titrated blank}) \times 0.1 \times \frac{1000}{10}
\]

**Lactone acidity** (L.A.) corresponds to the combined acidity which is not directly titratable: \[
\text{L.A}_{(\text{meq/kg})} = (\text{ml of 0.1M NaOH titrated}) - (\text{ml of 0.1M HCl titrated}) \times \text{HCl Normality factor} \times \frac{1000}{10}
\]

**Total acidity** (T.A) is the sum of the free and lactone acidities.

\[
\text{T.A.}_{(\text{meq/kg})} = \text{F.A.}_{(\text{meq/kg})} + \text{L.A.}_{(\text{meq/kg})}
\]
2.2.10. Determination of Protein Contents

The protein content of honey was measured according to Lowry's method (Lowry et al., 1951). Briefly, BSA solutions were prepared by diluting a stock BSA solution (1 mg/mL) to 5 mL. BSA concentrations ranged from 0.05 to 1.00 mg/mL. Based on these dilutions, 0.2 mL of protein solution was placed in different test tubes and 2 mL of alkaline copper sulfate reagent (analytical reagent) was added. After the resulting solution was mixed properly, it was incubated at room temperature for 10 min. Then, 0.2 mL of Folin–Ciocalteu reagent solution was added to each tube and incubated for 30 min. The colorimeter was calibrated with a blank and the absorbance was measured at 660 nm.

2.2.11. Determination of Total Phenolic Content

The concentration of phenolics in the honey samples was estimated using a modified spectrophotometric Folin–Ciocalteu method (Singleton et al., 1999). Briefly, 2 g of honey was mixed with distilled water up to 10 mL. About 1 mL (0.2 g/mL) of honey extract was mixed with 1 mL of Folin and Ciocalteu’s phenol reagent. After 3 min, 1 mL of 10% Na₂CO₃ solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm using a T 60 UV/VIS spectrophotometer (PG Instruments Ltd, UK). Gallic acid was used to calculate a standard curve (20, 40, 60, 80 and 100 μg/mL; r² = 0.9970). The results were reported as the mean ± standard deviation and expressed as mg of gallic acid equivalents (GAEs) per kg of honey.

2.2.12. Determination of Total Flavonoid Content

The total flavonoid content in each honey sample was measured using the colorimetric assay developed by Zhishen et al. (1999). Briefly, 2 g of honey was mixed with distilled water up to 10 mL. Honey extract (1 mL) was mixed with 4 mL of distilled water. At the baseline, 0.3 mL of NaNO₂ (5% w/v) was added. After 5 min, 0.3 mL of AlCl₃ (10% w/v) was added followed by the addition of 2 mL of NaOH (1 M) six min later. The volume was increased to 10 mL by adding 2.4 mL distilled water. The mixture was vigorously shaken to ensure adequate mixing and the absorbance was read at 510 nm. A calibration curve was created using a standard solution of catechin (20, 40, 60, 80 and 100 μg/mL; r² = 0.9880). The results were expressed as mg catechin equivalents (CEQ) per kg of honey.
2.2.13. Determination of Proline Content

The proline content in the honey samples was measured using a method established by the IHC (USDA, 1985). Briefly, approximately 5 g of honey was transferred into a beaker and was dissolved in 50 ml water. The solution was quantitatively transferred to a 100 mL volumetric flask before further dilution to 100 ml with distilled water. After that, approximately 0.5 mL of the sample solution was transferred into a tube while 0.5 mL of water (blank test) was transferred into a second tube and 0.5 mL of proline standard solution will be taken into three other tubes. To each tube, about 1 mL of formic acid and 1 mL of ninhydrin solution was added each. The tubes was capped carefully and shaken vigorously for 15 min. The tubes were then placed in a boiling water bath for 15 min and were immersed below the level of the solution. The tubes were further transferred to another water bath and incubated at 70°C for 10 min. About 5 mL of the 2-propanol water solution was added to each tube followed by immediate capping. The tubes were left to cool for about 45 min after its removal from the 70°C water bath and the absorbance was measured at 510 nm (near maximum).

2.2.14. Determination of Ascorbic Acid Content

The ascorbic acid content was measured using the method described by (Ferreira et al., 2009). A sample of the honey (100 mg) was extracted with 10 mL of 1% metaphosphoric acid at room temperature for 45 min and filtered through Whatman No. 4 filter paper. The filtrate (1 mL) was mixed with 9 mL of 0.005% 2,6-dichlorophenolindophenol (DCPIP) and the absorbance of the mixture was measured within 30 min at 515 nm against a blank. The ascorbic acid content was calculated based on a calibration curve of pure L-ascorbic acid (50, 100, 200 and 400 μg/mL; \(Y = 3.2453X - 0.0703; r^2 = 0.9440\)). The results are expressed as mg of ascorbic acid/kg of honey.

2.3. Antimicrobial Activity Tests

2.3.1. Preparation of Honey and Solutions

The uncontaminated samples was used for this study. The three honey samples were sterilized by filtering through a 0.22μm membrane filter (Acrodisc, Pall Corporation, USA) (Al-Somal et al., 1994) into separate sterile labelled bijou bottles. The honey solutions were prepared immediately prior to testing by diluting to the different required concentrations with sterile distilled water to: 100 (undiluted honey), 80, 60, 40, 20 and 10 (% v/v) for antimicrobial activity test. From the 50% (v/v)
honey solution, 6 serial 1:1 (2-fold dilution) dilutions were made, resulting in final concentrations of: 50%, 25%, 12.5%, 6.3%, 3.1% and 1.6% for minimum inhibitory concentration (MIC) determination.

2.3.2. Collection of Test Microorganisms

For bacteria, three Gram-positive organisms namely MDR *Staphylococcus aureus* (gentamycin, chloramphenicol, norfloxacin, levofloxacin and clindamycin), *Bacillus cereus* and *Klebsiella pneumonia*, and three Gram-negative organisms namely *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 25783, and MDR *S. enterica* (ampicillin, co-trimoxazole and chloramphenicol) were tested. In addition, four fungal isolates were also tested: *Aspergillus niger*, *Candidatropicalis* and *Candida albicans* SC 5314. The typed bacterium and fungus were used as control strains. The test microorganisms were collected from the Department of Microbiology, University of Nigeria, Nsukka while the typed culture were purchased from culture collection centre. The cultures were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Turbidity standard for inoculum preparation

In order to standardize the inoculum density for all antimicrobial tests that were conducted in this work, a BaSO₄ turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent were used. A BaSO₄ 0.5 McFarland standards was prepared as follows: a 0.5-ml aliquot of 0.048 mol/L BaCl₂ (1.175% w/v BaCl₂. 2H₂O) was added to 99.5 ml of 0.18 mol/L H₂SO₄ (1.0% v/v), the absorbance at 625 nm was 0.008 to 0.10 for the 0.5 McFarland standard (equivalent to 1x10⁸ CFU/mL). The barium sulphate suspension was transferred into screw-cap tubes that have the same size and same volume of those that were used in growing the needed broth culture. Tubes was tightly sealed to prevent any loss by evaporation and stored in the dark at room temperature (Andrews, 2001).

2.3.3. Preparation of Standard Inocula

The inocula were prepared and standardized according to Clinical and Laboratory Standards Institute Approved Standard for bacteria (CLSI, 2006) and fungi (CLSI, 2002) by comparing with 0.5 McFarland Standard. Stock inoculum suspensions were prepared by taking five colonies (>1 mm in diameter) from 24h cultures (37°C) into 5 ml sterile saline). Each suspension was shaken for 15 s and density adjusted visually to 0.5 McFarland turbidity standards. This was done by adding more organisms (incubating the organism) when the suspension is too light or
diluting with sterile distilled water when it is too heavy. The turbidity of each suspension was compared by holding both the standard and the inoculums tubes side by side in front of a white paper with black lines. (Dilutions of these suspensions were also sub-cultured on nutrient agar (Oxoid Ltd, UK) to determine the number of cfu/ml) (Sutton, 2011).

Also, fresh mould and yeast cultures were prepared by sub-culturing into SDA and incubate at 35°C for 3-6 days and 24-48h respectively in order to maintain approximately uniform growth rate of each organism. The test mould was washed out with 5-10mL sterile normal saline (0.9%) and filtered through sterile cotton wool to obtain the spore suspension. The yeast suspensions were obtained by taking five colonies (>1 mm diameter) from cultures grown on Sabouraud agar into 5 mL of sterile saline water (0.9%). Each inoculum suspensions was shaken for 15 s and standardized to 10^5 spores or cells/ml respectively (by adjusting the turbidity with sterile saline water or by serial dilution). The viability of each isolate was confirmed by inoculation into Sabouraud dextrose agar (SDA) (Oxoid Ltd, UK) plates and counting the number of cfu/ml (Sutton, 2011; Anyanwu, 2012).

2.3.4. Antimicrobial Susceptibility Testing of Honey

The effect of different honey varieties on the several bacterial and fungal species was assayed by agar well diffusion method. The minimal inhibitory concentration (MIC) was determined visually. Each assay will be carried out in triplicates for each honey sample against each test organism.

Agar Well Diffusion Method

Nutrient agar (Oxoid Ltd, UK) and Sabouraud dextrose agar (SDA) (Oxoid Ltd, UK) plates were prepared aseptically according to manufacturer’s specification and allowed to solidify. A micropipette (or 1ml sterile syringe) was used to introduce 100µL (0.1ml) of the standard inoculum of the previously prepared bacterial and fungal isolates onto agar plates respectively, and spread with a sterile glass spreader. The plates were allowed to dry for 3-5 minutes. With the aid of the sterile cork borer, 6 radial wells of 6 mm diameter were punched equidistantly at different sites on the plates. One hundred microlitre (0.1ml) of each of the honey concentrations (100% (undiluted honey), 80%, 60%, 40%, 20% and 10%, (v/v) was placed onto six of the bored wells. The distilled water and different concentrations of antibiotics (500 – 31.3μg/ml of ciprofloxacin (Bharat Parenterals LTD) and 400 – 12.5μg/ml of ketoconazole (Fidson Pharmaceutical LTD) for Pseudomonas aeruginosa ATCC
25783 and Candida albicans SC 5314 respectively) served as negative and positive controls respectively. These processes were repeated for each honey sample viz Apis mellifera honey, ‘Okotobo’ and ‘Ifufu’ honeys; and for each of the organisms. The plates were left on the bench for 40 minutes for pre-diffusion. The culture plates containing bacteria and yeasts were incubated at 37°C for 24h while other culture plates containing the mould (Aspergillus niger) were incubated at room temperature (30± 2) for 72h. After incubation, the plates were examined and diameter of zones of inhibition were measured (in millimetres) for the different concentrations of the honeys and mean values recorded (Allen et al., 1991; Sommeijer et al., 1995; Moussa et al., 2012).

2.3.5. Determination of Minimum Inhibitory Concentration (MIC)

Following the initial antimicrobial screening tests, the minimum inhibitory concentration of each honey was determined by using the broth tube microdilution method as described by Barrow and Feltham (2004), Andrews (2001), and Patton et al. (2006) which is in accordance with Clinical Laboratory Standards Institute Approved Standard for bacteria (CLSI, 2006) and fungi (CLSI, 2002). Serial dilutions of each honey sample were made in eppendorf tubes containing 700µL of mueller hinton broth (MHB) (Oxoid Ltd, UK) and sabouraud dextrose broth (SDB) medium for bacteria and fungi respectively, to give a final concentration of 50%, 25%, 12.5%, 6.3%, 3.1% and 1.6% (achieved by adding 700µL of honey to 700µL of MHB or SDB and then serially transferring 700µL from it to the next tube and so on). 700µL was removed from the last tube. About 10µL of the standardized test organisms will be dispensed into the tubes. The negative control tubes were MHB or SDA with different concentrations of each honey samples, with no organism. Positive control tubes contained only 700µL broth medium and each of the organisms but no honey. Also, different concentrations of Ciprofloxacin and ketoconazole as above, for Pseudomonas aeruginosa ATCC 25783 and Candida albicans SC 5314 respectively were used as positive control drugs. The tubes containing bacteria and yeasts were incubated in the dark at 37°C for 24h while other tubes containing moulds were incubated in the dark at room temperature (30± 2°C) for 72h, all with constant shaking (at 250 rpm), to prevent adherence and clumping. The MIC was determined by visually inspecting the tubes for turbidity post-incubation (matching the mueller hinton broth and sabouraud dextrose broth respectively with the corresponding negative control tube of the same concentration). The MIC was reported as the lowest
concentration of test material which results in 100% inhibition of growth of the test organism. These was done in triplicates and all MIC values were expressed in % (vol/vol).

2.3.6. Determination of Minimum Biocidal Concentration

The minimum biocidal concentration (MBC) of the honey varieties were determined by further sub-culturing from the tubes which showed no visible growth in the MIC assay onto fresh sterile Nutrient agar and Sabouraud dextrose agar plates respectively. The culture plates containing bacteria and yeasts were incubated at 37°C for 24h while other culture plates containing the mould were incubated at room temperature (30± 2°C) for 72h. The plates were incubated until growth is seen in the growth control subculture. The MBC was therefore taken as the lowest concentration or highest dilution of honey that did not show any visible growth on the sub-cultured NA and SDA plates (Andrews, 2001).

2.4.7. Determination of Non-Peroxide Antimicrobial Activities of Honey Varieties

In order to determine non-peroxidase antimicrobial activities of the honey varieties, honey dilutions (50-1.6% v/v) were prepared in MHB/SDB containing catalase solution (Sigma, C-40) at a final concentration of 0.2% (w/v) (2mg of catalase in 10ml of MHB/SDB). The assay was conducted similar to the MIC determination as previously described. Control tube received broth, catalase and corresponding honey concentrations (negative control) only; and test isolate, broth and catalase (positive control) (Allen et al., 1991). After incubation, MBC and MFC were also determined as described previously.

2.4. Statistical Analyses

Results were reported as the mean ± standard deviation of triplicate experiments. Several parameters such as pH, proline, ascorbic acid, colour intensity, flavonoid and phenolic contents were correlated. Analysis of variance (ANOVA), Games-Howell and Turkey post hoc multi-comparison test, including Kruskal Wallis (KW) and Mann Whitney U-test were used for comparison of means using a significant level of p<0.05 (SPSS version 20). Correlation between parameters was obtained using Pearson’s correlation coefficient (r) (p < 0.01) and was ran with the same statistical package, and Microsoft Excel 2013.
CHAPTER THREE: RESULTS

3.1. Physicochemical Analyses

The parameters like pH, electrical conductivity (mS/cm), total dissolved solid (ppm), colour characteristic, colour intensity (ABS$_{450}$) (mm), moisture contents (%), free, total and lactone acidities (meq/kg), total protein content (g/kg), hydroxymethylfurfural (HMF) (mg/kg), total sugar and reducing sugar contents (%(g/100g)), total flavonoid (mg$_{CEQ}$/kg) and phenolic contents (mg$_{GAE}$/kg), proline (mg/kg) and ascorbic acid contents (mg/kg) were determined.

3.1.1. pH of the Honey Samples

The pH of the honey samples varied from 4.04± 0.124 to 4.46± 0.095 for A. mellifera honey (Table 1); 3.65 ± 0.036 to 3.85 ± 0.111 for Hypotrigona sp. honey (Table 2) and 3.80 ± 0.027 to 4.65 ± 0.053 for Melipona sp. honey (Table 3). All the honey samples tested were acidic. The mean pH of the honey varieties ranged from 3.75± 0.11 to 4.24± 0.20 and they were found to be significantly different from each other (One Way ANOVA, F$_{(2, 24)}$ = 30.99, p< 0.05) (Table 4). Hypotrigona sp. honey had the lowest mean pH (3.75 ± 0.105) and it was significantly different from mean pH of A. mellifera (4.24 ± 0.20, p = 0.003) and Melipona sp. (4.21 ± 0.371, p<0.05) honeys (Table 4). The mean pH of Melipona sp. honeys was not significantly different from mean pH of A. mellifera honey (p = 0.975).

3.1.2. Moisture Content
The moisture contents in the investigated honey samples were between 11.537± 0.454 and 11.870± 0.703 % for *A. mellifera* honey (Table 1); 17.053± 0.489 and 18.310± 0.900 % for *Hypotrigona* sp. honey (Table 2) and 12.863± 0.221 and 15.203± 0.186 % for *Melipona* sp. honey (Table 3). The mean moisture contents for *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys were 11.74 ± 0.47 %, 17.50 ± 0.80 %, and 13.86 ± 1.06 % respectively, which were significantly different from each other (One Way ANOVA, \( F_{(2, 24)} = 115.013, p < 0.05 \)) (Table 4). *Hypotrigona* sp. honeys had the highest mean moisture content and it was significantly different from *A. mellifera* (*p* < 0.05) and *Melipona* sp. (*p* = 0.001) honeys (Table 4). *A. mellifera* honeys had the least mean moisture content and it was significantly different from *Melipona* sp. honeys (*p* < 0.05).

**3.1.3. Electrical Conductivity**

In the investigated honey samples, the electrical conductivities (EC) were between 0.163 ± 0.0153 – 0.240 ± 0.020 mS/cm for *A. mellifera* honey (Table 1); 0.270 ± 0.020 – 0.323 ± 0.049 mS/cm for *Hypotrigona* sp. honey (Table 2) and 0.217 ± 0.015 – 0.257 ± 0.015 mS/cm for *Melipona* sp. honey (Table 3). The mean electrical conductivities of the honey varieties were 0.207 ± 0.04, 0.303 ± 0.04 and 0.238 ± 0.02 respectively (Table 4). There were statistically significant differences between the mean electrical conductivities of the honey varieties (One Way ANOVA, \( F_{(2, 24)} = 20.908, p < 0.05 \)), (Table 4). The mean EC of *Hypotrigona* sp. honeys was significantly different from mean EC of *A. mellifera* (*p*<0.05) and *Melipona* sp. (*p*<0.05) honeys. While the mean EC of *A. mellifera* was not significantly different from *Melipona* sp. (*p* = 0.109) honeys.

**3.1.4. Total Dissolved Solids (TDS)**

The TDS in the investigated honey samples ranged from 264.1± 0.59 to 316.3±0.06 ppm for *A. mellifera* honey (Table 1); 349.0± 0.36 to 399.1± 0.35 ppm for *Hypotrigona* sp. honey (Table 2) and 209.0± 0.31 – 221.2± 0.15 ppm for *Melipona* sp. honey (Table 3). The mean TDS of *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys were 287.46 ± 22.95, 370.01 ± 22.51 and 214.00 ± 5.55 ppm respectively (Table 4). There were significant differences between the mean TDS of the honey varieties (One Way ANOVA, \( F_{(2, 24)} = 154.591, p < 0.05 \)) (Table 4). *Hypotrigona* sp. honey had the highest mean TDS and it was significantly different from mean TDS of *A. mellifera* and *Melipona* sp. honeys (*p* < 0.05). *A. mellifera* mean TDS was significantly different from *Melipona* sp. honeys TDS content (*p* < 0.05) (Table 4).
3.1.5. Colour Characteristics and Colour Intensity (ABS\textsubscript{450})

The absorbance of the honey samples at 560 nm were between 1.986 ± 0.001 to 2.108 ± 0.001 nm for \textit{A. mellifera} honey (Table 1); 1.861 ± 0.001 to 2.103 ± 0.001 nm for \textit{Hypotrigona} sp. honey (Table 2) and 1.533 ± 0.000 to 2.012 ± 0.001 nm for \textit{Melipona} sp. honey (Table 3). The mean absorbance of \textit{A. mellifera}, \textit{Hypotrigona} sp. and \textit{Melipona} sp. honeys were 2.065 ± 0.06, 1.950 ± 0.12 and 1.726 ± 0.22 nm respectively (Table 4). When compared with Pfund scale table use for grading the colour of honeys (Table 5), \textit{A. mellifera} honey was amber in colour and whereas the other two honey varieties were light amber in colour.

ABS\textsubscript{450} of the investigated samples ranged from 961.33 ± 0.58 to 1006.33 ± 1.53 µAU for \textit{A. mellifera} honey (Table 1); 630.00 ± 1.73 to 724.33 ± 0.58 µAU for \textit{Hypotrigona} sp. honey (Table 2) and 762.33 ± 2.31 to 805.33 ± 0.58 µAU for \textit{Melipona} sp. honey (Table 3). The mean colour intensities of \textit{A. mellifera}, \textit{Hypotrigona} sp. and \textit{Melipona} sp. honeys were 985.33 ± 19.64, 679.00 ± 40.95 and 786.67 ± 19.14 µAU respectively (Table 4). There were statistically significant differences between the mean ABS\textsubscript{450} of the honeys (One Way ANOVA, $F_{(2, 24)} = 268.417$, $p < 0.05$). \textit{A. mellifera} honeys had the highest mean ABS\textsubscript{450} (985.33 ± 19.64 µAU), which was significantly different from \textit{Hypotrigona} sp. (679.00 ± 40.95 µAU) and \textit{Melipona} sp. honey (786.67 ± 19.14 µAU) ($p < 0.05$) (Table 4). The later was significantly different from mean ABS\textsubscript{450} of \textit{Hypotrigona} sp. honey.

3.1.6. Total Sugars, Reducing Sugars and Sucrose Contents

The percentage total sugars of the honeys ranged from 68.32 ± 0.01 to 79.61 ± 0.03 % (g/100g) for \textit{A. mellifera} honey (Table 1); 57.94 ± 0.03 to 66.32 ± 0.01% (g/100g) for \textit{Hypotrigona} sp. honey (Table 2) and 79.55 ± 0.44 to 82.04 ± 0.19% (g/100g) for \textit{Melipona} sp. honey (Table 3). There were statistically significant differences between the mean total sugar contents of the honey varieties (One way ANOVA, $F_{(2, 24)} = 53.765$, $p < 0.05$) (Table 4). The mean total sugar content of \textit{Melipona} sp. honey samples (80.71 ± 1.37 g/100g) was significantly higher than that of \textit{A. mellifera} (72.70 ± 7.50 g/100g) and \textit{Hypotrigona} sp. honey (62.32 ± 5.25 g/100g) samples ($p < 0.05$) (Table 4). The mean total sugar content of \textit{A. mellifera} honey was also significantly different from that of \textit{Hypotrigona} sp. honey ($p < 0.05$).

The reducing sugar contents of the honey varieties ranged from 66.00 ± 0.02 to 77.26 ± 0.03 % (g/100g) for \textit{A. mellifera} honey (Table 1); 56.12 ± 0.04 to 64.64 ± 0.02% (g/100g) for \textit{Hypotrigona} sp. honey (Table 2) and 73.72 ± 0.46 to 77.47 ±
0.20% (g/100g) for Melipona sp. honey (Table 3). The percentage mean reducing sugar contents of the honey samples were significantly different from each other (One way ANOVA, F (2, 24) = 35.303, \( p < 0.05 \)). The Melipona sp. honey had significantly the highest mean reducing sugar contents \((75.64 \pm 1.99 \text{ g/100g}, p< 0.05)\) and Hypotrigona sp. honey had significantly the least mean reducing sugar contents \((60.49 \pm 5.21 \text{ g/100g})\) (Table 4).

The sucrose contents of A. mellifera, Hypotrigona sp. and Melipona sp., honeys ranged from 2.32 ± 0.01 to 2.42 ± 0.02 (g/100g) (Table 1); 1.67± 0.02 to 1.99 ± 0.02 (g/100g) (Table 2), 4.57 ± 0.02 to 5.82 ± 0.02\%(g/100g)(Table 3) respectively. The percentage mean sucrose contents of the honeys were found to be significantly different from each other (One way ANOVA, F (2, 24) = 140.198, \( p <0.05 \)). The mean sucrose contents of Melipona sp., A. mellifera and Hypotrigona sp. honeys were 5.06 ± 0.75 g/100g, 2.36 ± 0.05 g/100g and 1.83 ± 0.14 g/100g respectively (Table 4). The Melipona sp. honey had significantly the highest mean sucrose contents \((p< 0.05)\) and Hypotrigona sp. honey had significantly the least mean sucrose contents \((p < 0.05)\) (Table 4).

### 3.1.7 Hydromethylfurfural (HMF) of the honeys

The HMF of the honeys ranged from 11.97 ± 0.05 to 16.12 ± 0.12 mg/kg for A. mellifera honey (Table 1); 16.29 ± 0.04 to 17.07 ± 0.05 mg/kg for Hypotrigona sp. honey (Table 2) and 5.35 ± 0.12 to 5.65 ± 0.04 mg/kg for Melipona sp. honey (Table 3). The results showed that there were significant differences between the mean HMF of the honeys (One way ANOVA, F (2, 24) = 250.031, \( p < 0.05 \)). The Melipona sp. honey had the lowest mean HMF content \((5.50 \pm 1.15 \text{ mg/kg}, p < 0.05)\) and it was significantly different from the mean HMF of Hypotrigona sp. honey\((16.58 \pm 0.37 \text{ mg/kg}, p < 0.05)\) and A. mellifera honey \((13.75 \pm 1.85 \text{ mg/kg}, p < 0.05)\) (Table 4). Also, the mean HMF of Hypotrigona sp. honey was significantly different from that of A. mellifera honey.

### 3.1.8 Total, free and lactone acidities of the different honey samples

The total acidities of the investigated honey samples ranged from 20.96 ± 0.32 to 22.07 ± 0.10 meq/kg for A. mellifera honey (Table 1); 35.08± 0.30 to 35.93± 0.07meq/kg for Hypotrigona sp. honey (Table 2) and 12.40± 0.03 to 12.95± 0.07meq/kg for Melipona sp. honey (Table 3). The mean total acidities of A. mellifera, Hypotrigona sp. and Melipona sp. honeys were 21.65 ± 0.55, 35.57 ± 0.42 and 12.57 ± 0.27meq/kg respectively (Table 4). There were significant differences between the
mean total acidities of these honey varieties (One Way ANOVA, \( F_{(2, 24)} = 6492.145, p < 0.05 \)).

The free acidities of the honey varieties ranged from 18.24 ± 0.25 to 19.46 ± 0.43 meq/kg for *A. mellifera* honey (Table 1); 30.34 ± 0.03 to 30.89 ± 0.22 meq/kg for *Hypotrigona* sp. honey (Table 2) and 10.63 ± 0.16 to 11.65 ± 0.12 meq/kg for *Melipona* sp. honey (Table 3). There were significant differences between the mean free acidities of the honeys (One Way ANOVA, \( F_{(2, 24)} = 3524.638, p < 0.05 \)) (Table 4).

The lactone acidities of the honeys ranged from 2.71 ± 0.07 to 3.62 ± 0.09 meq/kg for *A. mellifera* honey (Table 1); 4.19 ± 0.19 to 5.59 ± 0.06 meq/kg for *Hypotrigona* sp. honey (Table 2) and 1.04 ± 0.07 to 1.80 ± 0.07 meq/kg for *Melipona* sp. honey (Table 3). There were significant differences between the mean lactone acidities of the honeys (One Way ANOVA, \( F_{(2, 24)} = 123.790, p < 0.05 \)) (Table 4).

The *Hypotrigona* sp. honey had significantly the highest mean total, free and lactone acidities of 35.57 ± 0.42, 30.69 ± 0.32 and 4.88 ± 0.61 meq/kg (\( p < 0.05 \)) respectively. *Melipona* sp. honey had significantly the lowest mean total, free and lactone acidities of 12.59 ± 0.27, 11.23 ± 0.47 and 1.39 ± 0.34 meq/kg (\( p < 0.05 \)) respectively.

### 3.1.9. Protein contents of the honey samples

In the investigated honey samples, the protein contents of *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys varied from 3.05 ± 0.05 to 3.94 ± 0.01 g/kg (Table 1), 4.99 ± 0.04 to 6.58 ± 0.03 g/kg (Table 2) and 2.97 ± 0.04 to 3.94 ± 0.01 g/kg (Table 3) respectively. *Hypotrigona* sp. honey had highest mean protein content of 5.75 ± 0.69 g/kg, while *A. mellifera* and *Melipona* sp. honeys had mean protein content of 3.37 ± 0.43 g/kg and 3.56 ± 0.45 g/kg respectively. There were statistically significant differences between the mean protein contents of the honeys (One Way ANOVA, \( F_{(2, 24)} = 54.292, p < 0.05 \)) (Table 4). *Hypotrigona* sp. honey samples had the highest mean protein content, which was significantly different from *A. mellifera* and *Melipona* sp. honey mean protein contents (\( p < 0.05 \)). There was no significant difference between the mean protein contents of *A. mellifera* and *Melipona* sp. honey (\( p > 0.05 \)) (Table 4).

### 3.1.10. Total polyphenol content

The total polyphenol contents of the honeys ranged from 409.73 ± 1.95 to 475.68 ± 0.41 mg gallic acid/kg (mg GAE/kg) for *A. mellifera* honey (Table 1); 375.82 ± 0.25 to 531.10 ± 0.05 mg GAE/kg for *Hypotrigona* sp. honey (Table 2) and 354.03 ±
0.08 to 386.09 ± 0.11 mg GAE/kg for *Melipona* sp. honey (Table 3). The mean polyphenol contents of *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys were 439.16 ± 29.06, 527.41 ± 3.60, and 371.98 ± 14.18 mg GAE/kg respectively, which were significantly different from each other (One Way ANOVA, $F_{(2, 24)} = 154.982, p < 0.05$) (Table 4). *Hypotrigona* sp. honey total phenolic acid content was significantly different from *A. mellifera* and *Melipona* sp. honeys ($p < 0.05$). There was significant difference between the mean polyphenol contents of *A. mellifera* and *Melipona* sp. honeys ($p < 0.05$).

### 3.1.11. Flavonoid Content

The flavonoid contents of the investigated honey samples ranged from 57.00 ± 0.16 to 65.82 ± 0.29 mg of catechin per kg (mg CEQ/kg) for *A. mellifera* honey (Table 1); 28.1 ± 0.03 to 52.52 ± 0.03 mg CEQ/kg for *Hypotrigona* sp. honey (Table 2) and 82.78 ± 0.39 to 92.60 ± 0.10 mg CEQ/kg for *Melipona* sp. honey (Table 3). The mean flavonoid content was significantly different from each other (One Way ANOVA, $F_{(2, 24)} = 91.210, p < 0.05$). The *Melipona* sp. honey had statistically the highest mean flavonoid content (86.39 ± 4.69 mg CEQ/kg) and it was significantly different from the mean flavonoid contents of *Hypotrigona* sp. (41.37 ± 10.65 mg CEQ/kg, $p < 0.05$) and *A. mellifera* (61.72 ± 3.89 mg CEQ/kg, $p < 0.05$) honeys. There was significant difference between the mean flavonoid contents of *A. mellifera* and *Hypotrigona* sp. honeys ($p < 0.05$).

### 3.1.12. Proline content

The proline contents of the honey samples varied from 338.89 ± 0.13 to 481.27 ± 0.03 mg/kg for *A. mellifera* honey (Table 1); 390.73 ± 0.25 to 498.52 ± 0.04 mg/kg for *Hypotrigona* sp. honey (Table 2) and 276.11 ± 0.03 to 338.84 ± 0.07 mg/kg for *Melipona* sp. honey (Table 3). There were significant differences between the mean proline contents of the honey varieties (One-way ANOVA, $F_{(2, 24)} = 14.039, p < 0.05$). The mean proline content of *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys were 386.46 ± 71.11 mg/kg, 430.17 ± 51.45 mg/kg and 298.74 ± 30.16 mg/kg respectively (Table 4). The mean proline contents of the *Hypotrigona* sp. honey was not significantly different from that of *A. mellifera* honey ($p < 0.05$). But both were significantly different from *Melipona* sp. honey sample ($p < 0.05$).

### 3.1.13. Ascorbic acid content

The ascorbic acid contents of the honeys ranged from 149.48 ± 0.28 to 161.96 ± 0.05 mg/kg for *A. mellifera* honey (Table 1); 153.80 ± 0.07 to 169.27 ± 0.04 mg/kg for
for *Hypotrigona* sp. honey (Table 2) and 144.31 ± 0.08 to 151.62 ± 0.34 mg/kg for *Melipona* sp. honey (Table 3). There were statistically significant differences between the mean ascorbic acid contents of the honey varieties (One Way ANOVA, $F_{(2, 24)} = 13.767, p < 0.05$). The mean ascorbic acid contents of *Melipona* sp. honey samples (148.54 ± 3.29 mg/kg,) was significantly different from *A. mellifera* (156.29 ± 5.48 mg/kg, $p < 0.05$) and *Hypotrigona* sp. (161.69 ± 6.70 mg/kg, $p < 0.05$) honeys. There was no significant difference between the mean ascorbic acid of *A. mellifera* and *Hypotrigona* sp. honey samples ($p > 0.05$) (Table 4).
<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Sample I mean ± SD</th>
<th>Sample II mean ± SD</th>
<th>Sample III mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.46 ± 0.095</td>
<td>4.22 ± 0.077</td>
<td>4.04 ± 0.124</td>
</tr>
<tr>
<td>Moisture Contents (%)</td>
<td>11.870 ± 0.703</td>
<td>11.537 ± 0.454</td>
<td>11.817 ± 0.301</td>
</tr>
<tr>
<td>Electrical Conductivity (EC) (mS/cm)</td>
<td>0.163 ± 0.0153</td>
<td>0.217 ± 0.1155</td>
<td>0.240 ± 0.0200</td>
</tr>
<tr>
<td>Total Dissolved Solids (TDS) (ppm)</td>
<td>264.1 ± 0.59</td>
<td>282.0 ± 0.25</td>
<td>316.3 ± 0.06</td>
</tr>
<tr>
<td>Colour (nm)</td>
<td>1.986 ± 0.001</td>
<td>2.102 ± 0.001</td>
<td>2.108 ± 0.001</td>
</tr>
<tr>
<td>Colour Intensity (ABS&lt;sub&gt;450&lt;/sub&gt;) (µAU; 50w/v)</td>
<td>961.33 ± 0.58</td>
<td>988.33 ± 1.15</td>
<td>1006.33 ± 1.53</td>
</tr>
<tr>
<td>Total Sugar Contents ((%) g/g)</td>
<td>79.61 ± 0.03</td>
<td>70.17 ± 0.21</td>
<td>68.32 ± 0.01</td>
</tr>
<tr>
<td>Reducing sugar ((%) g/g)</td>
<td>77.26 ± 0.03</td>
<td>67.75 ± 0.19</td>
<td>66.00 ± 0.02</td>
</tr>
<tr>
<td>Sucrose Contents ((%) g/g)</td>
<td>2.35 ± 0.01</td>
<td>2.42 ± 0.02</td>
<td>2.32 ± 0.01</td>
</tr>
<tr>
<td>HMF (mg/kg)</td>
<td>13.17 ± 0.05</td>
<td>11.97 ± 0.05</td>
<td>16.12 ± 0.12</td>
</tr>
<tr>
<td>Total acidity (meq/kg)</td>
<td>21.93 ± 0.03</td>
<td>22.07 ± 0.10</td>
<td>20.96 ± 0.32</td>
</tr>
<tr>
<td>Free acidity (meq/kg)</td>
<td>18.31 ± 0.11</td>
<td>19.46 ± 0.43</td>
<td>18.24 ± 0.25</td>
</tr>
<tr>
<td>Lactone acidity (meq/kg)</td>
<td>3.62 ± 0.09</td>
<td>2.94 ± 0.08</td>
<td>2.71 ± 0.07</td>
</tr>
<tr>
<td>Total Protein Contents (g/kg)</td>
<td>3.05 ± 0.05</td>
<td>3.11 ± 0.02</td>
<td>3.94 ± 0.01</td>
</tr>
<tr>
<td>Total Polyphenols Contents (mg&lt;sub&gt;GAE&lt;/sub&gt;/kg)</td>
<td>409.73 ± 1.95</td>
<td>432.07 ± 0.10</td>
<td>475.68 ± 0.41</td>
</tr>
<tr>
<td>Total Flavonoids Contents (mg&lt;sub&gt;CEQ&lt;/sub&gt;/kg)</td>
<td>57.00 ± 0.16</td>
<td>62.35 ± 1.09</td>
<td>65.82 ± 0.29</td>
</tr>
<tr>
<td>Proline Contents (mg/kg)</td>
<td>338.89 ± 0.13</td>
<td>339.21 ± 0.01</td>
<td>481.27 ± 0.03</td>
</tr>
<tr>
<td>Ascorbic Acid Contents (mg/kg)</td>
<td>157.44 ± 0.49</td>
<td>161.96 ± 0.05</td>
<td>149.48 ± 0.28</td>
</tr>
</tbody>
</table>

Mean ± standard deviation of duplicate experiments. GAE – Gallic acid equivalent; CEQ – Catechin equivalent.
Table 2: Physicochemical analysis of *Hypotrigona sp.* honey

<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Sample I mean ± SD</th>
<th>Sample II mean ± SD</th>
<th>Sample III mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.85 ± 0.111</td>
<td>3.65 ± 0.036</td>
<td>3.75 ± 0.042</td>
</tr>
<tr>
<td>Moisture Contents (%)</td>
<td>18.31 ± 0.900</td>
<td>17.05 ± 0.489</td>
<td>17.12 ± 0.239</td>
</tr>
<tr>
<td>Electrical Conductivity (EC) (mS/cm)</td>
<td>0.317 ± 0.0058</td>
<td>0.270 ± 0.0200</td>
<td>0.323 ± 0.0493</td>
</tr>
<tr>
<td>Total Dissolved Solids (TDS) (ppm)</td>
<td>399.1 ± 0.35</td>
<td>349.0 ± 0.36</td>
<td>362.0 ± 0.15</td>
</tr>
<tr>
<td>Colour (nm)</td>
<td>2.10 ± 0.001</td>
<td>1.86 ± 0.001</td>
<td>1.88 ± 0.001</td>
</tr>
<tr>
<td>Colour Intensity (ABS$_{450}$) (µAU; 50w/v)</td>
<td>724.33 ± 0.58</td>
<td>630.00 ± 1.73</td>
<td>682.67 ± 0.58</td>
</tr>
<tr>
<td>Total Sugar Contents ((%) g/g)</td>
<td>62.72 ± 0.01</td>
<td>66.32 ± 0.01</td>
<td>57.94 ± 0.03</td>
</tr>
<tr>
<td>Reducing sugar ((%) g/g)</td>
<td>60.72 ± 0.02</td>
<td>64.64 ± 0.02</td>
<td>56.12 ± 0.04</td>
</tr>
<tr>
<td>Sucrose Contents ((%) g/g)</td>
<td>1.99 ± 0.02</td>
<td>1.67 ± 0.02</td>
<td>1.82 ± 0.02</td>
</tr>
<tr>
<td>HMF (mg/kg)</td>
<td>16.39 ± 0.08</td>
<td>16.29 ± 0.04</td>
<td>17.07 ± 0.05</td>
</tr>
<tr>
<td>Total acidity (meq/kg)</td>
<td>35.70 ± 0.22</td>
<td>35.08 ± 0.30</td>
<td>35.93 ± 0.07</td>
</tr>
<tr>
<td>Free acidity (meq/kg)</td>
<td>30.85 ± 0.29</td>
<td>30.89 ± 0.22</td>
<td>30.34 ± 0.03</td>
</tr>
<tr>
<td>Lactone acidity (meq/kg)</td>
<td>4.85 ± 0.10</td>
<td>4.19 ± 0.19</td>
<td>5.59 ± 0.06</td>
</tr>
<tr>
<td>Total Protein Contents (g/kg)</td>
<td>6.58 ± 0.03</td>
<td>4.99 ± 0.04</td>
<td>5.67 ± 0.02</td>
</tr>
<tr>
<td>Total Polyphenols Contents (mg$_{GAE}$/kg)</td>
<td>531.10 ± 0.05</td>
<td>522.91 ± 0.10</td>
<td>375.82 ± 0.25</td>
</tr>
<tr>
<td>Total Flavonoids Contents (mg$_{CEQ}$/kg)</td>
<td>52.52 ± 0.03</td>
<td>28.19 ± 0.03</td>
<td>43.41 ± 0.22</td>
</tr>
<tr>
<td>Proline Contents (mg/kg)</td>
<td>498.52 ± 0.04</td>
<td>401.26 ± 0.26</td>
<td>390.73 ± 0.25</td>
</tr>
<tr>
<td>Ascorbic Acid Contents (mg/kg)</td>
<td>169.27 ± 0.04</td>
<td>153.80 ± 0.07</td>
<td>162.01 ± 0.23</td>
</tr>
</tbody>
</table>

Mean ± standard deviation of triplicate experiments. GAE – Gallic acid equivalent; CEQ – Catechin equivalent.
Table 3: Physicochemical analysis of *Melipona* sp. honey

<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Sample I mean ± SD</th>
<th>Sample II mean ± SD</th>
<th>Sample III mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.80 ± 0.027</td>
<td>4.18 ± 0.066</td>
<td>4.65 ± 0.053</td>
</tr>
<tr>
<td>Moisture Contents (%)</td>
<td>13.507 ± 0.047</td>
<td>15.203 ± 0.186</td>
<td>12.863 ± 0.221</td>
</tr>
<tr>
<td>Electrical Conductivity (EC) (mS/cm)</td>
<td>0.217 ± 0.0152</td>
<td>0.240 ± 0.0100</td>
<td>0.257 ± 0.0153</td>
</tr>
<tr>
<td>Total Dissolved Solids (TDS) (ppm)</td>
<td>209.0 ± 0.31</td>
<td>211.7 ± 0.15</td>
<td>221.2 ± 0.15</td>
</tr>
<tr>
<td>Colour (nm)</td>
<td>1.533 ± 0.000</td>
<td>2.012 ± 0.001</td>
<td>1.632 ± 0.001</td>
</tr>
<tr>
<td>Colour Intensity (ABS&lt;sub&gt;450&lt;/sub&gt;) (µAU; 50w/v)</td>
<td>762.33 ± 2.31</td>
<td>792.33 ± 1.15</td>
<td>805.33 ± 0.58</td>
</tr>
<tr>
<td>Total Sugar Contents ((%) g/g)</td>
<td>80.53 ± 1.59</td>
<td>79.55 ± 0.44</td>
<td>82.04 ± 0.19</td>
</tr>
<tr>
<td>Reducing sugar ((%) g/g)</td>
<td>75.73 ± 2.25</td>
<td>73.72 ± 0.46</td>
<td>77.47 ± 0.20</td>
</tr>
<tr>
<td>Sucrose Contents ((%) g/g)</td>
<td>4.79 ± 0.95</td>
<td>5.82 ± 0.02</td>
<td>4.57 ± 0.02</td>
</tr>
<tr>
<td>HMF (mg/kg)</td>
<td>5.50 ± 0.03</td>
<td>5.65 ± 0.04</td>
<td>5.35 ± 0.12</td>
</tr>
<tr>
<td>Total acidity (meq/kg)</td>
<td>12.43 ± 0.10</td>
<td>12.95 ± 0.07</td>
<td>12.40 ± 0.03</td>
</tr>
<tr>
<td>Free acidity (meq/kg)</td>
<td>10.63 ± 0.16</td>
<td>11.62 ± 0.12</td>
<td>11.43 ± 0.05</td>
</tr>
<tr>
<td>Lactone acidity (meq/kg)</td>
<td>1.80 ± 0.07</td>
<td>1.32 ± 0.07</td>
<td>1.04 ± 0.07</td>
</tr>
<tr>
<td>Total Protein Contents (g/kg)</td>
<td>2.97 ± 0.04</td>
<td>3.94 ± 0.01</td>
<td>3.78 ± 0.01</td>
</tr>
<tr>
<td>Total Polyphenols Contents (mg&lt;sub&gt;GAE&lt;/sub&gt;/kg)</td>
<td>386.09 ± 0.11</td>
<td>354.03 ± 0.08</td>
<td>375.82 ± 0.25</td>
</tr>
<tr>
<td>Total Flavonoids Contents (mg&lt;sub&gt;CEQ&lt;/sub&gt;/kg)</td>
<td>82.78 ± 0.39</td>
<td>83.79 ± 0.06</td>
<td>92.60 ± 0.10</td>
</tr>
<tr>
<td>Proline Contents (mg/kg)</td>
<td>281.28 ± 0.06</td>
<td>276.11 ± 0.03</td>
<td>338.84 ± 0.07</td>
</tr>
<tr>
<td>Ascorbic Acid Contents (mg/kg)</td>
<td>149.69 ± 0.27</td>
<td>151.62 ± 0.34</td>
<td>144.31 ± 0.08</td>
</tr>
</tbody>
</table>

Mean ± standard deviation of triplicate experiments. GAE – Gallic acid equivalent; CEQ – Catechin equivalent
Table 4: Comparison of physicochemical parameters of honeys from *Apis mellifera*, *Hypotrigona* sp. and *Melipona* sp.

<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Samples</th>
<th>Limits of Int’l Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMS mean ± SD</td>
<td>HYPS mean ± SD</td>
</tr>
<tr>
<td>pH</td>
<td>4.24 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.75 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Moisture Contents (%)</td>
<td>11.74 ± 0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.50 ± 0.80&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Electrical Conductivity (EC) (mS/cm)</td>
<td>0.207 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.303 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Dissolved Solids (TDS) (ppm)</td>
<td>287.46 ± 22.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>370.01 ± 22.51&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colour (nm)</td>
<td>2.065 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.950 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colour Intensity (ABS&lt;sub&gt;450&lt;/sub&gt;) (µAU; 50w/v)</td>
<td>985.33 ± 19.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>679.00 ± 40.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Sugar Contents ((%) g/g)</td>
<td>72.70 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.32 ± 5.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reducing sugar ((%) g/g)</td>
<td>70.34 ± 7.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.49 ± 5.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sucrose Contents ((%) g/g)</td>
<td>2.36 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.83 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HMF (mg/kg)</td>
<td>13.75 ± 1.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.58 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total acidity (meq/kg)</td>
<td>21.65 ± 0.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.57 ± 0.42&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lactone acidity (meq/kg)</td>
<td>18.67 ± 0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.69 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Protein Contents (g/kg)</td>
<td>3.09 ± 0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.88 ± 0.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Polyphenols Contents (mg&lt;sub&gt;gallic acid/kg&lt;/sub&gt;)</td>
<td>439.16 ± 29.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>527.41 ± 3.60&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Flavonoids Contents (mg&lt;sub&gt;catechin/kg&lt;/sub&gt;)</td>
<td>61.72 ± 3.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.37 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proline Contents (mg/kg)</td>
<td>386.46 ± 71.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>430.17 ± 51.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ascorbic Acid Contents (mg/kg)</td>
<td>156.29 ± 5.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>161.69 ± 6.70&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means were compared by using one way ANOVA and Tukey Post Hoc Multiple Comparisons. In each row, values with different letters (superscripts) indicate significant differences (*p*< 0.05). (AMHS = *Apis mellifera* honey; HYHS = *Hypotrigona* sp. honey; and MEPSH = *Melipona* sp. honey) NFL - No fixed limit.
<table>
<thead>
<tr>
<th>Colour</th>
<th>Colour range</th>
<th>Pfund scale (mm)</th>
<th>Mid-range absorbance at 560 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water White</td>
<td>Very light colour</td>
<td>8.0 or less</td>
<td>0.0945</td>
</tr>
<tr>
<td>Extra White</td>
<td>Darker than water white</td>
<td>9-17</td>
<td>0.189</td>
</tr>
<tr>
<td>White</td>
<td>Darker than extra white</td>
<td>18-34</td>
<td>0.378</td>
</tr>
<tr>
<td>Extra Light</td>
<td>Darker than white</td>
<td>35-50</td>
<td>0.595</td>
</tr>
<tr>
<td>Amber</td>
<td>Darker than extra light amber</td>
<td>51-85</td>
<td>1.389</td>
</tr>
<tr>
<td>Light Amber</td>
<td>Darker than extra light amber</td>
<td>86-114</td>
<td>1.39-3.008</td>
</tr>
<tr>
<td>Amber</td>
<td>Darker than light amber</td>
<td>114</td>
<td>&gt;3.1</td>
</tr>
</tbody>
</table>

3.1.14. Correlation among some physicochemical parameters

The correlation matrixes showed significant correlations between some of the physicochemical parameters. In A. mellifera honey samples, strong positive correlations were found between the colour intensity of honey samples and some physicochemical parameters like phenolics, flavonoid, protein and proline contents at 0.954, 0.990, 0.833, and 0.803 respectively (Table 6). Ascorbic acid had strong negative correlations with protein (r = -0.911) and proline (r = -0.932). The proline content also had strong positive correlations with \( \text{ABS}_{450} \) (r = 0.999), protein (r = 0.996), total phenol content (r = 0.943), and flavonoid content (r = 0.792).

Also, in correlation matrixes of Hypotrigona sp. honey samples, strong positive correlations were found between the colour intensity of honey samples and phenolics, flavonoid, protein, ascorbic acid and proline contents at 0.995, 0.997, 0.989, 0.999 and 0.778 respectively (Table 7). Ascorbic acid had strong positive correlations with \( \text{ABS}_{450} \) (r = 0.999), protein (r = 0.992), total phenol content (r = 0.991), proline content (r = 0.797) and flavonoid content (r = 0.994). While proline content had positive correlations with \( \text{ABS}_{450} \) (r = 0.778), protein (r = 0.864), total phenol content (r = 0.710), ascorbic acid content (r = 0.797) and flavonoid content (r = 0.727).

Strong positive correlations were found between the colour intensity of Melipona sp. honey samples and some physicochemical parameters like flavonoid, protein and proline at 0.792, 0.898 and 0.679, respectively (Table 8). Generally, the colour intensity of the honeys increases with increases in the phenolic and flavonoid contents. Unlike in A. mellifera and Hypotrigona sp. honeys, ascorbic acid had strong negative correlations with flavonoid (r = -0.935) and proline (r = -0.981). Also, a strong negative correlation was observed between the protein content and total phenolic contents (r = -0.839).
Table 6: Correlation matrix showing the interrelation among phenolics, flavonoids, ascorbic acid, proline, ABS<sub>450</sub> and protein from *Apis mellifera* honey.

<table>
<thead>
<tr>
<th></th>
<th>ABS&lt;sub&gt;450&lt;/sub&gt; (µAU,50w/v)</th>
<th>Protein content (g/kg)</th>
<th>Total phenol content (mg GAE/kg)</th>
<th>Total flavonoid content (mg CEQ/kg)</th>
<th>Proline content (mg/kg)</th>
<th>Ascorbic acid content (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS&lt;sub&gt;450&lt;/sub&gt; (µAU,50w/v)</td>
<td>1.000</td>
<td>0.833**</td>
<td>0.954**</td>
<td>0.990**</td>
<td>0.803**</td>
<td>-0.536</td>
</tr>
<tr>
<td>Protein content (g/kg)</td>
<td>0.833**</td>
<td>1.000</td>
<td>0.957**</td>
<td>0.821**</td>
<td>0.996**</td>
<td>-0.911**</td>
</tr>
<tr>
<td>Total phenol content (mg GAE/kg)</td>
<td>0.954**</td>
<td>0.957**</td>
<td>1.000</td>
<td>0.943**</td>
<td>0.943**</td>
<td>-0.759*</td>
</tr>
<tr>
<td>Total flavonoid content (mg CEQ/kg)</td>
<td>0.990**</td>
<td>0.821**</td>
<td>0.943**</td>
<td>1.000</td>
<td>0.792*</td>
<td>-0.525</td>
</tr>
<tr>
<td>Proline content (mg/kg)</td>
<td>0.803**</td>
<td>0.996**</td>
<td>0.943**</td>
<td>0.792*</td>
<td>1.000</td>
<td>-0.932**</td>
</tr>
<tr>
<td>Ascorbic acid content (mg/kg)</td>
<td>-0.536</td>
<td>-0.911**</td>
<td>-0.759*</td>
<td>-0.525</td>
<td>-0.932**</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).
Table 7: Correlation matrix showing the interrelation among phenolics, flavonoids, ascorbic acid, proline, ABS$_{450}$ and protein from *Hypotrigona* sp. honey.

<table>
<thead>
<tr>
<th></th>
<th>ABS$_{450}$ (µAU,50w/v)</th>
<th>Protein content (g/kg)</th>
<th>Total phenol content (mg GAE/kg)</th>
<th>Total flavonoid content (mg CEQ/kg)</th>
<th>Proline content (mg/kg)</th>
<th>Ascorbic acid content (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS$_{450}$ (µAU,50w/v)</td>
<td>1.000</td>
<td></td>
<td>0.997**</td>
<td>0.997**</td>
<td>0.778</td>
<td>0.999**</td>
</tr>
<tr>
<td>Protein content (g/kg)</td>
<td>0.987**</td>
<td>1.000</td>
<td>0.967**</td>
<td>0.973**</td>
<td>0.864**</td>
<td>0.992**</td>
</tr>
<tr>
<td>Total phenol content (mg GAE/kg)</td>
<td>0.995**</td>
<td>0.967**</td>
<td>1.000</td>
<td>1.000**</td>
<td>0.710*</td>
<td>0.991**</td>
</tr>
<tr>
<td>Total flavonoid content (mg CEQ/kg)</td>
<td>0.997**</td>
<td>0.973**</td>
<td>1.000**</td>
<td>1.000</td>
<td>0.727*</td>
<td>0.994**</td>
</tr>
<tr>
<td>Proline content (mg/kg)</td>
<td>0.778*</td>
<td>0.864**</td>
<td>0.710*</td>
<td>0.727*</td>
<td>1.000</td>
<td>0.797*</td>
</tr>
<tr>
<td>Ascorbic acid content (mg/kg)</td>
<td>0.999**</td>
<td>0.992**</td>
<td>0.991**</td>
<td>0.994**</td>
<td>0.797*</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).
Table 8: Correlation matrix showing the interrelation among phenolics, flavonoids, ascorbic acid, proline, ABS450 and protein from *Melipona* sp. honey.

<table>
<thead>
<tr>
<th></th>
<th>ABS\textsubscript{450} (µAU,50w/v)</th>
<th>Protein content (g/kg)</th>
<th>Total phenol content (mg GAE/kg)</th>
<th>Total flavonoid content (mg CEQ/kg)</th>
<th>Proline content (mg/kg)</th>
<th>Ascorbic acid content (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS\textsubscript{450} (µAU,50w/v)</td>
<td>1.000</td>
<td>0.898**</td>
<td>-0.515</td>
<td>0.792*</td>
<td>0.679</td>
<td>-0.530</td>
</tr>
<tr>
<td>Protein content (g/kg)</td>
<td>0.898**</td>
<td>1.000</td>
<td>-0.839**</td>
<td>0.446</td>
<td>0.290</td>
<td>-0.109</td>
</tr>
<tr>
<td>Total phenol content (mg GAE/kg)</td>
<td>-0.515</td>
<td>-0.839**</td>
<td>1.000</td>
<td>0.111</td>
<td>0.275</td>
<td>-0.445</td>
</tr>
<tr>
<td>Total flavonoid content (mg CEQ/kg)</td>
<td>0.792*</td>
<td>0.446</td>
<td>0.111</td>
<td>1.000</td>
<td>0.985**</td>
<td>-0.935**</td>
</tr>
<tr>
<td>Proline content (mg/kg)</td>
<td>0.679*</td>
<td>0.290</td>
<td>0.275</td>
<td>0.985**</td>
<td>1.000</td>
<td>-0.981**</td>
</tr>
<tr>
<td>Ascorbic acid content (mg/kg)</td>
<td>-0.530</td>
<td>-0.109</td>
<td>-0.445</td>
<td>-0.935**</td>
<td>-0.981**</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed).
3.2. Antimicrobial Activity screening of the Honey Varieties

The initial antimicrobial activity screening of the honey samples was done using agar-well diffusion assay through the determination of zones of inhibition of honey samples.

3.2.1. Antimicrobial Activity of Apis mellifera Honey Samples

The three samples of Apis mellifera honey showed antimicrobial activity against all the tested organisms at a concentration range of 10 - 60% (v/v). Ten percent (v/v) of each of the honey samples did not show inhibition zones against B. cereus (Figure 1; Plate 1) and MDR S. aureus (Figure 2). Klebsiella pneumonia and E. coli were inhibited at least concentrations of 60% (Figure 3) and 10% (v/v) (Figure 4) respectively. However, 20% of the honey samples showed no inhibition zones against P. aeruginosa (ATCC 25783) (Figure 5), MDR S. enterica (Figure 6) and A. niger (Figure 7). Also, 10% (v/v) of the honey samples showed inhibition zones against C. albicans (Figure 8) and C. tropicalis (Figure 9).

3.2.2. Antimicrobial Activity of Hypotrigona sp. Honey Samples

All the three Hypotrigona sp. honey samples showed antimicrobial activity against the tested organisms at a concentration range of 10 – 40% (v/v). Except for C. albicans (SC5314) (Figure 8), the three honey samples inhibited all the test organisms at a concentration of 10% (v/v) (Figure 1 – 9; Plate 2). Hypotrigona sp. honey sample I, II and III showed inhibition zones against C. albicans at concentrations of 20%, 40% and 40% (v/v) respectively (Figure 8).

3.2.3. Antimicrobial Activity of Melipona sp. Honey Samples

The honey samples showed antibacterial activity against all the tested organisms at a concentration range of 10 - 40% (v/v). Ten percent (v/v) of the three honey samples each showed inhibition zones against B. cereus (Figure 1), while 20% (v/v) of the honey samples inhibited MDR S. aureus (Figure 2; Plate 3), Klebsiella pneumonia (Figure 3), E. coli (Figure 4), MDR S. enterica (Figure 6), C. albicans (SC5314) (Figure 8), and C. tropicalis (Figure 9). A. niger (Figure 7) and P. aeruginosa (ATCC 25783) (Figure 5) were both inhibited by 40% (v/v) concentration of the honeys.
Figure 1: Zones of inhibition diameter (mm) of the honey samples against *B. cereus* (Mean ± SD)

(AMI – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 2: Zones of inhibition diameter (mm) of the honey samples against *Staphylococcus aureus* (Mean ± SD)

(AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 3: Zones of inhibition diameter (mm) of the honey samples against *Klebsiella pneumonia* (Mean ± SD)

(AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 4: Zones of inhibition diameter (mm) of the honey samples against *Escherichia coli* (Mean ± SD)

(AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
**Figure 5:** Zones of inhibition diameter (mm) of the honey samples against *Pseudomonas aeruginosa* (ATCC 25783)(Mean ± SD)

(AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 6: Zones of inhibition diameter (mm) of the honey samples against MDR *Salmonella enterica* (Mean ± SD)

(AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 7: Zones of inhibition diameter (mm) of the honey samples against *Aspergillus niger* (Mean ± SD)

(AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 8: Zones of inhibition diameter (mm) of the honey samples against *Candida albicans* (SC5314) (Mean ± SD) (AM I – III, HY I – III and MEP I -III stand for *Apis Mellifera* honey, *Hypotrigona* sp. and *Melipona* sp. respectively)
Figure 9: Zones of inhibition diameter (mm) of the honey samples against Candida tropicalis (Mean ± SD)
(AMI – III, HY I – III and MEP I -III stand for Apis Mellifera honey, Hypotrigona sp. and Melipona sp. respectively)
Plate 1: Zones of inhibition of *A. mellifera* honey against *B. cereus* (on nutrient agar plate) (concentration of 100, 80, 60, 40, 20, and 10% v/v).
Plate 2: Zones of inhibition of *Hypotrigona* sp. honey against *B. cereus* (on nutrient agar plate) (concentration of 10 – 100% v/v)
Plate 3: Zones of inhibition of *Melipona* sp. honey (10 – 100% v/v) against *S. aureus* (on nutrient agar plate) (concentration of 10 – 100% v/v)
3.3. Mean Antibacterial Activities of the Different Honey Varieties

SPSS statistical programme version twenty was used to analyse our data and comparisons between means were made using the one-way ANOVA and Games – Howell post hoc test at 0.05 probabilities. There were statistically significant differences between the mean inhibition zone diameters (mm) of *Apis Mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys against *Bacillus cereus* ($F_{(2,51)}= 3.494, p = 0.038$), *Staphylococcus aureus* ($F_{(2,51)}= 5.523, p = 0.007$), *Klebsiella pneumonia* ($F_{(2,51)}= 3.868, p = 0.027$), *Escherichia coli* ($F_{(2,51)}= 8.609, p = 0.001$), *Pseudomonas aeruginosa* ATCC 25783 ($F_{(2,51)}= 8.621, p = 0.001$), MDR *Salmonella enterica* ($F_{(2,51)}= 3.691, p = 0.032$) and *Aspergillus niger* ($F_{(2,51)}= 15.214, p = 0.000007$). There were no significant differences between the mean zones of inhibition of the honeys against *C. albicans* (SC5314) ($F_{(2,51)}= 0.609, p = 0.548$) and *C. tropicalis*(F$_{(2,51)}$= 0.746, $p = 0.480$) (Table 9 and Appendix C).

A Games - Howell post-hoc test revealed that the mean zones of inhibition(mm) of *Apis Mellifera* honey against *B. cereus* (10.01 ±6.58) and *E. coli* (12.13 ± 5.88) were significantly higher than the mean inhibition zones of *Melipona* sp. honey against these organisms (5.71 ± 3.64, $p = 0.031$ and 5.37 ± 4.30, $p = 0.00039$ respectively). There were no statistically significant differences between the mean zones of inhibition of *Apis Mellifera* honey (against *B. cereus* and *E. coli*) and *Hypotrigona* sp. honey (8.37 ± 4.05, $p = 0.051$ respectively). Also, there were no statistically significant differences between the mean inhibition zones of *Hypotrigona* sp. and *Melipona* sp. honey against *B. cereus* ($p = 0.247$) and *E. coli* ($p = 0.207$). The mean zones of inhibition diameter of *Apis Mellifera* and *Melipona* sp. honeys were significantly lower against MDR *S. aureus* (3.37 ± 3.16, $p = 0.009$ and 3.89 ± 3.74, $p = 0.029$ respectively for the honeys), *K. pneumonia* (4.07 ± 4.71, $p = 0.022$ and 5.49 ± 3.87, $p = 0.201$ respectively), *P. aeruginosa*(ATCC 25783) (5.49 ± 4.64, $p = 0.012$ and 4.04 ± 3.60, $p = 0.001$ respectively) and MDR *S. enterica* (3.95 ± 3.94, $p = 0.051$ and 4.09 ± 3.22, $p = 0.065$ respectively) compared to the *Hypotrigona* sp. honey (7.14 ± 4.11, 7.92 ± 3.96, 9.77 ± 4.58 and 6.96 ± 4.03 respectively for the organisms). There were no statistically significant differences between the mean inhibition zones of *Apis Mellifera* and *Melipona* sp. honeys against these organisms ($p = 0.904$, $p = 0.573$, $p = 0.575$ and $p = 0.993$ respectively). However, *Hypotrigona* sp. honey had the highest mean zone of inhibition against *A. niger* (10.12 ±5.51) which was significantly different from *Apis
Mellifera (5.51 ± 5.00, p = 0.08) and Melipona sp. (1.99 ± 1.91, p = 0.000004) honeys. Also, Melipona sp. honey had the least mean ZID which was significant different from Apis Mellifera honey mean ZID against A. niger (p = 0.054). Therefore, Hypotrigona sp. honey had the highest mean zone of inhibition against MDR S. aureus, K. pneumonia, P aeruginosa (ATCC 25783), MDRS. enterica serovar Typhi, and A. niger as compared to other honey samples. Also, A. mellifera honey had the highest mean zone of inhibition against B. cereus, E. coli, C. albicans (SC5314) and C. tropicalis.

In addition, sterile distilled water (negative control) did not produce any zone of inhibition; whereas positive controls i.e. ciprofloxacin (500 – 15.6μg/ml) and Ketoconazole (400 – 12.56μg/ml) produced respectively 20 ± 0.88 - 10 ± 0.29 and 22 ± 0.87 - 9 ± 0.87 mm mean inhibition zone against reference strains respectively (Table 10).
### Table 9: Comparison of mean zones of inhibition diameter (mm) of *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp. honey samples against the test microorganisms

<table>
<thead>
<tr>
<th>Test Organism</th>
<th>Apis mellifera Honey (n=3)</th>
<th>Hypotrigona sp. Honey (n=3)</th>
<th>Melipona sp. Honey (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em></td>
<td>10.01 ± 6.58b</td>
<td>8.37 ± 4.05ab</td>
<td>5.71 ± 3.64a</td>
</tr>
<tr>
<td>MDR S. aureus</td>
<td>3.37 ± 3.16a</td>
<td>7.14 ± 4.11b</td>
<td>3.89 ± 3.74a</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>4.07 ± 4.71a</td>
<td>7.92 ± 3.96b</td>
<td>5.49 ± 3.87a</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>12.13 ± 5.88b</td>
<td>8.19 ± 4.41ab</td>
<td>5.37 ± 4.30a</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>5.49 ± 4.64a</td>
<td>9.77 ± 4.58b</td>
<td>4.04 ± 3.60a</td>
</tr>
<tr>
<td>ATCC 25783</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDR S. enterica</td>
<td>3.95 ± 3.94a</td>
<td>6.96 ± 4.03b</td>
<td>4.09 ± 3.22a</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>5.51 ± 5.00b</td>
<td>10.12 ± 5.51c</td>
<td>1.99 ± 1.91a</td>
</tr>
<tr>
<td><em>C. albicans SC</em></td>
<td>6.31 ± 4.64a</td>
<td>5.09 ± 4.40a</td>
<td>4.86 ± 3.53a</td>
</tr>
<tr>
<td>5314</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>7.38 ± 5.46a</td>
<td>6.76 ± 3.66a</td>
<td>5.61 ± 3.86a</td>
</tr>
</tbody>
</table>

*Hypotrigona* sp. and *Melipona* sp. honey samples against the test microorganisms

Means were compared by using one-way ANOVA and Games - Howell Post Hoc Multiple Comparisons. In each row, values with different letters (superscripts) indicate significant differences (*p* < 0.05). Mean zones of inhibition diameter (mm) ± Standard deviation.
Table 10: Zones of inhibition diameter for control drugs and sterile distilled water
(Mean value ± Standard Error (mm))

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Concentration (µg/ml)</th>
<th>Mean ZID ± Standard Error (mm)</th>
<th>Sterile Distilled Water (Negative Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pseudomonas</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aeruginosa</em> ATCC 25783</td>
<td>500</td>
<td>20 ± 0.88</td>
<td>NZI</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>20 ± 0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>17 ± 0.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>15 ± 0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31.3</td>
<td>12 ± 0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>10 ± 0.29</td>
<td></td>
</tr>
<tr>
<td><strong>Candida</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>albicans SC 5314</em></td>
<td>400</td>
<td>22 ± 0.87</td>
<td>NZI</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19 ± 0.87</td>
<td></td>
</tr>
<tr>
<td><strong>NZI = No zones of inhibition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NZI = No zones of inhibition
3.4. Minimum Inhibitory Concentration of investigated Honey Samples

The minimum inhibitory concentrations (MIC) of the honey varieties were determined using micro-dilution methods.

3.4.1. MIC of *Apis mellifera* Honey Samples

*Apis Mellifera* honey samples (I – III) inhibited all isolates tested at MIC range of 12.5 – 25.0% (v/v) (Table 11). *A. mellifera* honey sample I had MIC of 12.5% (v/v) against *B. cereus* and MDR *S. aureus* while *E. coli* and *P. aeruginosa* (ATCC 25783) were both inhibited at MIC of 6.3% (v/v). MIC of 25.0% (v/v) inhibited MDR *S. enterica*, *A. niger*, *K. pneumonia*, *C. albicans* (SC5314) and *C. tropicalis*. *A. mellifera* honey sample II had MICs just like sample I except that *P. aeruginosa* (ATCC 25783), *C. albicans* (SC5314) and *C. tropicalis* were inhibited at MIC of 12.5% (v/v). Also, sample III had similar MICs with sample I except that *K. pneumonia*, *C. albicans* (SC5314) and *C. tropicalis* were inhibited at MIC of 12.5% (v/v).

3.4.2. MIC of *Hypotrigona* sp. Honey Samples

*Hypotrigona* sp. honey samples (I – III) inhibited all isolates tested at MIC range of 12.5 – 25.0% (v/v) (Table 12). Honey sample I had MIC of 3.1% (v/v) against *B. cereus* and *C. tropicalis* while the rest of the test isolates were inhibited at MIC of 6.3% (v/v). In honey sample II, MIC of 6.3% (v/v) inhibited all the test isolates except *A. niger* and *C. tropicalis* that were inhibited at MICs of 12.5% and 3.1% (v/v) respectively. *Hypotrigona* sp. honey sample III had MIC of 3.1% against *B. cereus* while *P. aeruginosa* (ATCC 25783) and *A. niger* were both inhibited at MIC of 12.5% (v/v). The same honey sample inhibited the rest of the test isolates at MIC of 6.3% (v/v).

3.4.3. MIC of *Melipona* sp. Honey Samples

*Melipona* sp. honey samples (MEP I - III) also inhibited all the tested isolates at concentration range of 6.3–25.0% (v/v) (Table 13). The honey sample I had MIC of 6.3% against *B. cereus*, *K. pneumonia* and *C. tropicalis*. *A. niger* was inhibited at MIC of 25.0% while the rest of the test isolates were inhibited at MIC of 12.5% (v/v). Honey sample II had similar MICs with sample I except that *P. aeruginosa* (ATCC 25783) was inhibited at MIC of 6.3% (v/v). In honey sample III, similar MICs were observed except that *P. aeruginosa* (ATCC 25783) and *E. coli* were inhibited at MIC of 6.3% (v/v).
Table 11: Minimum inhibitory concentration (MIC) of three *Apis mellifera* honey samples with and without addition of catalase.

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Sample I (MIC without catalase)</th>
<th>Sample II (MIC without catalase)</th>
<th>Sample III (MIC without catalase)</th>
<th>Sample I (MIC with catalase)</th>
<th>Sample II (MIC with catalase)</th>
<th>Sample III (MIC with catalase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>MDR S. aureus</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>25.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>E. coli</td>
<td>6.3</td>
<td>6.3</td>
<td>&gt;50.0</td>
<td>6.3</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>P. aeruginosa ATCC 25783</td>
<td>6.3</td>
<td>6.3</td>
<td>&gt;50.0</td>
<td>6.3</td>
<td>6.3</td>
<td>6.3</td>
</tr>
<tr>
<td>MDR S. enterica</td>
<td>25.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>A. niger</td>
<td>25.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>C. albicans SC 5314</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Median of triplicate experiments. MIC in % (v/v)
Table 12: Minimum inhibitory concentration of three *Hypotrigona* sp. honey samples with and without addition of catalase.

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (without catalase)</td>
<td>MIC (with catalase)</td>
<td>MIC (without catalase)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>3.1 12.5</td>
<td>6.3 25.0</td>
<td>3.1 12.5</td>
</tr>
<tr>
<td>MDR <em>S. aureus</em></td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>6.3 12.5</td>
<td>6.3 12.5</td>
<td>6.3 25.0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 25783</td>
<td>3.1 6.3</td>
<td>6.3 12.5</td>
<td>6.3 12.5</td>
</tr>
<tr>
<td>MDR <em>S. enterica</em></td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
<td>6.3 12.5</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>6.3 12.5</td>
<td>12.5 25.0</td>
<td>12.5 25.0</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
<td>6.3 25.0</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>3.1 25.0</td>
<td>3.1 25.0</td>
<td>6.3 25.0</td>
</tr>
</tbody>
</table>

Median of triplicate experiments. MIC in % (v/v)
Table 13: Minimum inhibitory concentration of three *Melipona* sp. honey samples with and without addition of catalase.

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Sample I (without catalase)</th>
<th>Sample I (with catalase)</th>
<th>Sample II (without catalase)</th>
<th>Sample II (with catalase)</th>
<th>Sample III (without catalase)</th>
<th>Sample III (with catalase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
</tr>
<tr>
<td></td>
<td>(without catalase)</td>
<td>(with catalase)</td>
<td>(without catalase)</td>
<td>(with catalase)</td>
<td>(without catalase)</td>
<td>(with catalase)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>6.3</td>
<td>12.5</td>
<td>6.3</td>
<td>25.0</td>
<td>6.3</td>
<td>12.5</td>
</tr>
<tr>
<td>MDR <em>S. aureus</em></td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>12.5</td>
<td>50.0</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>6.3</td>
<td>25.0</td>
<td>6.3</td>
<td>25.0</td>
<td>6.3</td>
<td>12.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>12.5</td>
<td>25.0</td>
<td>12.5</td>
<td>25.0</td>
<td>6.3</td>
<td>12.5</td>
</tr>
<tr>
<td><em>P. aeruginosa ATCC 25783</em></td>
<td>12.5</td>
<td>25.0</td>
<td>6.3</td>
<td>25.0</td>
<td>6.3</td>
<td>12.5</td>
</tr>
<tr>
<td>MDR <em>S. enterica</em></td>
<td>12.5</td>
<td>50.0</td>
<td>12.5</td>
<td>25.0</td>
<td>12.5</td>
<td>25.0</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td><em>C. albicans SC 5314</em></td>
<td>12.5</td>
<td>25.0</td>
<td>12.5</td>
<td>25.0</td>
<td>12.5</td>
<td>25.0</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>6.3</td>
<td>12.5</td>
<td>6.3</td>
<td>25.0</td>
<td>6.3</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Median of triplicate experiments. MIC in % (v/v)
3.4.4. MIC of non-peroxidase Activities of the Honey Varieties

Honey samples were diluted with and without catalase to determine whether their antimicrobial activities were solely due to generation of hydrogen peroxide. The *Apis mellifera* honey samples’ non-peroxidase MIC were within the range of 12.5 – 50.0% (v/v) (Table 11). *Apis mellifera* honey sample I had MIC of 25% against *B. cereus*, *MDR S. aureus*, and *E. coli* while *P. aeruginosa* was inhibited at MIC of 12.5% (v/v). Also, *A. mellifera* honey sample I at highest concentration of 50.0% (v/v) did inhibit *K. pneumonia*, *MDR S. enterica*, *C. albicans* and *C. tropicalis* while *A. niger* was not inhibited (Table 11). *Apis mellifera* honey sample II had MIC of 12.5 and 50.0% against *E. coli* and *A. niger* respectively. *K. pneumonia* and *MDR S. enterica* were not inhibited at 50.0% of this honey sample used. The rest of the isolates were inhibited at MIC of 25.0% (v/v). 25.0% was the MIC of honey sample III against *B. cereus*, *MDR S. aureus*, *C. albicans* and *C. tropicalis* while *E. coli* and *P. aeruginosa* was inhibited at MIC of 12.5% (v/v) (Table 11).

The three honey samples from *Hypotrigona* sp. (I - III) had non-peroxidase MIC range of 6.3 – 25% (v/v) (Table 12). The non-peroxidase MIC of honey sample I against *B. cereus*, *E. coli*, and *A. niger* was 12.5% while *P. aeruginosa* was inhibited at MIC of 6.25% (v/v). The rest were inhibited at concentration of 25% (v/v) (Sample I). Honey sample II inhibited all the test isolates at MIC of 25% (v/v) except for *P. aeruginosa*, and *E. coli* that were inhibited at concentration of 12.5% (v/v). *Hypotrigona* sp. honey sample III inhibited all the isolates tested at MIC of 25% except for *B. cereus*, *A. niger* and *MDR S. enterica* that were inhibited at MIC of 12.5% (v/v) (Table 12).

Honey samples (I – III) from *Melipona* sp. had non-peroxidase MIC range of 12.5 – 50% (v/v) against the test isolates (Table 13). *Melipona* sp. honey sample I non-peroxidase MIC against *B. cereus* and *C. tropicalis* was 12.5% (v/v) while *MDR S. enterica* was inhibited at MIC of 50% (v/v). The rest of the isolates tested were inhibited at MIC of 12.5% (Sample I) except *MDR S. aureus* and *A. niger* were not inhibited even at highest concentration of 50% used. MEP II honey inhibited all the isolates at MIC of 25% (v/v) except those organisms that were not inhibited by honey sample I. *Melipona* sp. honey sample III also inhibited all the organisms at MIC of 12.5 % (v/v) except for *MDR S. aureus*, *MDR S. enterica* and *C. albicans* SC 5314 that were inhibited at MIC of 50%, 25% and 25% (v/v) respectively. *A. niger* was not inhibited at the highest concentration of 50% (v/v) used.
3.5. Mean MIC of *Apis mellifera*, *Hypotrigona* sp. and *Melipona* sp. Honeys

The mean minimum inhibitory concentrations of the honey varieties were analysed with SPSS statistical programme version twenty using Kruskal-Wallis (KW) test and Mann-Whitney U test at 0.05 probabilities.

Kruskal-Wallis (KW) test revealed that there were statistically significant differences between the mean MICs of *Apis Mellifera*, *Hypotrigona* sp. and *Melipona* sp. honeys against *Bacillus cereus* (KW test, df = 2, \(p = 0.000078\)), *Staphylococcus aureus* (KW test, df = 2, \(p = 0.003\)), *Klebsiella pneumonia* (KW test, df = 2, \(p = 0.000024\)), *Escherichia coli* (KW test, df = 2, \(p = 0.003\)), *MDR Salmonella enterica* (KW test, df = 2, \(p = 0.000075\)), *Aspergillus niger* (KW test, df = 2, \(p = 0.0001\)), *C. albicans* (SC5314) (KW test, df = 2, \(p = 0.001\)) and *C. tropicalis* (KW test, df = 2, \(p = 0.000356\)). There were no significant differences between the mean MIC of the honeys against *Pseudomonas aeruginosa* ATCC 25783 (KW test, df = 2, \(p = 0.624\)) (Table 14 and Appendix D 1a).

Mann-Whitney U-test pairwise comparisons revealed that the mean MIC of *Apis Mellifera* honey against *B. cereus* (11.11 ± 2.70) was significantly different from mean MIC of *Melipona* sp. honey (6.25 ± 0.00, \(U = 9.000, p = 0.001\)) (Appendix D 2b) and *Hypotrigona* sp. honey (4.15 ± 1.58, \(U = 3.000, p = 0.000433\)) (Appendix D 2a). The *Hypotrigona* sp. honey mean MIC against *B. cereus* was significantly different from mean MIC of *Melipona* sp. honey (\(U = 13.500, p = 0.004\)) (Appendix D 2c) (Table 14).

The mean MIC of *Hypotrigona* sp. honey against MDR *S. aureus* (7.29 ± 3.13) was significantly different from mean MIC of *Apis Mellifera* honey (11.81 ± 2.08, \(U = 13.000, p = 0.006\)) (Table 14 and Appendix D 2a) and *Melipona* sp. honey (13.19 ± 489, \(U = 12.000, p = 0.005\)) (Appendix D 2c). The mean MIC of *Apis Mellifera* was not significantly different from mean MIC of *Melipona* sp. honey (\(U = 36.500, p = 0.586\)) (Appendix D 2b) (Table 14).

The mean MIC of *Hypotrigona* sp. honey against *K. pneumonia* (18.75 ± 7.65) was the highest and was significantly different from mean MIC of *Hypotrigona* sp. (6.25 ± 0.00, \(U = 4.500, p = 0.000395\)) (Appendix D 2a) and *Melipona* sp. honey (6.25.00 ± 0.00, \(U = 4.500, p = 0.005\)) (Appendix D 2b). There was no statistically significant differences between the mean MIC of *Hypotrigona* sp. and *Melipona* sp. honey (\(U = 40.500, p = 1.000\)) (Appendix D 2c) (Table 14).
Melipona sp. honey had the highest mean MIC against E. coli (9.72 ± 3.29) (Table 14) and was significantly different from mean MIC of Hypotrigona sp. (6.25 ± 0.00, U = 18.000, p = 0.011) (Appendix D 2c) and Apis Mellifera (5.56 ± 1.38, U = 31.500, p = 0.004) (Appendix D 2b) honeys. There was no statistically significant differences between the mean MIC of Hypotrigona sp. and Apis Mellifera honey (U = 31.500, p = 0.145) (Appendix D 2a).

As with B. cereus (Table 14), the mean MIC of Apis Mellifera honey against MDR S. enterica (23.60 ± 4.20) was significantly different from mean MIC of Melipona sp. honey (13.19 ± 4.89, U = 12.000, p = 0.005) (Appendix D 2b) and Hypotrigona sp. honey (6.59 ± 2.45, U = 1.000, p = 0.000163) (Appendix D 2a). The Hypotrigona sp. honey mean MIC was significantly different from mean MIC of Melipona sp. honey (U = 8.000, p = 0.002) (Appendix D 2c).

The mean MIC of Hypotrigona sp. honey (Table 14) against A. niger (11.11 ± 2.76) was significantly different from mean MIC of Apis Mellifera honey (22.22 ± 5.51, U = 7.000, p = 0.001) (Appendix D 2a) and Melipona sp. honey (22.22 ± 5.51, U = 7.000, p = 0.001) (Appendix D 2c). The mean MIC of Apis Mellifera was not significantly different from mean MIC of Melipona sp. honey (U = 40.500, p = 1.000) (Appendix D 2b).

In C. albicans (SC5314) (Table 14), the mean MIC of Hypotrigona sp. (6.94 ± 2.08) was significantly different from mean MIC of Apis Mellifera honey (13.89 ± 6.83, U = 12.500, p = 0.005) (Appendix D 2a) and Melipona sp. honey (12.5 ± 0.00, U = 4.500, p = 0.000226) (Appendix D 2c). The mean MIC of Apis Mellifera honey was not significantly different from mean MIC of Melipona sp. honey (U = 12.500, p = 1.000) (Appendix D 2b).

In C. tropicalis, the mean MIC of Hypotrigona sp. honey (4.50 ± 1.66) was significantly different from mean MIC of Apis Mellifera honey (13.19 ± 7.29, U = 6.000, p = 0.001) (Appendix D 2a) and Melipona sp. honey (6.25 ± 0.00, U = 18.000, p = 0.011) (Appendix D 2c). The mean MIC of Apis Mellifera honey was not significantly different from mean MIC of Melipona sp. honey (U = 13.500, p = 0.004) (Appendix D 2b) (Table 14).
Table 14: Comparison of the mean MIC (%v/v) of honey samples from *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp.

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th><em>A. mellifera</em> Honey (n=3)</th>
<th><em>Hypotrigona</em> sp. Honey (n=3)</th>
<th><em>Melipona</em> sp. Honey (n=3)</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>12.5</td>
<td>11.11 ± 2.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1</td>
<td>4.15 ± 1.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>MDR S. aureus</em></td>
<td>12.5</td>
<td>11.81 ± 2.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3</td>
<td>7.29 ± 3.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>25.0</td>
<td>18.75 ± 7.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3</td>
<td>6.25 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>6.3</td>
<td>5.56 ± 1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3</td>
<td>6.25 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 25783</td>
<td>6.3</td>
<td>7.64 ± 2.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.3</td>
<td>7.28 ± 4.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>MDR S. enterica</em></td>
<td>25.0</td>
<td>23.60 ± 4.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.3</td>
<td>6.59 ± 2.45&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>25.0</td>
<td>22.22 ± 5.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5</td>
<td>11.11 ± 2.76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>12.5</td>
<td>13.89 ± 6.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3</td>
<td>6.94 ± 2.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>12.5</td>
<td>13.19 ± 7.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1</td>
<td>4.50 ± 1.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Median of triplicate experiments of three honey samples (MIC = Minimum Inhibitory Concentration) (a>b>c increase in potency)  
Means were compared using Kruskal Wallis (KW) test and Mann Whitney U-test. In each row, values with different letters (superscripts) indicate significant differences (p< 0.05).
3.6. Minimum Biocidal Concentration of investigated Honey Varieties

3.6.1. MBC of *Apis mellifera* Honey Samples

*Apis Mellifera* honey samples (I – III) were biocidal to all isolates tested at MBC range of 6.3 – 50.0% (v/v) (Table 15). 6.3% (v/v) of honey sample I was bactericidal to *E. coli* while 25% (v/v) of the same honey sample was bactericidal to *B. cereus* and *P. aeruginosa*. Honey sample I was not biocidal to *A. niger* and MDR *S. enterica* at concentration of 50% but it was biocidal to the rest of the test organisms at the same concentration. The MBC of honey sample II was just like that of sample I except that *E. coli* was killed at MBC of 12.5% and it was biocidal to *K. pneumonia*, and MDR *S. enterica* at 50% concentration.6.3% and 12.5% (v/v) of honey sample III were bactericidal to *E. coli* and *P. aeruginosa* respectively. Honey sample III also was not biocidal to *A. niger* and MDR *S. enterica* at concentration of 50% while 25% was biocidal to the rest of the test isolates. The MBC of sample I and III were the same as MIC against *E. coli* (6.3%).

3.6.2. MBC of *Hypotrigona* sp. Honey Samples

*Hypotrigona* sp. honey samples (I-III) was biocidal to all the test organisms at MBC range of 3.1 – 25 %, (v/v) (Table 16). Honey sample I was biocidal to *B. cereus*, *E. coli* and *C. tropicalis* at MBC of 3.1%, 6.3% and 6.3% (v/v) respectively. The MBC of 12.5% (v/v) of sample I killed MDR *S. aureus*, *P. aeruginosa* and *A. niger* while the remaining three isolates were killed at MBC of 25%. Honey sample II was biocidal to *B. cereus*, *E. coli* and *C. tropicalis* at MBC of 12.5%. Except for MDR *S. enterica* killed at MBC of 50% (sample II), the rest test isolates were kill at MBC of 25% (v/v). Honey sample III killed the test isolates just like honey sample II, except that *B. cereus* and MDR *S. enterica* were killed at MBC of 3.1% and 25% respectively. The MBCs of sample I against *B. cereus* and *E. coli*, and MBC of sample III against *B. cereus* were the same as their respective MICs.

3.6.3. MBC of *Melipona* sp. Honey Samples

*Melipona* sp. honey samples (I-III) was biocidal to all the isolates tested at concentration range of 6.3– 50.0% (v/v) (Table 17). The three honey samples were not fungicidal to *A. niger*. Honey sample I was biocidal to *B. cereus*, *K. pneumonia*, *E. coli* and *C. tropicalis* at MBC of 12.5% (v/v). MDR *S. aureus* was killed at MBC of 25.0% and the rest isolates were killed at highest concentration of 50% used. Honey sample II was biocidal to all the isolates at MBC of 25% except for *K. pneumonia* killed at MBC of 12.5%. Also, honey sample III was biocidal to *B. cereus*,*K. pneumonia*,
pneumonia, *P. aeruginosa* and *C. tropicalis* at MBC of 12.5% (v/v). MDR *S. aureus*, MDR *S. enterica* and *E. coli* were inhibited at MBC of 50, 25 and 6.25% (v/v) respectively.
Table 15: Minimum biocidal concentration (MBC) and non-peroxidase antimicrobial activity of *Apis mellifera* honey

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBC</td>
<td>MBC</td>
<td>MBC</td>
</tr>
<tr>
<td></td>
<td>(without catalase)</td>
<td>(with catalase)</td>
<td>(without catalase)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
</tr>
<tr>
<td>MDR <em>S. aureus</em></td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>6.3</td>
<td>50.0</td>
<td>12.5</td>
</tr>
<tr>
<td><em>P. aeruginosa ATCC 25783</em></td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>MDR <em>S. enterica</em></td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>C. albicans SC 5314</em></td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

MBC in % (v/v)
### Table 16: Minimum biocidal concentration (MBC) and non-peroxidase antimicrobial activity of *Hypotrigona* sp.honey

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Sample I MBC (without catalase)</th>
<th>Sample I MBC (with catalase)</th>
<th>Sample II MBC (without catalase)</th>
<th>Sample II MBC (with catalase)</th>
<th>Sample III MBC (without catalase)</th>
<th>Sample III MBC (with catalase)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em></td>
<td>3.1</td>
<td>25.0</td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>3.1</td>
<td>50.0</td>
</tr>
<tr>
<td>MDR <em>S. aureus</em></td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>25.0</td>
<td>50.0</td>
<td>25.0</td>
<td>50.0</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>6.3</td>
<td>12.5</td>
<td>12.5</td>
<td>25.0</td>
<td>12.5</td>
<td>50.0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 25783</td>
<td>12.5</td>
<td>25.0</td>
<td>25.0</td>
<td>50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>MDR <em>S. enterica</em></td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>12.5</td>
<td>50.0</td>
<td>25.0</td>
<td>50.0</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>6.3</td>
<td>&gt;50.0</td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>12.5</td>
<td>&gt;50.0</td>
</tr>
</tbody>
</table>

MBC in % (v/v)
Table 17: Minimum biocidal concentration (MBC) and non-peroxidase antimicrobial activity of *Melipona* sp.honey

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Sample I</th>
<th>Sample II</th>
<th>Sample III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MBC (without catalase)</td>
<td>MBC (with catalase)</td>
<td>MBC (without catalase)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>12.5</td>
<td>50.0</td>
<td>25.0</td>
</tr>
<tr>
<td>MDR <em>S. aureus</em></td>
<td>25.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>12.5</td>
<td>&gt;50.0</td>
<td>12.5</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>12.5</td>
<td>50.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 25783</td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
</tr>
<tr>
<td>MDR <em>S. enterica</em></td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>50.0</td>
<td>&gt;50.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>12.5</td>
<td>50.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

MBC in % (v/v)
3.6.4. MBC of non-peroxidase Activities of the Honey Varieties

The minimum biocidal concentration of catalasetreated *Apis mellifera* honey samples was within the range of 25 – 50% (v/v) (Table 15). The *Apis mellifera* honey samples I was only biocidal to *E. coli* and *P. aeruginosa* at concentration of 50 and 25 % (v/v) respectively. Honey sample II at concentration of 50% was biocidal to only these two organisms while sample III was biocidal to the same organisms at concentrations of 25% (v/v).

The non-peroxidase MBCs of *Hypotrigona* sp. honey samples were within the range of 12.5 – 50% (Table 16). *Hypotrigona* sp. honey sample I was only biocidal to *B. cereus, K. pneumonia, E. coli, P. aeruginosa* and *A. niger* at concentration of 25%, 50%, 12.5%, 25% and 50% (v/v) respectively. Honey sample II was only biocidal to *K. pneumonia, E. coli, P. aeruginosa* and *A. niger* at concentration of 50%, 25%, 50% and 50% (v/v). While sample III was not biocidal to MDR *S. aureus, P. aeruginosa, C. tropicalis* and *C. albicans SC 5314* (Table 16).

The non-peroxidase MBCs of *Melipona* sp. honey samples were within the range of 25 -50% (v/v) (Table 17). *Melipona* sp. honey sample I was biocidal to only *B. cereus, E. coli* and *C. tropicalis* at concentrations of 50% (v/v). Honey sample II was biocidal to *B. cereus* and *C. tropicalis* only at concentration of 50% (v/v). Also, sample III was biocidal to *B. cereus, K. pneumonia, E. coli, P. aeruginosa, and C. tropicalis* at concentrations of 25%, 50%, 25%, 50%, and 50% (v/v) respectively.

3.7. Mean MBC of *Apis mellifera, Hypotrigona sp. and Melipona sp. Honeys*

The mean minimum biocidal concentrations of the honey varieties were analysed with SPSS statistical programme version twenty using Kruskal-Wallis (KW) test and Mann-Whitney U test at 0.05 probabilities. Kruskal-Wallis (KW) Test revealed that there were statistically significant differences between the mean MBCs of *Apis Mellifera, Hypotrigona sp. and Melipona sp.* honeys against *Bacillus cereus* (KW test, df = 2, \( p = 0.001 \)), *Staphylococcus aureus* (KW test, df = 2, \( p = 0.006 \)), *Escherichia coli* (KW test, df = 2, \( p = 0.003 \)), MDR *Salmonella enterica* (KW test, df = 2, \( p = 0.015 \)) and *C. tropicalis* (KW test, df = 2, \( p = 0.002 \) (Table 18). There were no significant differences between the mean MBC of the honeys against *Pseudomonas aeruginosa* ATCC 25783 (KW test, df = 2, \( p = 0.794 \)), *Klebsiella pneumonia* (KW test, df = 2, \( p = 0.630 \)), *Aspergillus niger* (KW test, df = 2, \( p = 0.521 \)) and *C. albicans* (SC5314) (KW test, df = 2, \( p = 0.153 \)) (Appendix E 1 and Table 18).
Mann-Whitney U-test pairwise comparisons revealed that the mean MBC of Hypotrigona sp. honey against B. cereus (6.23 ± 4.70) was significantly different from mean MBC of Melipona sp. honey (25.69 ± 15.45, U = 6.000, p = 0.002) and A. mellifera honey (25.00 ± 10.83, U = 3.000, p = 0.001). The A. mellifera honey mean MBC was not significantly different from mean MBC of Melipona sp. honey (U = 39.000, p = 0.883) (Appendix E 1 and Table 18). These results showed that Hypotrigona sp. honey was the most effective antibactericidal agent against B. cereus, followed by A. mellifera and Melipona sp. honeys.

The mean MBC of Apis Mellifera honey against MDR S. aureus (36.11 ± 13.18) was significantly different from mean MBC of Melipona sp. honey (15.28 ± 12.15, U = 12.500, p = 0.006) and Hypotrigona sp. honey (22.22 ± 5.51, U = 17.500, p = 0.015). The Hypotrigona sp. honey mean MBC was not significantly different from mean MBC of Melipona sp. honey (U = 28.500, p = 0.204). These results showed that Hypotrigona sp. honey was also the most effective antibactericidal agent against MDR S. aureus, followed by A. mellifera and Melipona sp. honeys (Table 18 and Appendix E 2a–2c).

Melipona sp. honey had the highest mean MBC against E. coli (24.31 ± 16.07) and it was significantly different from the mean MBC of Hypotrigona sp. (9.72 ± 3.29, U = 14.500, p = 0.015) and Apis Mellifera honey (7.64 ± 2.76, U = 8.500, p = 0.003). There was no statistically significant difference between the mean MBC of Hypotrigona sp. and Apis Mellifera honey (U = 27.000, p = 0.159). These results showed that Hypotrigona sp. and Apis Mellifera honeys were the most effective antibactericidal agent against E. coli (Table 18 and Appendix E 2a–2c).

The mean MBC of Apis Mellifera honey against MDR S. enterica (13.89 ± 22.05) was significantly different from mean MBC of Melipona sp. honey (33.33 ± 12.50, U = 13.500, p = 0.010) and Hypotrigona sp. honey (41.67 ± 12.50, U = 18.000, p = 0.035). The Hypotrigona sp. honey mean MBC was not significantly different from mean MBC of Melipona sp. honey (U = 27.000, p = 0.169). These results showed that A. mellifera honey was the most effective antibactericidal agent against MDR S. aureus, followed by Hypotrigona sp. and Melipona sp. honeys (Table 18 and Appendix E 2a–2c).

The mean MBC of Apis Mellifera honey against C. tropicalis (29.17 ± 12.50) was significantly different from mean MBC of Melipona sp. honey (12.50 ± 10.83, U = 13.500, p = 0.010) and Hypotrigona sp. honey (10.42 ± 3.13, U = 3.000, p =
0.00005). The *Hypotrigona* sp. honey mean MBC was not significantly different from mean MBC of *Melipona* sp. honey (*U* = 36.000, *p* = 0.669). These results showed that *Hypotrigona* sp. and *Melipona* sp. honeys were the most effective antifungicidal agent against *C. tropicalis*, followed by *A. mellifera* honeys (Table 18 and Appendix E 2a – 2c).
Table 18: Comparison of the mean MBC (% v/v) of honey varieties from *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp.

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th><em>A. mellifera</em> Honey (n=3)</th>
<th><em>Hypotrigona</em> sp. Honey (n=3)</th>
<th><em>Melipona</em> sp. Honey (n=3)</th>
<th>P -value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>25.0</td>
<td>25.00 ± 10.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.1</td>
<td>6.23 ± 4.70&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>MDR S. aureus</em></td>
<td>25.0</td>
<td>36.11 ± 13.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.0</td>
<td>22.22 ± 5.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>25.0</td>
<td>26.39 ± 20.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0</td>
<td>25.00 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>6.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.64 ± 2.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.5</td>
<td>9.72 ± 3.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>ATCC 25783</td>
<td>25.0</td>
<td>19.44 ± 6.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0</td>
</tr>
<tr>
<td><em>MDR S. enterica</em></td>
<td>50.0</td>
<td>13.89 ± 22.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0</td>
<td>33.33 ± 12.50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>50.0</td>
<td>25.00 ± 25.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0</td>
<td>22.22 ± 5.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>25.0</td>
<td>36.11 ± 13.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0</td>
<td>23.61 ± 4.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>25.0</td>
<td>29.17 ± 12.50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5</td>
<td>10.42 ± 3.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Median and mean of triplicate experiments (MBC = Minimum Biocidal Concentration) (a>b>c increase in activity)

*Median MBC values were the same as MIC. Means were compared using Kruskal Wallis (KW) test and Mann Whitney U-test. In each row, values with different letters (superscripts) indicate significant differences.
3.8. Mean of non-peroxidase MIC and MBC

There were no statistically significant differences between the mean non-peroxidase MICs of the honey varieties. The mean non-peroxidase MBCs were not possible to compare since some of them did not inhibit the test isolate. *A. melliferahoney did not have mean non-peroxidase MIC against only MDR *S. enterica* (Table 19). The same honey had meannon-peroxidase MBC against only *B. cereus*, *E. coli* and *P. aeruginosa*. *Hypotrigona* sp. honey had mean MIC against all the isolates tested and also it had mean MBC against MDR *S. aureus*, MDR *S. enterica*, *C. albicans* SC 5314 and *C. tropicalis*(Table 19).*Melipona* sp. honey had mean non-peroxidase MIC against all isolates except MDR *S. aureus* and *A. niger*, and it was only biocidal to only *B. cereus*, *E. coli* and *C. tropicalis*. 
Table 19: Mean of non-peroxidase MIC and MBCC (%v/v) of honey varieties from *A. mellifera*, *Hypotrigona* sp. and *Melipona* sp.

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th><em>A. mellifera</em> Honey (n=3)</th>
<th><em>Hypotrigona</em> sp. Honey (n=3)</th>
<th><em>Melipona</em> sp. Honey (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MICC Median(Mean)</td>
<td>MBCC Median</td>
<td>MICC Median(Mean)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>25.0(20.83 ± 6.25)</td>
<td>50.0</td>
<td>12.5(14.58 ± 6.25)</td>
</tr>
<tr>
<td>MDR <em>S. aureus</em></td>
<td>25.0(23.61 ± 4.17)</td>
<td>&gt;50.0</td>
<td>25.0(22.22 ± 5.51)</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>50.0(25.00 ± 25.00)</td>
<td>&gt;50.0</td>
<td>25.0(23.61 ± 4.17)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>12.5(14.58 ± 6.25)</td>
<td>25.0</td>
<td>12.5(16.67 ± 8.27)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em>ATCC 25783</td>
<td>12.5(14.58 ± 6.25)</td>
<td>25.0</td>
<td>12.5(14.58 ± 8.27)</td>
</tr>
<tr>
<td>MDR <em>S. enterica</em></td>
<td>&gt;50.0</td>
<td>&gt;50.0</td>
<td>25.0(18.75 ± 7.65)</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>50.0(13.89 ± 22.05)</td>
<td>&gt;50.0</td>
<td>12.5(16.67 ± 6.25)</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>25.0(30.56 ± 15.45)</td>
<td>&gt;50.0</td>
<td>25.0(20.83 ± 6.25)</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>25.0(33.33 ± 12.5)</td>
<td>&gt;50.0</td>
<td>25.0(20.83 ± 6.25)</td>
</tr>
</tbody>
</table>
In each row, values with different letters (superscripts) indicate significant differences ($p < 0.05$). Mean and median of triplicate experiments (MICC and MBCC: Minimum Inhibitory Concentration and Minimum Biocidal Concentration with catalase respectively)
3.9. MICs and MBCs of control drugs

The MICs for the control drugs were 15.63 and 12.5 (µg/ml) against the P. aeruginosa (ATCC 25783) and C. albicans (SC5314) respectively. While the MBCs for the control drugs were 125 and 200 (µg/ml) against the P. aeruginosa (ATCC 25783) and C. albicans (SC5314) respectively (Table 20).
<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> ATCC 25783</td>
<td>15.63</td>
<td>125</td>
</tr>
<tr>
<td><em>C. albicans</em> SC 5314</td>
<td>12.5</td>
<td>200</td>
</tr>
</tbody>
</table>

Ciprofloxacin(500mg) and Ketoconazole (200mg) for bacterium and fungus respectively.
CHAPTER FOUR: DISCUSSION

4.1. Discussion

4.1.1. Physicochemical Properties of the Honeys

All the honey samples tested were acidic, with pH values ranging from 4.04 to 4.46 for *A. mellifera* honey; 3.65 to 3.85 for *Hypotrigna* sp. honey and 3.80 to 4.65 for *Melipona* sp. honey. The observed pH values in this study are similar to those previously reported for Nigerian honey samples from different regions, which had pH values between 3.49 and 4.90 (Adebiyi *et al*., 2004; Omafuvbe and Akanbi, 2009; Adenekan *et al*., 2010; Buba *et al*., 2013; Eleazu *et al*., 2013). Published data from other countries reported that pH of honey may range between 3.49 and 4.70 (Chefrour *et al*., 2009; Makawi *et al*., 2009; Ouchemoukh *et al*., 2007; Azeredo *et al*., 2003; Kayacier and Karaman, 2008; Adams *et al*., 2008; Saxena *et al*., 2010) and our findings are within this range. Low pH of honey is attributed to the presence of organic acids such as gluconic, pyruvic, malic and citric acids (Terrab *et al*., 2002), and the variation in pH of different honey samples is said to be due to floristic composition and floral diversity of the regions (Azeredo *et al*., 2003; Makawi *et al*., 2009). *Meliponas* sp. honey had the highest pH that might be due to the presence of alkaline contents in the honey extracts. Similar findings were reported for *Melipona* sp. honey by Boorn *et al*., (2010).

The moisture contents of the investigated honey samples ranged from 11.537 to 11.870 % for *A. mellifera* honey; 17.053 to 18.310 % for *Hypotrigna* sp. honey and 12.863 to 15.203 % for *Melipona* sp. honey. In this study, the highest moisture contents were found in the *Hypotrigna* sp. honey (18.310 ± 0.900 %) and all the investigated honey samples are within the limit (<21%) recommended by the international quality regulations (Codex Alimentarius, 2001; European Commission, 2002). Almost similar findings have been reported by Omafuvbe and Akanbi (2009), Omoya *et al*., (2014), Pimentel *et al*., (2013) and Eleazu *et al*., (2013), who found the moisture contents in their tested honey samples ranging between 11.6 and 19.2 %. The moisture content of honey is widely related to the harvest season and the level of maturity in the hive (Mandal and Mandal, 2011). Water content is very important for the shelf life of honey during storage because high water content can lead to undesirable honey fermentation due to osmotolerant yeasts, which form ethyl alcohol and carbon dioxide (Molan, 1992).
Honey samples from *Apis mellifera*, *Hypotrigona* sp. and *Melipona* sp. had mean electrical conductivities (EC) of $0.207 \pm 0.04$, $0.303 \pm 0.04$ and $0.238 \pm 0.03$ mS/cm respectively. The EC values of the investigated honey samples are within the allowed limit of international standards (lower than 0.8 mS/cm) (Codex Alimentarius, 2001). The ECs of the honeys are similar to the findings previously reported by Adebiyi *et al.* (2004), Omafuvbe and Akanbi (2009), Buba *et al.* (2013) and Pimentel *et al.* (2013) on Nigerian honey (EC range between 0.21 and 1.61 mS/cm). Electrical conductivity (EC) is one of the most important factors for determining the physical characteristics of honey (Serrano *et al.*, 2004). It is also an important physicochemical measurement for the authentication of unifloral honeys (Mateo and Bosch-Reig, 1998). The electrical conductivity depends upon the mineral contents of the honey. It is very often used in routine honey testing instead of the ash content. The relationship between the two parameters have been shown by several authors (Piazza *et al.*, 1991; Sancho *et al.*, 1991). This parameter was recently included in the international standards, replacing the determination of ash content (Codex Alimentarius, 2001).

The total dissolved solids (TDS) of the investigated honey samples ranged from 264.1 – 316.3 ppm for *A. mellifera* honey; 349.0 – 399.1 ppm for *Hypotrigona* sp. honey and 209.0 – 221.2 ppm for *Melipona* sp. honey. According to Lawal *et al.* (2009) and Akoh (1991), the TDS values of Nigerian honeys ranged between 204.32 and 388.54 ppm and the results of our findings are within this range. TDS is a measure of combined content of all inorganic and organic substances in honey in the molecular, ionized or micro-granular (colloidal solution) suspended forms (Serrano *et al.*, 2004). *Hypotrigona* sp. honey had the highest mean TDS content ($370.01 \pm 22.51$ ppm), which indicates that it is rich in both organic and inorganic substances.

In this study, the colours of all honey samples were between light amber and amber. The *A. mellifera* honey showed the amber colour, similar to reports by Adebiyi *et al.* (2004) and Buba *et al.* (2013). According to Diez *et al.* (2004) honey colour varies naturally in a wide range of tones, ranging from light yellow to amber, dark amber and black, in extreme cases, and sometimes even green or red hues may occur. Honey darkens with age, and other changes in colour may result from the beekeeper’s interventions and different ways of conservation, such as the use of old honeycombs, contact with metals, and exposure to high temperatures or light (Codex Alimentarius,
2001). Also, the variations maybe entirely due to the plant source of the honey, although heat may modify the colour of honey by darkening action (Molan, 2007).

In the analysed honey samples, A. mellifera honey had the highest colour intensity (ABS$_{450}$) range between 961.33 and 1006.33µAU. This is the first report on colour intensity of Nigerian honey. Similarly, the ABS$_{450}$ values for Italian, Slovenian, Algerian and Indian honeys were reported to be 25–3413 µAU, 70–495 µAU, 724 -1188µAU and 524–1678 µAU, respectively (Khalil et al., 2012; Ouchemoukhet et al., 2007; Bertoncel et al., 2007; Bogdanov, 2009). ABS$_{450}$ is a reliable parameter for confirming the presence of pigments that have antioxidant activities such as carotenoids and some flavonoids and; it is usually correlated with the phenolic levels and flavonoid content of the honey (Frankel et al., 1998; Terrab et al. 2002). These were also observed in this study in which honey samples with higher phenolic and flavonoid content tend to have significantly higher colour intensities, as observed in A. mellifera and Hypotrigona sp. honeys.

From the analysed honey samples, total sugar contents ranged between 57.94 and 82.04 %. The total sugar contents of the analysed honeys are within the range previously reported on honeys from both stingless bees (70.03 – 80.90 %) and A. mellifera (50.61 – 75.69 %) (Souza et al., 2006; Onyenso and Akachukwu, 2011; Pimentel et al., 2013; Buba et al., 2013; Omoya et al., 2014). The mean reducing sugar contents in honey samples from A. mellifera, Melipona sp. and Hypotrigona sp. were 72.70 ± 7.50 g/100g, 62.32 ± 5.25 g/100g and 80.71 ± 1.37 g/100g respectively. Regarding reducing sugars (fructose and glucose), the EC Directive 2001/110 imposes that the amount of reducing sugars should be ≥ 60 g/100g, with the exception of honeydew honey, which has a lower limit (≥ 45 g/100 g). The reducing sugars of the analysed honey samples were above 60% except for some Hypotrigona sp. honey samples. Although, there is no international standards for stingless bee honey (Codex Alimentarius Commission, 2001). Omoya et al. (2014) and Ezeazu et al. (2013) have reported similar reducing sugar contents of Nigerian honey samples to be between 49 and 76.9%, and 60 and 76% respectively. Onyenso and Akachukwu (2011), and Boorn et al. (2010) reported both total sugar and reducing sugar contents for stingless bees in the range of 66.0 – 89.0% and 66.0 – 79.20% respectively, similar to our findings. Melipona sp. honey had the highest mean sucrose contents (5.06 ± 0.75), slightly above the standard limit (≤ 5g/100g). Agbagwa et al.(2011) and Silva et al.
(2009) reported that the sucrose contents of honeys were between 0.2 and 4.8 %, similar to our findings. According to Azeredo et al. (2003), the high sucrose content means, in most cases, a premature harvesting of honey, that is, a product in which sucrose was not thoroughly transformed into glucose and fructose by the action of the invertase enzyme. Honey contains about 85% of sugars mainly fructose and glucose produced by hydrolyses of sucrose (Bogdanov, 2009). The sugar content of honey is a quality criteria which is influenced by honey storage and heating and thus is an indicator of honey freshness and overheating (Chen et al., 2012). The sucrose content is also important in knowing whether the bees fed on sugar or if the honey was adulterated by direct addition of sucrose (Agbagwa et al., 2011). Taking into account the composition of stingless bee honey, mainly in terms of the reducing sugars that are higher and lower moisture compared to honey of Apis, this product cannot undergo fermentation quickly if properly stored after the harvesting (Onyenso and Akachukwu, 2011).

In this study, the hydromethylfurfural (HMF) ranged from 11.97 ± 0.05 - 16.12 ± 0.12, 16.29 ± 0.04 - 17.07 ± 0.05 and 5.35 ± 0.12 - 5.65 ± 0.04 mg/kg for A. mellifera, Melipona sp. and Hypotrigona sp. honeys respectively. These results were within the recommended range set by Codex Alimentarius and EU (maximum of 80mg/kg). These findings are similar to other published levels for HMF (5.0 – 50 mg/kg) (Boorn et al., 2010; Onyenso and Akachukwu, 2011; Agbagwa et al., 2011). Persano et al. (2004) reported low HMF concentrations of two unprocessed Australian honey samples (1.43 to 15.23 mg/kg), similar to our findings. HMF formation results from the acid-catalyzed dehydration of hexose sugars with fructose being particularly susceptible. In addition, HMF is only present in trace amounts in fresh honey, and its concentration has been reported to increase with storage and the prolonged heating of the honey. HMF is thus an essential parameter used to indicate honey purity (Fasasi, 2012). High HMF formation may occur due to overheating, exposure to high temperatures or the type of sugar present in the honey, as well as the fructose/glucose ratio (Ajlouni and Sujirapinyokul, 2010; Fallico et al., 2004). Overall, the low HMF concentrations of the analysed honey samples confirmed that these samples are of good quality.

The total acidity of the analysed honey samples ranged between 12.40 and 35.93 meq/kg. In this study, Hypotrigona sp. honey had the highest total acidity values.
compared to others (Table 4). Similarly, Fasasi (2012) reported the range of total acidity in local honeys from 23.55-58.52 meq/kg while according to Buba et al. (2013) a range of 14.25 to 36.67 meq/kg was reported. In contrast to our findings, Oliveira et al. (2013) reported a total acidity values of 69.06 meq kg-1 for stingless bee (T. angustula) and a range from 92.09 to 102.10 meq kg-1 for another stingless bee (S. depilis). Souza et al. (2006) who investigated 152 samples of honeys of various Meliponinae species from eight countries reported acidity values ranging from 5.9 to 109.0 meq kg-1. According to Vit et al. (2004), acidity can be directly related to the maturation state of honey, and it increases with fermentation. Hypotrigona sp. honey had the highest mean lactone contents. The free acidities of the analysed honey samples were within the international standard (≤ 50 meq/kg).

The protein contents in the three honey varieties used in this study ranged between 2.97 and 6.58 g/kg. The Hypotrigona sp. honey had the highest mean protein content (5.75 ±0.69 g/kg) and lowest mean protein content was found in A. mellifera honey (3.37 ± 0.43 g/kg). A high protein concentration has also been reported in some Nigeria honeys (3.7 to 9.4 g/kg) (Onyenso and Akachuku, 2011). The protein content values of Algerian and Malaysian honey samples are similar to values observed in this study (5.56 and 2.29 g/kg respectively) (Ouchemoukh et al., 2007; Khalil et al., 2011). Protein content in honey generally ranges from 2 to 5 g/kg according to Bogdanov (2009) and the results of these studies are within this range. The concentrations of proteins and amino acids in honeys vary depending on their botanical or geographical origin and storage time. Protein content in honey samples are reported to consist of mainly enzymes (White, 1975). It is possible that the plant sources produce large amounts of pollen and nectar, which can contribute to the protein content in the honey samples. This requires further investigation according to Onyenso and Akachuku (2011). Proline was detected in high concentrations (298 - 430 mg/kg) in all the analysed honey samples. Some authors have also reported that high concentrations of proline are typical for honeydew honeys (Moniruzzaman et al., 2013). Proline is an important amino acid that originates mostly from the salivary secretions of Apis mellifera during the conversion of nectar into honey. Proline content is an indication of honey ripeness and, in some cases, sugar adulteration (Oliveira et al., 2013).
The phenolic acids in investigated honey varieties ranged between 354.03 and 531.10 mg GAE/kg. The *Hypotrigona* sp. honey had the highest mean phenol content (527.41 ± 3.60 mg GAE/kg) and the lowest mean phenol content was found in *Melipona* sp. honey (371.98 ± 14.18 mg GAE/kg). The total polyphenol content of analysed *A. mellifera* honey samples are similar to reporton Nigerian honey samples (402.98 – 531.01mg GAE/kg) by Lawal *et al.*(2009). In contrast, Ouchemoukh *et al.*(2007) and Fasasi *et al.*(2012) reported lower phenolic content of Algerian and Malaysian honey samples compared to *Hypotrigona* sp. honeys. The phenolics or polyphenols are one of the most important classes of compounds found in honey. The total concentration of phenols in honey is highly dependent on its plant source. Because the content of phenolic compounds is usually lower in light-coloured honey compared to that of dark honeys, the high levels of polyphenols in all honeys may contribute to its darker colour (Jasicka-Misiak *et al.*, 2011). The high level of polyphenols in the studied *Hypotrigona* sp. honeys further indicates their higher antioxidant properties (Vela et al., 2007; Socha et al., 2009).

In the analysed honey varieties, *Melipona* sp. honey had the highest mean flavonoid contents (86.39 ± 4.69 mg CEQ/kg) and the lowest mean flavonoid contents was found in *Hypotrigona* sp. honey (41.37 ± 10.65 mg CEQ/kg). In contrast, Eleazu *et al.*(2013) reported lower flavonoid contents values between 10.154 and 10.250 mg CEQ/kg for Nigerian honey. Similarly, Khalil *et al.*(2012) also reported flavonoid contents values between 27.07 and 71.78 mg CEQ/kg for Malaysian honeys. While Özkök *et al.*(2010) reported flavonoid contents values between 28.17 and 87.01 mg CEQ/kg for Turkish honeys. Flavonoids are low molecular weight phenolic compounds that are vital components for the aroma and antioxidant properties of honey (Kroyer and Hegedus, 2001). Flavonoids stabilize reactive oxygen species by neutralizing with the reactive element of the radical (Nijveldt *et al.*, 2001). Therefore, honey containing higher flavonoid concentrations is desirable due to their purported antioxidant potential.

The ascorbic acid contents of the analysed honey varieties ranged between 144.31 and 169.27 mg/kg. This is the first report on ascorbic acid content of Nigerian stingless bee honeys and there is also scanty of report on ascorbic acid content of Nigerian honey. The investigated honey samples have higher ascorbic acid than honeys from some countries like Bangladeshi honey (129.8 to 154.3 mg/kg) (Islam *et
al., 2012), Portuguese honey (140 to 145 mg/kg) (Ferreira et al., 2009), Indian forest honey (120 to 260.90 mg/kg) (Kishore et al., 2011), Algerian honey (236.80 to 315.90 mg/kg) (Khalil et al., 2012) and Malaysian pineapple honey (129.14 to 146.40 mg/kg) (Kishore et al., 2011; Moniruzzaman et al., 2013). Apart from polyphenols, ascorbic acid is one of the non-enzymatic substances present in honey that is a known antioxidant (Kroyer and Hegedus, 2001). It has also been reported that when honey is stored for a long duration, the concentrations of several other compounds may also decrease, which can affect both ascorbic acid and enzyme levels (Wang et al., 2006). These factors can lead to variations in the ascorbic acid concentrations of honey.

In the investigated A. mellifera honey samples, strong correlation was found between the colour intensity (ABS₄₅₀) of honey samples and some physicochemical parameters, like phenolics, flavonoid, protein and proline contents at 0.954, 0.990, 0.833, and 0.803, respectively (Table 6). While in Hypotrigona sp., a strong correlation was found between the colour intensity of honey samples and some physicochemical parameters, like phenolics, flavonoid, protein, ascorbic acid and proline contents at 0.995, 0.997, 0.989, 0.999 and 0.778 respectively (Table 7). Also, in Melipona sp. honey, a strong correlation was found between the colour intensity of honey samples and some physicochemical parameters, like flavonoid, protein and proline at 0.792, 0.898 and 0.679, respectively (Table 8). Comparatively, a smaller strong correlation was found between the colour intensity of Malaysian honey samples and the other antioxidant parameters, like flavonoid, proline and protein contents at 0.735, 0.701, 0.938 and 0.873, respectively (Moniruzzaman et al., 2013). Khalil et al. (2012) also reported a smaller strong correlation between the colour intensity of Algerian honey samples and the other antioxidant parameters, like flavonoid, proline and protein contents at 0.968, 0.934 and 0.876 respectively. In Indian honeys, the correlation between ABS₄₅₀ and phenolic contents was reported to be 0.830, smaller than the one reported in this research (Saxena, et al., 2010). Thus, the higher correlation observed in this study indicates that (A. mellifera and Hypotrigona sp. honeys) these honeys may have a stronger antioxidant capacity compared to Malaysian, Algerian and Indian honeys. But Melipona sp. honeys did not show any correlation between phenolic contents and ABS₄₅₀, which mean that other compounds maybe responsible for the colour intensity of the honey. However, in future, it will be good to confirm these findings with larger number of samples.
4.1.2. Antibacterial Activities of the Honey Varieties

All the organisms tested showed clear zones of inhibition in response to different concentration of the honey varieties. For multidrug resistant (MDR) *Staphylococcus aureus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* ATCC 25783, MDR *S. enterica* and *Aspergillus niger*, *Hypotrigona* sp. honey showed comparatively higher activity than other honey varieties. For *Bacillus cereus*, *Escherichia coli* and *Aspergillus niger*, *A. mellifera* honey showed higher activity than *Melipona* sp. honey. The three honey varieties had comparatively similar activities against *Candida tropicalis* and *Candida albicans* SC 5314. Agbaje *et al.* (2006) and Mohapatra *et al.* (2011) reported similar results for Nigerian honeys against *E. coli* and *K. pneumonia*. *A. mellifera* honey had the least inhibitory effect against *K. pneumonia* (3 - 12 mm), which is similar to report by Agbagwa and Okolo (2012) (7 - 10 mm). Also in Nigeria, Omoya *et al.* (2014) reported a similar result on antimicrobial activity of honeys against *B. cereus* (9 − 10 mm), *E. coli* (13 − 20 mm) and *Salmonella* sp. (8 − 18 mm) except against *A. niger* (1 − 3 mm). Anyanwu (2012) reported a similar result for the antifungal activity of *A. mellifera* honey against *A. niger* (3 − 10 mm) and *C. albicans* (4 − 10 mm). Omafuvbe and Akanbi (2009) found similar results through well diffusion method that Nigerian honey showed activity against *Salmonella* sp. (12 − 22 mm), *B. cereus* (12 − 29 mm), *K. pneumonia* (9 − 15 mm) and *E. coli* (19 − 38 mm). In contrast, the same author reported that honeys from different regions in Nigeria were not active against *S. aureus*, *C. albicans* and *P. aeruginosa*. Similarly, the antimicrobial activities of *Melipona* sp. and *Trigona* sp. honeys (3 − 22 mm) have been reported by Minisha and Shyamapide (2011) and Cortopassi-Laurino and Gelly (1991).

Almost all the honey varieties used in this study inhibited most of the test isolates at a lower MIC. *A. mellifera* and *Melipona* sp. honey had mean MIC range between 6.3 and 25% against all the isolates tested. The *A. mellifera* honey inhibited *E. coli* and *P. aeruginosa* at MIC of 6.3 % (v/v). *Hypotrigona* sp. honey had mean MIC range between 3.1 and 12.5 % (v/v) against the test isolates. Recently, similar findings were reported by Boorn *et al.* (2010), Chen *et al.* (2012), Hegazi and Fyrouz (2012) and Fahim *et al.* (2014), who showed that the concentration of honey they used to inhibit similar isolates did not exceed 40%. Similar to these studies, Boom *et al.* (2010) and Pimentel *et al.* (2013) reported lower MIC values for Meliponinae bee
tribes (Stingless bees) (2.5 – 16 %) and Apini bee tribes (25 – 40 %) honey against bacterial and fungal isolates. According to Halawani and Shohayeb(2011) and Postmes et al. (1993), the variable results observed between honeys from the same insect could possibly be due to the different floral sources utilized by the bees, the harvesting season and the geographical factors like temperature, humidity, e.t.c where the honey was produced. Melissa et al. (2004) reported that the dilution of honey enhances hydrogen peroxide mediated antimicrobial activity which may explain some discrepancies observed with antimicrobial activity of these honeys.

The minimum biocidal concentration(MBC) of A. mellifera and Meliona sp.honey samples against all isolates tested were between 6.3% and 50%. While Hypotrigona sp. honey had MBC range between 3.1 and 50% against all isolates tested. The MBC of the investigated honey samples corroborated with the findings of Oyeleke et al. (2010), who also reported MBC range between 6.25% and >50% against similar isolates. The present findings are supported by Othman(2010) who showed that MBC values of Yemeni honey samples were in the range of 20 to 40 %. Similarly, Othman(2014) reported that E. coli was the most susceptible to antimicrobial activity of honey. Jantakee and Tragoolpua(2015) reported a similar results that 49 isolates of S. aureus were completely inhibited by the 50% (v/v) longan honey and the MBC values ranged from 25% to >50%. Kwakman et al.(2010) also reported on MBC of pasture honey (20 – 40 %) against many organisms, similar to our findings. Anwanwu (2012) reported that the minimum fungicidal concentration of Nigerian honeys ranged between 12.5 and 50% (v/v) against Aspergillus niger, and Candida albicans. Hypotrigona sp. honey was the most effective against most isolates tested, which also reflected in their physicochemical properties.

When the honey samples were treated with catalase to eliminate the effects of hydrogen peroxide, the results showed that MIC and MBC values increased. In the absence of hydrogen peroxide, these three honey varieties showed more effectiveness against B. cereus and E. coli. Hypotrigona sp. honey was more effective against K. pneumonia and A. niger. Melipona sp. honey was also more effective against C. tropicalis. This is the first report on non-peroxidase antimicrobial activity of Nigerian honey. These results were similar to findings of Fahim et al. (2014), who investigated the non-peroxidase activity of honeys indigenous to Pakistan against similar
organisms (MBC range between 15% and >50%). Allen et al. (1991) and Molan (2006) also reported non-peroxidase activity of manuka honey and showed that the use of a catalase concentration up to 100-fold failed to remove the activity. Some researcher suggested that the non-hydrogen peroxidase activity of honeys could be due to high level of hydrogen peroxide concentration that the normal catalase treatment is not sufficient to eliminate (Weston, 2000; Patton et al., 2006; Kwakman et al., 2010). Even in the absence of hydrogen peroxide, other physicochemical properties of the honey maybe responsible for the antimicrobial activity of honey. Therefore, more research is still needed to harmonize the way antimicrobial activity of honey is determined.

4.2. Conclusions

This research has shown that the honey varieties varied significantly in their physicochemical properties and antimicrobial potentials. Hypotrigona sp. honeys had the highest mean moisture, total dissolved solids, hydromethylfurfural, total and lactone acidities, protein, and phenol contents. Melipona sp. honey had the highest mean free acidity, flavonoids, total sugar and reducing sugar contents. Hypotrigona sp. and Melipona sp. honey varieties have shown to possess antimicrobial properties similar to widely used A. mellifera honey. This study scientifically authenticates the potentials use of these stingless bee honeys as an alternative therapeutic agent.

Hypotrigona sp. (Okotobo) and Melipona sp. (Ifufu) honeys that are not consumed as widely as regular bee honey have shown to contain bioactive compounds and have antimicrobial properties similar to those of regular bee honey.

4.3. Recommendations

The observed differences between Hypotrigona sp., Melipona sp., and Apis mellifera honey varieties have reinforced the need for specific quality patterns for the management of honey from these native bees. There is need for further research on the antimicrobial activity and wound healing potential of stingless bee honeys in Nigeria. Further research is still needed for identification and characterization of the active principle(s) of these honeys, which will provide valuable information on their quality and possible therapeutic potential.

Stingless bee farming (Meliponiculture) should be encouraged in Nigeria just like in other countries like New Zealand, Brazil, Australia, Ghana, etc. Since it is rapidly advancing in both curiosity and consideration as a potentially-viable source of
secondary income streams, even though stingless bee rearing is quite new and just started to be embraced by many people. Perhaps this is due to the unique character and nature of the melopine bee hive, which, in nature, occurs in the trunk of a tree (tree-trunk) and is easily accessible by persons with fewer disposable resources or funds used to culture other bee species requiring specially-built, expensive bee hives. Thus, meliponiculture could result in advancements in socio-economic status for many stakeholders. As an industry in infancy, meliponiculture is a potential source of income revenue that is readily accessible to the majority of people irrespective of regional factors or income levels. Another attractive feature of meliponiculture is that the meliponine bees are ‘stingless’. Unlike the situation in keeping the European honey bee, beekeepers need not purchase expensive, protective clothing in order to manage and handle hives or to harvest and collect products (honey, propolis, beebread) with melipona.