

**ANTIMICROBIAL EFFECTS OF HONEY AND ITS SPECIFIC ACTIONS ON  
CELL WALLS, MEMBRANES AND ENZYMES OF SOME MICROBIAL  
PATHOGENS**

**BY**

**ATTAH, Friday  
MTech/SLS/2017/6703**

**DEPARTMENT OF MICROBIOLOGY  
FEDERAL UNIVERSITY OF TECHNOLOGY  
MINNA**

**JUNE, 2021**

# ANTIMICROBIAL EFFECTS OF HONEY AND ITS SPECIFIC ACTIONS ON CELL WALLS, MEMBRANES AND ENZYMES OF SOME MICROBIAL PATHOGENS

## ABSTRACT

Antimicrobial agents of plant origin have enormous therapeutic potentials. Honey, which is a product of plant, is a sugary substance produced by bee from the nectar of flower. It has been an age long antimicrobial therapy for wounds and burns. The aim of this study was to determine the antimicrobial effects of honey and its specific actions on cell walls, membranes and enzymes of the following organisms: *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Trichophyton verrucosum*, *Trichophyton equinum* and *Escherichia coli* using agar well diffusion method. *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Escherichia coli* were tested at 100 %, 80 % and 60 % (v/v) honey concentration and *Trichophyton verrucosum* and *Trichophyton equinum* were tested at 100 % honey concentration only. *Escherichia coli* had the highest zone of inhibition of 29.0 mm, 27.0 mm and 19.0 mm at 100 %, 80 % and 60 % respectively followed by *Bacillus subtilis*, which had 15.0 mm, 1.0 mm and 8.0 mm zones of inhibition while *Pseudomonas aeruginosa* had 13.7 mm, 11.0 mm and 6.3 mm zones of inhibition as the least. At 100 %, *T. verrucosum* and *T. equinum* had zone of inhibition of 14.0 mm and 17.0 mm respectively. The minimum inhibitory concentrations recorded for *Escherichia coli*, *Bacillus subtilis* and *P. aeruginosa* were 10 %, 80 % and 100 % (v/v) respectively. The minimum bactericidal concentrations for *E. coli* and *B. subtilis* were 20 % and 100 % (v/v) respectively. The effect of honey on bacterial isolates after incubation for one hour and two hours revealed that *E. coli* had 22.0 and 35.3 µg/ml protein leakage; *Bacillus subtilis* had 31.0 and 49.0 (µg/ml) while *Pseudomonas aeruginosa* had 49.7 and 60.0 (µg/ml) respectively. The result of enzymatic inhibition showed that honey had activity against the cells treated compared to the control: *E. coli* had 11.0 and 14.0 (mm); *Bacillus subtilis* had 30.0 and 40.0 (mm) while *Pseudomonas aeruginosa* had 31.7 and 45.0 (mm) for the treated and untreated cells respectively. The result of this study showed that the honey had a broad spectrum antimicrobial activities and could be recommended for antibiotics alternative therapy.

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background to the Study

Antimicrobial agents possess immense curative prospects and are very effective in controlling disease causing organisms (Mahato and Sharma, 2018). Prior to the discovery of microorganisms, it was generally believed that certain medicinal herbs had curative properties and, without a doubt, contained what we now refer to as antimicrobial principles (Mahato and Sharma, 2018). Common infectious diseases have been cured by the use of plants, and this form part of the customary management of different health challenges, some of these conventional medicines are still included. The rich sources of antimicrobial agents are medicinal plants (Jindal and Vashist, 2013).

Medicinal plants are rich sources of antimicrobial agents (Jindal and Vashist, 2013). According to World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs and 80 % of world population is dependent on traditional medicine and a major part of traditional therapies involves the use of plant extracts or their active constituents. Yet a scientific study to determine their antimicrobial active compounds is a comparatively new field. (Parmar and Rawat, 2012).

One of the earliest medicines used by traditionalist for both infectious and non-infectious diseases and was also recommended for management of burns and wounds was honey, a plant product according to the report of Olatunji *et al.* (2018).

Honey is a sugary and thick material formed by bees (Olatunji *et al.*, 2018). It is made up of flower nectars (floral honey) and sweet plant deposits (non-floral honey), as well as an enzyme secreted by honeybees. These sugary substances are gathered by bees, who

then improve them through their own materials before processing them in beehives (Manyi-Loh *et al.*, 2011; Süerdem and Akyalçin, 2017).

Honey is a popular sweetener, non-toxic, non-irritant and a common household product (Usman *et al.*, 2015). Ayurveda, an ancient Indian System of Health Care treats honey as food for health while recommending it as a medicine for some conditions using it externally as well as orally (Khandal *et al.*, 2010). Ayurveda, which means science of long life, is believed to have originated over 6000 years ago and was designed to promote good health and long life rather than to fight disease and was practiced by physicians and surgeons (called Bhesaja or vaidya) but recently herbal medicine has attracted much attention as alternative medicines useful for treating or preventing life-style related disorders (Agyare *et al.*, 2009).

Honey is a well-researched oldest medicine for a variety of pathogenic microbes, as well as an active antibacterial agent for burn injuries (Brudzynski, 2006). After topically applied to wounds, osmosis would be expected to draw water from the wound into the honey helping to dry the infected tissue and reduce bacteria growth (Al-Naama, 2009). Honey's antibacterial properties have been demonstrated in various reports and clinical trials against a wide range of microorganisms, including multi-antibiotic drug resistant strains (Kumarasamy and Mahendran, 2015).

Laboratory studies and clinical trials have shown that honey is an effective broad-spectrum antimicrobial agent. Honey has been reported to have inhibitory effect on several bacteria including aerobes and anaerobes, Gram-positive and Gram-negative and is effective against methicillin resistant *Staphylococcus aureus* (MRSA),  $\beta$ -hemolytic *Streptococci* and vancomycin-resistant *enterococci* (VRE) (Allen *et al.*, 2000; Kingsley, 2001).

The use of honey for wound infections treatment dated over 2000 years prior to bacterial discovery to be the cause of infections (Sushila *et al.*, 2012). Olatunji *et al.* (2018) reported that the antimicrobial activity of honey was first recognized in 1892, and this was then accompanied by detailed studies to further substantiate this argument and to show factors leading to the activity of antimicrobials.

Natural medicinal products have been used for millennia in the treatment of multiple ailments (Manyi-Loh, *et al.*, 2011). Although many have been superseded by conventional pharmaceutical approaches, there is currently, resurgence in interest in the use of honey and honey products by the populace. This choice of honey for therapeutic purpose is a branch of medicine called apitherapy (Ghosh and Playford, 2003).

Honey can be classified based on where the bee got resources for honey make up: Floral and non-floral honeys. Floral honeys can either be unifloral or multifloral, depending whether the honey collected is from the nectar of the same flower or from nectar of flowers of various types (Manyi-Loh, *et al.*, 2011). Non floral honey (honey dew) is made by bees that extract sugars from the living tissues of plants or fruits, and/or scavenge the excretions of insects (aphids) that tap the veins of higher plants (Subrahmanyam, 2007).

The antimicrobial effects of honey 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 non-peroxide components (Taormina *et al.*, 2001; Al-Naama, 2009).

Among the possible therapeutic alternatives that are approved, non-toxic and with a wide range of antimicrobial spectrum of action, honey is considered. This may be a potential alternative or replacement for antimicrobial agents, but its use is constrained by certain factors. Inadequate knowledge of antimicrobial properties and lack of adequate

information have reduced the clinical applicability of honey (Malik *et al.*, 2010; Mandal and Mandal, 2011).

In Nigeria, honey is accepted to be important in traditional treatment of respiratory ailments, skin infections, diarrhoea and other diseases as captured by Eleazu *et al.* (2013). There are numerous reports on the physico - chemical, antimicrobial, microbiological and medicinal properties of honey from many parts of the world, including North America, Europe, Asia, Australia, and South Africa (Gomes *et al.*, 2010; Mandal and Mandal, 2011; Fahim *et al.*, 2014).

Data on Nigerian honey is limited; however, some physical properties, antibacterial and chemical properties had been documented from honey in Nigeria (Adebiyi *et al.*, 2004; Omafuvbe and Akanbi 2009; Anyanwu, 2012; Eleazu *et al.*, 2013; Buba *et al.*, 2013). Although, the antifungal study on Nigerian honey is minimal, nevertheless, separate study conducted by Akujobi and Njoku (2010), Anyanwu (2012), Eleazu *et al.* (2013) and Buba *et al.* (2013) indicated that Nigeria honey could kill fungi and also inhibit the growth of fungi

## **1.2 Statement of Research Problem**

Attention had been drawn nowadays in serious search for antimicrobial compound all over the world owing to the failure of the already existing antibiotics (Ibrahim and Aliyu, 2015). With the irrational and massive use of antibiotics in underdeveloped and developing countries, resistance develops and spread beyond human imagination. As a result, the effectiveness of the antibiotics is reduced (Zakaria, 2015). Rural dwellers and disadvantaged people who are unable to have enough money to access good health care services relied on conventional medicines which they are familiar with for their treatment (Krishnan, 2018).

The World Health Organization (WHO) has assessed that up to 80 percent of individuals in the developing nations rely on local medicinal prescriptions because of their easy accessibility, wide affordability and cultural familiarity. Indeed, about 40 percent population of the world's poor have no good hospital, hence, they depend on local medicinal prescription (Krishnan, 2018).

Clinical acceptability of honey has been slowed down by curtailed knowledge of the antimicrobial activity and short accurate mechanisms for determine the type of action of honey and variations of honey (Malik *et al.*, 2010). According to Elijah *et al.* (2015), the structural existence of honey may be linked to geographic origin, and the antibacterial function of floral sources may play a key role. In Nigeria, there is no comprehensive record that honey from all the States have antimicrobial activity and of course, Nigeria has different climatic zones

### **1.3 Justification for the Study**

The antimicrobial information of traditional medicine is a panacea for battling antibiotic resistant microorganisms in the developing nation like Nigeria. Many traditional medicines had been hawked without antimicrobial authentication of which honey was part of. Moreover, the specific of action of any antimicrobial agents is very important as it gives specific information about its activity.

The antimicrobial activities of honey from different countries has been reported in recognition of the medicinal properties of honey (Olajuyigbe *et al.*, 2017). In Nigeria, the therapeutic ability and the antibacterial activities of various honey samples widely marketed for consumption need to be validated. Previous researched works on honey dwell on the antimicrobial activity

Therefore, for honey to be recommended as an alternative antimicrobial agent, like other conventional medicines, there is need for laboratory investigation on the specific action of honey on bacterial cell and its enzymes.

#### **1.4 Aim and Objectives of the Study**

The aim of this study was to determine the antimicrobial effects of honey and its specific actions on cell walls, membranes and enzymes of some microbial pathogens.

The specific objectives were to:

- I Assess the antimicrobial activity of honey.
- II Determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).
- III Determine the bioactive component of the honey.
- IV Determine the effects of honey on the cell walls, membranes and enzymes of the test organisms.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Plant as Antimicrobial Agents

##### 2.1.1 Reason for reconsidering plant as antimicrobial agents

Nowadays, the world had observed the amazing success in improvement of technology, science and medical practices; however, there is futile effort in the control of dramatic spread of infectious diseases (Abdallah, 2011). Abdallah (2011) reported that the infectious diseases remain the second leading cause of death worldwide. Though, the need for new antimicrobial agents is greater than ever. Microbes had been existence in earth since million years ago, being one of the oldest creatures in this planet.

These microscopic organisms are adapted, developed and survived in this changing nature throughout the eras, while other advanced huge ancient animals and plants perished. Though, microbes considered as one of the most adaptive and successive creatures in nature. These microbes were already-since that time- subjected to antibiotics produced from other microorganisms such as *Penicillium notatum*, as example. And they produce antibiotic resistant mechanisms, naturally (Opal *et al*, 2000). It is therefore not shocking that organisms are securely developed against our manufactured and semi-synthetic antibiotics in the cutting edge time frame. On the other hand, after its arrival on earth, the battle between man and organisms has begun.

Historical evidence found that 60,000 people lived in Mesopotamia (Iraq) long before, using a therapeutic plant called Hollyhock (*Alcea rosea* L.) (Cowan, 1999). Perhaps the

best weapon used by old humans against diseases was plants. Man has been using anti-microbial medications against bacteria since time immemorial. The employment and development of these medications against microorganisms lingered across civilizations until the modern era.

Recently, the global problem of the rapid growth of bacterial resistance to chemotherapeutic agents has led researchers to explore the use of other natural antimicrobial products, such as medicinal plants (Abdallah, 2011)

### **2.1.2 Plants: An alternative source for antimicrobial agent.**

Traditionally, early civilization regarded herbal plants as the main source of antibiotics discovery as reported by McChesney *et al.* (2007). In Mesopotamia, according Newman *et al.* (2000), Egyptian had recorded about even hundred drugs mainly of plant origin between 1500 and 2900 BC. In what is known today as traditional medicine, as stated by Abdallah (2011), this ancient medical experience has been transferred to us. Until 1990s, plant roots, leaves and bark contributed about eighty percent of total of all treatments (McChesney *et al.*, 2007).

Each year, the processing and analysis of herbal plants and its products contribute majorly to the economic growth in India, both as a source of part-time and full employment. Plant is the most significant source of drug. Plants had been used for years as drugs in ancient times. In two distinct fields of health management, medicinal plants are commonly used worldwide; traditional medicine system and modern medicine system (Mohd *et al.*, 2012).

So many new drugs have been extracted for thousands of years from plants. Human beings were completely derived their care services from herbal plant prior to the discovery of antibiotics (Singh *et al.*, 2008). Herbal plants are known be the gift of nature to human beings to make well-life free of disease. The diverse tradition of India is a stable

and successful source of traditional medicines, many of which are of herbal origin. The medicinal properties of approximately 45,000 plant species (Grover *et al.*, 2002). People in olden days gathered knowledge concerning herbal plants and recognized it into herbal therapeutic categories (Rout *et al.*, 2009). Synthetic analogues are part of drug extracted from plants as uncovered in recent pharmacopoeia is more than 25 percent (Astin, 1998). Savant *et al.* (2014) noted that there are two reasons for clinical microbiologists to be involved in the subject of extract from plant for antimicrobial. Next, the bioconstituents in the extracts were used to make antibiotics; some are already being tested in humans. Alternative types are now being studied, especially plant sources. Second, awareness is created to many people on the issues with conventional antibiotics being over-prescribed and misused. Moreover, many individuals are interested in getting more control over their health care (Savant *et al.*, 2014).

### **2.1.3 Importance of antimicrobial agents from plant**

Thirty-eight plant-derived flavonoids representing seven distinct structural groups were checked for antibiotic-resistant bacteria activity by Xu and Lee (2001) using the disc-diffusion assay and broth dilution assay. Four flavonoids (myricetin, datiscetin, kaempferol, and quercetin) and two flavones (flavone and luteolin) demonstrated inhibitory activity against methicillin-resistant *Staphylococcus aureus* according to the findings. Myricetin has also been shown to prevent the growth of multidrug-resistant *Burkholderia cepacia*, vancomycin-resistant enterococci (VRE), and other medically important bacteria like *Klebsiella pneumoniae* and *Staphylococcus epidermidis*.

Traditional medicinal plants have been proposed by Ortega-Ramirez *et al.* (2014) and Seow *et al.* (2014) as a source of food additives and bioactive compounds, which have traditionally been used to manage health problems and preventing infection. Therapeutic plants produce anti-cancer terpenes and phenols, as well as oils derived from plants with

pharmacological functions such as antioxidant, anticancer, and antibacterial properties; use of these drugs as antimicrobial ingredients in food products; synergism between components affects their effectiveness. One hour of medicinal smoke therapy produced via wood burning and a combination of odoriferous and medicinal herbs resulted in a 94 percent reduction in bacterial counts according to Nautiyal *et al.* (2007). After 30 days, there were no pathogenic bacteria (*Corynebacterium urealyticum*, *Enterobacteraerogenes*, *Enterobacter aerogenes*, *Klebsiella mobilis*, *Kocuria rosea*, *Pseudomonas syringae pv*, *Persicae*, and *Staphylococcus lentus*) in the open space indicates that the medicinal smoke treatment is bactericidal. Herbal treatments of the natural smoke/inhalational approaches to drug delivery are possible with medicinal smoking.

According to Savant *et al.* (2014), majority of unexploited element of drugs is antimicrobials derived from plants. There must be continuous and more discovery of plant antimicrobials. Antimicrobial substances on plants have huge therapeutic potential. At the same time, they are successful in treating infectious diseases. In the treatment of infections, herbal medicine is successful, at the same time; many of the risk factors associated with naturally derived antibiotics are reduced. Effective, but gentle, they are. So many plants have tropisms that correspond to human structures.

Phytomedicines have a number of health consequences. Their therapies may also go beyond the disease's symptomatic care. A good example of this is *Hydrastis canadensis*. *Hydrastis* not only has antimicrobial properties, but it also improves splenic blood flow, allowing for optimum spleen activity and the development of facilitating compounds (Savant *et al.*, 2014).

#### **2.1.4 Traditional medicine**

Herbal medication is also known as phytomedicine (Attah *et al.* 2020). The use of the following plant part: roots, barks, seeds berries, flower for therapeutic reasons are referred to herbal drugs. Annuals, biennials and perennials can be medicinal plants. Annual plants either complete its life span within a year or within 6 Months after they have fruited and flower. The life cycle of biennial herbal plants is 1 to 2 years. Herbaceous plants have a half-year life cycle, which means they die entirely at the end of the growing season or after flowering and fruiting. Perennial plants spent many years. Sometime stem died during the stage of growth but the growth emerges (Krishnan, 2018). An analysis of annual growth rings in the secondary root xylem will determine the age of certain herbal plants.

Herbal remedies are becoming a significant trend, and studies show the importance of plants in preventing and treating. Therapeutically, the data collected scientifically on such plant derivatives can be used (Gupta, 1994). All over the world, there is a considerable interest in herbal medicine because herbs contain compounds that are therapeutically effective and are more safe and ideal for patients than pharmaceutical chemicals (Szabadi, 2006; Ang-Lee *et al.*, 2001).

In the early 19th century, when methods of phytochemical screening first became available, Parkash *et al.* (2018) reported that scientists began extracting and manipulating the active compounds from plants. Chemists began making their own formulation of plant compounds later, initiating the change towards raw plants to synthetic pharmaceuticals. The use of herbal medicines has decreased over time in favour of pharmaceutical products.

Unpurified bioactive compounds are widely used by practitioners of medicinal herbs. These unpurified plant extracts contain many different constituents. They suggest that they can work together synergistically in such a way that the impact of the constituents

as a whole is greater than the individual components as a whole. Toxicity is often minimized when whole herbs are used rather than individual components (Vickers *et al.*, 1999).

Historical and current studies and surveys signify that the Eastern region of the Mediterranean has been well-known throughout the generations with a rich inventory of natural medicinal herbs. Arab medicine has contributed greatly to the development of modern medicine in Europe and remains one of the closest forms of original European medicine. The rapid increase in consumption of herbal remedies worldwide has been stimulated by several factors, including the notion that all herbal products are safe and effective (Saad *et al.*, 2005).

In every area, use of traditional medicine is growing by the day. Alternative therapies are important to achieve the objective of "health for all," and a traditional medicine program has been in effect since 1978 (Rojas *et al.*, 2003). In the United States, traditional drugs are more natural, healthier and safer. Garlic, Echinacea, Ginkgo and many others products are widely marketed.

As of mid-1998, annual retail sales were close to \$4 billion, and medicinal herbs were used by 12 to 37 percent of US customers, according to recent surveys (Eisenberg *et al.*, 1998). Fifteen per cent to forty percent of customers have used herbal medicine to treat many illnesses, according to recent surveys and reports. The cost of prescription drugs has risen in tandem with a willingness to reuse natural or organic remedies, leading to an increase with the use of medicinal herbs in the United States over the last 25 years. Around 70 percent of German doctors recommend herbal medicines to patients (Parkash *et al.*, 2018).

### **2.1.5 Synopsis benefits of medicinal plants**

According to the report of Attah *et al.* (2020), medicinal plants provide people worldwide with a wide variety of subsistence, cultural and monetary benefits. They provide poor and disadvantaged people, especially in poor rural areas, with affordable means of primary health care. Given the fact that medicinal plants have several advantages, including:

- I strengthened healthcare delivery services
- II Increased guarantee of life
- III possibly feasible utilize of the biodiversity and
- IV progressed advantage sharing with neighborhood communities

According to Wang *et al.* (2002), the production capacity from natural sources is 8.5 million tons and the production of cultivated medicinal herbs in 2001-2002 was estimated at 0.3 million tons.

#### **2.1.6 Reports on the antibacterial activity of medicinal plants**

Combination of secondary metabolites present in plants has the beneficial medicinal effects. These secondary metabolites (steroids, fatty acid resins, alkaloids, hormones, tannins, are capable of inducing definite physiological effects on health as reported by Attah *et al.* (2020).

Nair *et al.* (2005) screened nine plants for potential antibacterial activity. The plants screened were *Sapindus emarginatus*, *Hibiscus rosa sinensis*, *Mirabilis jalapa*, *Rhoeo discolor*, *Nyctanthes arbor-tristis*, *Colocasia esculenta*, *Gracilaria corticata*, *Dictyota* sp. and *Pulicaria wightiana*. Antibacterial activity was tested against 6 bacterial strains, *Pseudomonas testosteroni*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Proteus morgani* and *Micrococcus flavus*. Two methods, Agar disc diffusion and Agar disc diffusion, were used to study the antibacterial activity of all these plants. *Pseudomonas testosteroni* and *Klebsiella pneumonia* were the most resistant bacterial strains. *Sapindus emarginatus* showed strong activity against the tested bacterial strains.

The tube diffusion method was used by Ramasamy and Manoharan (2004) to assess the antibacterial activity of useful compounds from different solvent extracts of *Anosomeles indica*, *Blumea lacera*, and *Melia azadirachta* against *E. coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Staphylococcus aureus*. Whereas petroleum ether and aqueous exhibited no effects. Relatively more sensitive were *Pseudomonas aeruginosa* and *Serratia marcescens*, respectively.

Astal *et al.* (2005) tested the aqueous extracts of sage and thyme had action against microorganisms. Antibacterial activity of phenolic extracts of sage and thyme against *Staphylococcus aureus* and *Enterococcus* species has been demonstrated. The ethanolic extract of parsley was more influenced by *Escherichia coli*. This extract, on the other hand, has no discernible effect on the Gram-positive bacteria examined. Antibacterial activity of synthetic oils of sage, thyme, and parsley against *Escherichia coli*, *Proteus mirabilis*, and *Salmonella typhi* was not detected. The results revealed that of the ten microorganisms studied, *Staphylococcus aureus* was the most susceptible to the majority of the three plant extracts.

Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) were tested against petroleum ether, benzene ethyl acetate and acetone extract leaves of *Galinsoga ciliate*. Gram positive bacteria were more susceptible than gram negative bacteria according to Poonkothai *et al.* (2005). It might be concluded that high phenolic content cause toxicity in microorganisms.

Deshpande *et al.* (2005) discovered that extracts from *Abrus precatorius*, *Boswellia serrata*, *Careya arborea*, *Emblica officinalis*, *Syzygium cumini*, *Woodfordia fruticosa*, and *Sphaeranthus indicus* had potent antibacterial activity against Gram-positive and

Gram-negative bacteria. Only Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) were used in the experiments against other plant extracts.

According to Tambekatr and Kharate (2005), *E. coli*, *Staphylococcus aureus*, *Proteus mirabilis*, *Salmonella typhi*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* were all inhibited by *Ocimum sanctum*. Antimicrobial activity was detected in leaves isolated from different plants, including "Tulsi, Pudina, and Beet." against *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella typhi*, *Vibrio cholerae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* while piper betel showed resistances to *Streptococcus pneumoniae*.

## **2.2 Definition and Description of Honey**

Honey is a thick watery substance prepared by bees from the nectar. It was made of water, glucose, fructose, proteins, vitamins and minerals (Al-Waili and Haq, 2004). It could be also described as the ordinary sweet substance created by honeybees from the nectar of blossoms or from the exudations of living parts of plants or excretions of plants sucking insects on the living parts of plants, which bees gather, convert and mixed with specific substances of their own, kept and keep in the honeycomb to mature as reported by Elijah *et al.* (2015).

Olakunle *et al.* (2013) reported that Honey predominantly comprises sugar and water. The sugar content is about 96 – 98 % of honey dry matter, mostly, are simple sugars, fructose (38.2 %) and glucose (31.3 %), which represents 85 – 95 % of total sugars. These are “simple” sugars, 6-carbon sugars. Other sugars include disaccharide such as maltose, sucrose and few oligosaccharides are also present.

Water was ranked second among the component of honey (Olakunle *et al.*, 2013). Its content is vital, as it affects honey storage. The ultimate water contained in honey

attributed to plethora of ecological influences, like temperature plus moisture in the hives at the time of processing, another thing was environment of the nectar and honey handling process at the point of harvest and preservation (Peter *et al.*, 2007). Gluconic acid is one of the 0.57 % organic acids in honey that is a consequence of the use of enzymatic glucose.

Natural antioxidants are liable for acidity in honey and add to its distinctive taste. Honey contained small amounts of minerals (0.17 percent) with calcium, copper, iron, manganese, and phosphorus as the most abundant. There are nitrogen components of which the enzymes come from the worker bees' salivary liberation. The principal enzymes present within honey are invertase, amylase, and glucose oxidase (Peter *et al.*, 2007). Singh *et al.* (2012) reported that honey also contains numerous other sugar sources, as well as acids, vitamins, proteins and minerals. In honey, alkaloids, anthraquinone glycosides, cardiac glycosides, flavonoids and reducing compounds were found (Rakhi *et al.*, 2010).

The colour of honey can vary from nearly colourless to dark brown and its consistency can be fluid, viscous or partly to entirely crystallized (Elijah *et al.*, 2015). The nectar origin encountered by the bees, as stated by Elijah *et al.* (2015), contributes to the variation of colour, taste and aroma. Honey is commonly marketed for its nutritional benefits, but was also use since ancient times as a medicinal agent, but recently, clinical education of honey as therapeutic aid was introduced (Molan, 1999).

Several substances are derived from the secretory glands of honey bees, as well as different herbal products, alone or even in various combinations. These compounds included royal jelly, bee wax, propolis, bee pollen, and bee venom (Cornara *et al.*, 2017). Most of them have been used by humans for dietary and health purposes since ancient times, according to a study by Cornara *et al.* (2017).

A wide range of scientific research has discovered many biological components in honeybee products, while numerous results have been devoted to summarizing medicinal applications and uses as nutritional supplement, cosmetic and pharmaceutical properties (Viuda-Martos *et al.*, 2008). Steps have been taken to make sure that some of the honey bee products have been manufactured in clinical environments, but their pharmaceutical and medical normalization, based on honey variations and plant - based products, is a challenge due to the high chemical inconsistency. Many compounds were isolated from honey including pharmaceutical substances indicating the usefulness of honey in production of antibiotics from plant sources (Cornara *et al.*, 2017).

### **2.2.1 Types of bee**

Honeybees of the genus *Apis* are social insects which distinguish honey and other substances of immense human value by their production and storage. Currently, two domesticated species are known, namely, the western *A. niger* and the eastern *A. niger*. *mellifera* is the scientific name for a plant that produces honey. *A. Cerana*, which is found in South and Southeast Asia, is native to Europe, Asia, and Africa and was imported to America and Eastern Africa (Cornara *et al.*, 2017). Honeybees were categorized into stingless bee and sting bee, according to the Rao *et al.* (2016) study. Stingless honey is produced from a sweet substance. The honey generated by stingless bees differs in colour, taste, and viscosity from that produced by *Apis* bees (honey bees) (Almeida-Muradian *et al.*, 2014). Honey developed by a variety of stingless bees has powerful antibacterial effects (Irish *et al.*, 2008; Boorn *et al.*, 2010). According to Rao *et al.*, (2016), this important bee product has been processed and used in a variety of therapeutic methods, both in conventional and alternative procedures where honey is collected directly from the forest, and in the more well-established *meliponary* as reported by Rao *et al.* (2016).

The honey comb of sting bee and honey pot of stingless bee honey were shown in Figure 2.1



A. Honey comb of sting bee honey



B. Honey pot of stingless bee honey

**Figure 2.1:** A and B: Honey Comb of Sting and Pot of Stingless Bee Honey.

**Source:** Rao *et al.* (2016)

### 2.2.2 Types of honey

Approximately 320 different varieties have been established, depending on some flower sources (Kaur *et al.*, 2017). The taste, shape, colour and smell of a specific form of honey depend on the different water sources of the bees visited by the plants and fauna. In terms of the season in which it is made, existing temperature, precipitation and changing climate, various kinds of honey are identical. Honey varies from light brown to dark brown in colour. According to Kaur *et al.* (2017) review, several popular honey categories are listed below:

- I. Manuka honey- is a common healing agent and acts as a wound antibiotic.
- II. Acacia honey- is useful for liver and digestive system cleansing.
- III. Buckwheat honey- The colour is darker and made of antioxidants.
- IV. Neem honey- is helpful in diabetes or high blood pressure (Kaur *et al.*, 2017).

Olatunji *et al.* (2015) reported that there are mainly two honey types; apiary honey and forest honey. Apiary honey is made in apiaries by both the honeybees, *Apis ceranaindica* and *Apis mellifera*, which are collected by the modern extraction process, while forest honey is manufactured by rock bee, *Apis dorsata*, or wild A. nests in forests. *Ceranaindica*, and are obtained by the crude technique of pressing the comb (Subrahmanyam, 2007).

Honey can also be graded according to its nectar quality. Floral (sweet deposit from flower) and non-floral (sweet deposit from plant) honeys are among them (Manyi-Loh *et al.*, 2011). Honeys can be uni-floral or multi-floral, based on whether the honey is obtained from the nectar of a single flower or a number of flowers (Manyi-Loh *et al.*, 2011). Bees that extract sugar from living plant or fruit tissue and/or scavenge insect excretions (aphids) that tap the veins of plant species are provided by non-floral honey (honey dew) (Subrahmanyam, 2007).

Another element that has contributed to the colour and shape of honey is volatile compounds. More than six hundred volatile organic compounds were found in honey. At standard ambient temperature, volatiles are chemical substances with high vapour pressure. Honey comprises aldehydes, ketones, acids, alcohols, esters, hydrocarbons, and cyclic molecules, among other things (Kaskoniene and Venskutonis, 2010). Honey contains a limited number of volatile organic compounds, but these compounds have an effect on its color stability; taste, smell, texture, and color are all influenced by the fruits and trees that bees come across (Manyi-Loh *et al.*, 2011). The majority of volatile organic compounds are obtained from flowers or nectar sources, while some are produced during storage and distribution time (Jerkovic *et al.*, 2011).

### **2.2.3 Production of honey**

The honey bee (*Apis mellifera*), a high-value nutritious product, is of great significance to humanity as a pest species of both domestic and commercial crops and a source of honey (Ratnieks and Carreck, 2010). Honey bee misfortune has led to a far-reaching concern about nectar bees' near-long-term capacity to provide services due to relationship generators of bugs and pathogens, introduction of agrochemicals, apicultural bad management, and the need for genetic discrepancies (Ratnieks and Carreck, 2010). The quality and nature of honey is affected by a variety of factors, including flower composition, hive location, bee health, and the temporary impact on native species and floral morphology (Galimberti *et al.*, 2014).

Honey is produced in a number of ways (pressed, centrifuged, drained, heat analyzed) and comes in a wide variety of physical forms (comb, chunk, crystallized or granulated, creamed). Inside a honey bee hive, there are three castes of bees: queens, workers, and drones according to Saranraj and Sivasakthi (2018). Honey production is possible due to a combined effort. Honey is made using nectar from fruit trees by honey bees, nectar is a sugar-rich fluid formed in glands called nectaries (Saranraj and Sivasakthi, 2018). In a study in Sicula, honey made by black local bees *Apis mellifera* species had approximately 10 times more polyphenolic content and antimicrobial activities than honey produced by other *Apis mellifera* bee species. (Tenore *et al.*, 2012).

### **2.3 History of Honey**

Honey has been identified since the creation of man as the longest sweeteners ever seen in the world, though the official timing of origin is far from certain (Nayik *et al.*, 2014). Honey's use and processing has a long and diverse history (Nayik *et al.*, 2014). This practice of honey medication had been in ancient prescriptions and present care of wound for therapeutic purposes is well known. The Egyptian utilized honey for other reason such as treatment of skin illness, application on wound and embalmment. In the treatment of

bruises and sores of the mouth and healed carbuncles and running injuries, Hippocrates (460-357 BC) found the comfort of honey. Aristotle (384-322 BC) stated that mild honey was a great balm for sore eyes (Al-Waili, 2003).

Saranraj and Sivasakthi (2018) reported that when there is an identifiable deficiency as a result of low glucose level inside the body, the old Greeks were thorough in using honey for rapid vitality recovery: competitors combined honey with water in a major athletic drinking opportunity. The Babylonian connected honey for the recuperating of eyes and ears diseases and treatment for topical application (Saranraj and Sivasakthi, 2018).

Honey has been used to treat various diseases since the ancient Egyptians, Assyrians, Greeks, and Romans used it alone and in supplement form, as well as plants and active ingredients, to treat burns, injuries, eye infections, and gastrointestinal disorders (Saranraj and Sivasakthi, 2018).

In order to treat snakebites, fever and laxative, honey is used by various African tribes. Moreover, as mentioned by Saranraj and Sivasakthi (2018), honey was used by Masai fighters to gain strength and capacity, most likely due to honey's high calorie content.

#### **2.4 Religious Significance of Honey**

Haile *et al.* (2017) reported that many religious books considered the uses of honey in different ways: The religion of Islam recommended the use of honey as food and medicine, and even named an entire chapter in the Holy Qur'an called Surah al-Nahl which mean chapter of Honeybee. The practice administering honey for therapeutic and healing purposes was firmly endorsed by the Prophet Muhammad in Hadith.

In Christendom, there are references made to the importance of honey in the scriptures, these include the Books of Exodus, Psalms, Mathew and Judges. In this Christian sacred book, the Bible, King Solomon instructed his son. "Eat honey my child, since it is good"

as cited by Haile *et al.* (2017). Honey is accepted by all cultural traditions. Honey is a beneficial nutritious liquid that has been embraced without reservation by all generations, traditions, and societies, both ancient and modern, as written in all holy books (Nayik *et al.*, 2014).

## **2.5 Antioxidant Property of Honey**

Honey has been used for a long time in both health control and household uses, but its antioxidant possessions have only recently entered public attention. As need for antioxidant requirements in food increases, honey is accepted as an antioxidant base, as stated by Abeshu and Geleta (2016). Lack of antioxidants in food induces oxidative stress, which has led to an unbalanced chemical change between the development of oxidative stress and our body's normal protective role resulting in cell damage and genetic composition disruption (Haile *et al.*, 2017).

Deep analysis has been conducted on the molecular mechanisms showing how normal cells undergo tumour promoter-induced transition to cancer cells. Experiments have shown, however, that the mitogen-activated protein (MAP) kinase signalling pathways are activated by different tumour promoters differentially. Survival, growth, proliferation, apoptosis, cell cycle control, inflammation, and differentiation may be biological responses (Mohammed and Babiker, 2009).

The antioxidants bind to the signalling pathways and escape the adverse effects indicated by cancer, cardiovascular disorders, inflammatory diseases, neurological degradation, tissue repair, bacterial infections and aging as stated by Abeshu and Geleta (2016). Honey contains major antioxidant activity in the form of glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids, and proteins (Hadjmohammadi and Nazari, 2010). The major antioxidants in honey are phenols like quercetin, hesperetin, and chrysin, as well as

maillard substances called melanoidins (Hadjmohammadi and Nazari, 2010). The phenol quercetin precisely attaches and actively prevents the activities of cellular transcription factors. The suppression of signalling pathways exceeds the mechanism of phosphorylation and activation, preventing the cellular reactive oxygen species. It also induces human osteosarcoma cell apoptosis and decreases levels of protein expression in human fibrosarcoma cells (Abeshu and Geleta, 2016).

## **2.6 Antibacterial Factors in Honey**

Matured honey has a higher proportion of six-carbon sugar, fructose in general, and some maltose and sucrose, and contains much less than 18 percent water (Paulus *et al.*, 2012). The excessive sugar concentration, mixed with a small of water, causes osmosis, which prohibits microorganisms from spoiling the honey. Yeast growth can already result in only minor concentration of honey, but the amount of sugar of honey is sufficient to maintain honey's antimicrobial activities if diluted to about 30-40 % (Paulus *et al.*, 2012). The antibacterial activity at higher dilutions is due to substances other than sugar (Paulus *et al.*, 2012).

Hydrogen peroxide was recognized as key antimicrobial substances of the honey as from 1960s according to a study by Paulus *et al.* (2012). The glucose oxidase enzyme, added by honey bees during honey processing, is activate by little addition of water to honey and transforms glucose into hydrogen peroxides and gluconic acid. Nevertheless, due to non-peroxide components, different honeys have substantial antibacterial activity. Recently, bee defensin-1 and methylglyoxal were recognized in both RS honey and manuka honey as antimicrobial substances (Adams *et al.*, 2008; Kwakman *et al.*, 2010). So many studies results showed variability in the sensitivity of low pH to the antimicrobial properties of honey (usually around 3.2 and 4.5), however it has shown conclusively that pH has a part to play in this feature (Kwakman *et al.*, 2010). In addition,

there are strong indications that additional honey antimicrobial components are present, the identity of which is not yet established.

### **2.6.1 Hydrogen peroxide in honey**

Glucose oxidase is one of the nutrient enzymes applied to honey by bees. It converts glucose into hydrogen peroxide and gluconic acid in the presence of oxygen (Bang *et al.*, 2003). When the concentration of sugar has not yet reached levels capable of stopping microbial proliferation, hydrogen peroxide is thought to play a role in stopping immature honey from spoiling. Glucose oxidase is inactivated for the duration of honey ripening, but re-gains activity on honey dilution. The concentration of H<sub>2</sub>O<sub>2</sub> is high. The importance of H<sub>2</sub>O<sub>2</sub> to honey's antimicrobial property will be determined by the impact of inactivating this compound by adding catalase. Dilution of H<sub>2</sub>O<sub>2</sub> reduces the antimicrobial property of several of these honey samples tested, suggesting the essential role of H<sub>2</sub>O<sub>2</sub>, however after H<sub>2</sub>O<sub>2</sub> inactivation, a significant number of honey maintain activity (Mundo *et al.*, 2004).

Paulus *et al.* (2012) reported that the factors known to affect H<sub>2</sub>O<sub>2</sub> accumulation are inactivation of the H<sub>2</sub>O<sub>2</sub>-producing enzyme glucose oxidase by exposure to heat or light or degradation of H<sub>2</sub>O<sub>2</sub> by honey. It has been suggested that catalase originating from pollen, nectar, or microorganisms would be responsible for the enzymatic H<sub>2</sub>O<sub>2</sub>-neutralizing activity of honey. As in honey, H<sub>2</sub>O<sub>2</sub> is also a chief antimicrobial defense system in plant nectar (Carter and Thornburg, 2004) and substantial variation in accumulation of H<sub>2</sub>O<sub>2</sub> also exists among nectar samples (Hillwig *et al.*, 2010). Peroxidases are by far the most protein in petunia honey, and the concentration of hydrogen peroxides in petunia and tobacco honey is indirectly related to the amount of peroxidase production in those nectars (Gonzalez-Teuber *et al.*, 2009).

### **2.6.2 Methylglyoxal (MGO) in honey**

Various honeys have significant non-peroxide antibacterial activity. Manuka honey has been most expansively subjected to identification of non-peroxide antimicrobial compounds. This honey is produced from nectar of the manuka tree (*Leptospermum scoparium*), a New Zealand indigenous plant known for its non-peroxide antibacterial activity. Of late, unusually high levels of the antimicrobial compound methylglyoxal (MGO) have been found in manuka honey (Adams *et al.*, 2008). MGO is derived from sugars in carbohydrate-containing foods and beverages (Weigel *et al.*, 2004). The high levels of methylglyoxal in manuka honey, on the other hand, are produced by the conversion of dihydroxyacetone (DHA), which is contained in extremely high concentrations in the nectar of *Leptospermum scoparium* flowers (Adams *et al.*, 2009). This conversion occurs non-enzymatically and at a slow pace while honey is processed. In such large quantities, the production of DHA in nectar and its function are unknown in manuka nectar trees. MGO concentrations in various foods have been estimated to range from 3-47 mg/kg, whereas manuka honey produces far higher amounts (ranging from 38 mg/kg to 1,541 mg/kg (0.74-30.0 mM) (Adams *et al.*, 2008).

Based on a clear correlation between MGO levels and the ability of honey to inhibit *Staphylococcus aureus* growth (Adams *et al.*, 2008), it has been suggested that MGO is fully responsible for the non-peroxide antibacterial activity of manuka honey. The treatment for *Staphylococcus aureus* has been discontinued, and the treatment for *B. subtilis* has been drastically reduced. On the other hand, it had no effect on *E. coli* and *Pseudomonas aeruginosa*.

### **2.6.3 Osmolarity factor in honey**

Osmolarity or osmotic concentration is the number of osmoles of a solute per litre of solution. It is expressed as Osm/L (Erstad, 2003). The surface tension of honey is usually high due to the high presence of sugar in honey, causing a decrease in water activity, which gives osmolarity a crucial role in the antimicrobial action of undiluted honey, because, for example, the development of many species of bacteria is completely subdued when the moisture content is between 0.94 and 0.99 as reported by Saranraj and Sivasakthi (2018).

#### **2.6.4 Hydroxymethylfurfuraldehyde in honey**

Honey also contains hydroxymethylfurfuraldehyde (HMF) in trace amounts. Although even fresh honeys have been shown to retain minimal quantities of HMF (Zappala *et al.*, 2005), which can easily be increased if the honey is kept at mild temperatures, HMF produced by fructose breakdown in the presence of acid was considered proof of honey adulteration. As a result, it's critical to keep honey refrigerated or in a cool place to keep HMF levels low, as HMF is one of the most important factors to consider when it comes to honey performance and marketing.

#### **2.6.5 Pollen, propolis and royal jelly of honey**

Along with nectar, bees gather plant pollen, supplying nutritional protein to the hive. In honey, there is still pollen present. Pollen from trees and plants pollinated by the wind is often present in honey (Bruni *et al.*, 2015). Pollen and phenolic compounds are present in carbohydrates, amino acids, DNA, vitamins, nucleic acids, protein, lipids, minerals, and flavonoids (Morais *et al.*, 2011).

Bees use propolis, which is made from tree exudates, to cover their nest in a protective layer against intruders (Viuda-Martos *et al.*, 2008). Propolis contains 50 percent resin, 30 percent wax, 10 % essential oils, 5 % pollen, as well as other organic matter (5 percent).

Propolis, phenolic compounds, esters, flavonoids, terpenes, and anthraquinones were among the 300 compounds present in honey (Bertrams *et al.*, 2013).

Royal jelly is a protein-based fluid developed naturally by glands in the hypopharynx of worker bees, according to Saranraj and Sivasakthi (2018); it is only for adult bees.

The main proteins of royal jelly are examined and analyzed because protein accounts for more than half of the dry mass of the jelly (Won *et al.*, 2009). Asthma, high blood pressure, and allergy symptoms are all treated with royal jelly as a dietary supplement.

The fatty portion is mainly composed of terminally and/or internally hydroxylated medium-chain fatty acids with terminal mono or dicarboxylic acid functions, which are either saturated or monounsaturated at two positions. The 10-carbon atoms of royal jelly-specific trans-10-hydroxy-2-decenoic acid (10-HDA) and 10-hydroxydecanoic acid are the primary components. There are also small numbers of sterols (Li *et al.*, 2013).

From the first to the third day of their lives, larvae consume this royal jelly before developing into female workers and male drones, or selected individuals who turn into queens before the end of the larval period. Adult queens have a special meal every day of their lives (Fujita *et al.*, 2013). Royal jelly has been used in herbal medicine for centuries, especially in Asia and Egypt. In the pharmaceutical and cosmetic industries, it is currently used and marketed as an over-the-counter functional food. Several researches looked at royal jelly's antimicrobial properties against bacteria, fungi, and viruses, as well as hypotensive, anti-tumor, anti-hypercholesterolemic, and anti-inflammatory properties in animal models (Ramadan and Al-Ghamdi, 2012).

#### **2.6.6 Acidity of honey**

One of the factors contributing to honey's antimicrobial activity is acidity. It was recently proposed that honey's antimicrobial properties were due to its acidity.

According to Mato *et al.* (2003), honey contains around thirty organic acids, but the most abundant is gluconic acid, which is formed by the enzyme glucose oxidase.

### **2.6.7 Phenolic compounds of honey**

Flavonoids: quercetin, pinocembrin, pinobanksin, chrysin, galangin, kaempferol, and luteolin are the most common phenolic compounds known in honey (Kaskonienė and Venskutonis, 2010). The aromatic acids play an aromatic ring role in organic acid. Examples of aromatic acids are phenolic compounds and organic carboxylic acid because of the presence of a phenolic ring feature. Many plants contained phenolic acids (Pinho *et al.*, 2014). Plant-specific metabolites, flavonoids which perform more than one function are also play a crucial role for symbiotic nitrogen fixation, UV filtration and plant pigmentation according to Dixon and Pasinetti (2010). Two-phenyl-1; 4-benzopyrone is the essential molecular structure, which is found in plants. Benzoic, ferulic, gallic, chlorogenic, caffeic, syringic acid, p-coumaric, and ellagic acids are examples of plant-derived phenolic acids. Phytochemical composition has an impact on honey's antibacterial activity (Kaskoniene and Venskutonis, 2010). Phenolic compounds have antibacterial, anti-inflammatory, and antioxidant properties.

### **2.6.8 Bee defensin-1**

Recently, in RS honey, defensin-1 has been recognized as the antimicrobial peptide bee (Kwakman *et al.*, 2010). This peptide was previously detected in the honeybee head and thoracic glands of honeybee hemolymph, the insect equivalent of blood (Klaudiny *et al.*, 2005). In honey bee, Bee defensin-1 has potent activity, but only against Gram-positive bacteria such as *B. Subtilis*, *S. Aureus*, and the *P. aenibacillus larvae* (*P. larvae*) (Kwakman *et al.*, 2010). As part of their innate immune system, invertebrates rely heavily on antimicrobial peptides to defend against microorganisms. According to Paulus *et al.*

(2012), each one of these AMPs has a separate spectrum of antibacterial properties, and these peptides cooperatively contain all the most significant microbe groups.

Foulbrood is a debilitating infection affecting larvae of bee in particular in America. The disease affected the digestive tract of larvae and contributes to significant larval mortality within the first 48 hours of egg hatching (Genersch, 2010). The presence of defensin-1 in royal jelly and honey can aid in the protection of bee brood against American Foulbrood, but this is speculation.

While RS honey of bee defensin-1 was readily detectable, it was not detected in manuka honey (Kwakman *et al.*, 2011a). The occurrence of defensin-1 in various honeys has not been extensively studied, and statistical data on the peptide's composition in honey is still lacking. For six of the 26 honeys, protein-based antibacterial compounds were reported earlier, but the identification of these proteins wasn't really accompanied (Mundo *et al.*, 2004). The reported protein-contained antibacterial spectrum of compounds for four of these honeys strongly matches the bee defensin-1 tested range that is a powerful activity against *Bacillus* spp. But, against *Staphylococcus aureus*, it has no activity.

The hypopharyngeal gland of the honeybee secretes bee defensin-1 (Kwakman *et al.*, 2010). Bees use hypopharyngeal gland secretions for the manufacture of royal jelly and honey, as stated by Paulus *et al.* (2012). In royal jellies and honeys, the quantity of bee defensin-1 varies greatly (Kwakman *et al.*, 2011b), with this form of peptide completely absent in certain samples. Since American foulbrood is caused by defensin-1, which is active against *Paenibacillus* larvae, it will be interesting to see whether variations in bee defensin-1 expression are related to honeybee infection susceptibility (Paulus *et al.*, 2012).

## **2.7 Health Benefit of Honey Intake**

### **2.7.1 Improves haematological parameters**

The different health benefits of metabolic precursors on haematological and blood levels are associated with the regular intake of natural honey: enzymes and minerals (Al-Waili, 2003). It has been shown that the use of natural honey in apitherapy improves anaemic symptoms and thus benefits patients. One dietary enhancement research reported an increased haematological advantage in mature experimental rat fed with forest honey from Nigeria when compared to controls (Ajibola *et al.*, 2007). Improved haemoglobin concentration; the improvement of haematocrit values and high count in red blood cell were found in rats that were fed with honey.

Improved haematological parameters and improved immunity in rats were also reported as a nutritional supplement with 10 % New Zealand forest honey in a comparable study from another laboratory (Chepulis, 2007). This researcher also documented a higher lymphocyte count and enhanced phagocytosis by neutrophils in rats fed natural honey relative to control rats. This aligns with a previous study that verified that prebiotics can improve immunity (Schley and Field, 2002) and that honey contains oligosaccharides and other prebiotics (Eteraf-Oskouei and Najafi, 2013).

In a clinical trial in California, human participants given either of two honey treatments in showed the benefits of haematoprotection and enhanced haematopoiesis (Schramm *et al.*, 2003). In addition, normal honey has immunoprotective ingredients. According to Al-Waili and Haq (2004), the oral consumption of Asian polyfloral honey from Al-Theed

City, UAE stimulates and increases antibody production during various immune responses against the T-cells antigens of the thymus- independent and dependent origin.

### **2.7.2 Boosting of the immune system**

Honey can help clear infections by inducing the immune system to attack infection, in addition to providing direct antibacterial action. The proliferation and activation of neutrophils by B-lymphocytes and T-lymphocytes in cell culture has been reported to stimulated by honey (Tonks *et al.*, 2003). A 5.8 kDA portion of manuka honey that stimulates the development of TNF- via Toll-like receptor in macrophages has recently been found by Tonks *et al.* (2007). Honey also supplies glucose, which is required for the "respiratory blast" of hydrogen peroxide-producing macrophages, which is the primary component of the microscopic organism action (Molan, 2001).

Furthermore, it provides glucose metabolism substrates, which is a critical pathway for macrophage power storage, allowing them to operate in environments where oxygen is scarce, such as damaged tissue and exudates. Because an acid pH inside the phagocytic vacuole is involved in killing ingested bacteria, the presence of acids in honey aids macrophages in their bacteria-destroying action. (Molan, 2001).

### **2.7.3 Anti-inflammatory activity of honey**

*Melipona marginata* is a Brazilian species of endangered stingless bee. With exceptional physicochemical properties and a special taste, it produces honey. When *M. marginata* honey was used in a study, it had anti-inflammatory effects on the human body (Borsato *et al.*, 2014). Manuka honey's antibacterial activities, as well as its components' anti-inflammatory characteristics, were documented. By exposing Manuka honey to human

monocytes, the development of various inflammatory cytokines was assessed (Tonks *et al.*, 2003).

According to the findings, honey activated the production of inflammatory cytokines such as interleukin-1 (IL-1) and IL-6, and also tumor necrosis factor-alpha (TNF-alpha) through a toll-like 4 (TLR4) receptor-dependent pathway (TNF-). A protein with a molecular weight of 5.8 kDa contained in Manuka honey has been shown to be important for stimulating various cytokine forms in human monocytes through the TLR4 pathway (Tonks *et al.*, 2007).

Animals have been shown to benefit from Tualang honey's anti-inflammatory properties. Tualang honey was used to treat a chemically induced corneal injury in rabbits and produced results that were comparable to standard treatment (Bashkaran *et al.*, 2011), indicating that it has the ability to cure eye problems.

#### **2.7.4 Effect of honey on fertility**

Honey seems to have a beneficial effect on fertility and raises hormones linked to fertility (Rao *et al.*, 2016). In a study conducted of rats exposed to auditory stress, researchers discovered that 0.2 mL of honey combined with water increased a decrease in fertility (Rao *et al.*, 2016) Noise is a natural teratogenic element which has a serious impact on health, reproductive fitness, and reproductive organ function. Honey was found to increase the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone in this study. Vitamin E's potential benefits for these variables have also been documented (Rao *et al.*, 2016). In many illnesses, conditions and disorders, this tension plays a critical role. Increased stress levels are also associated with changes in reproductive health. Tualang honey, given at 1.2 g/kg per day to restraint-stressed pregnant rats, improved corticosterone levels, pregnancy, and adrenal histomorphometry, according to one report (Haron *et al.*, 2014).

Mosavat *et al.* (2014) observed that 1 g/kg of honey supplementation had a major beneficial effect on female rats' altered gonadotropin level. Honey at a dose of 1.2 g/kg/day increased smoke-induced reproductive toxicity in rats, increasing the amount of active interference and ejaculation, according to other reports. As a result, there is a rise in fertility and mating rates (Mohammed *et al.*, 2013).

#### **2.7.5 Honey and diabetics**

Daily honey consumption lowered the glycemic index relative to sucrose and glucose in type I diabetic patients and healthy participants, and that honey does not have additional acute hyperglycemic effects on the isoglycemic volume of bread in type II diabetics at breakfast (Attah *et al.*, 2021)

Honey has lower glycemic and incremental indices when opposed to glucose and sucrose in type I diabetic patients (Abdulrhman *et al.*, 2011). In rabbits suffered from drug induced diabetes, the antihyperglycemic effects of honey were registered. In one experiment, various honey doses (as low as 5 ml/kg) were found to result in a substantial reduction in blood glucose and other related parameters (Rao *et al.*, 2016). Honey can be a healthy sugar replacement for diabetics even in low dosage (5 ml/kg). It has been found that honey and its ingredients have many long-term health benefits. In one study, honey resulted in weight gain and blood sugar level reductions (Rao *et al.*, 2016).

Honey contains a lot of fructose, which is a monosaccharide that can boost blood glucose levels by swallowing it through the mouth. It's also perplexing that scientists and nutritionists have recommended honey as a dietary supplement for diabetics (Adesoji and Oluwakemi, 2008).

#### **2.7.6 Anticancer activity of oral honey administration**

Among the most significant and dangerous diseases is cancer (Rao *et al.*, 2016). Several works on honey have been published that it has the possibility to deride and induce

angiogenic activity on the effectiveness of honey on different cancers according to Fauzi *et al.* (2011), Hawley *et al.* (2014) and Kustiawan *et al.* (2014). Research into the therapeutic potency of honey against cancer cells has shown substantial anti-angiogenic effects in terms of its durability, viability, and even cell proliferation (Fauzi *et al.*, 2011). Studies on Malaysian honey have shown that it has anti-cancer, anti-oral, anti-bladder, and anti-liver activity (Swellam *et al.*, 2003; Baig and Attique, 2014).

Manuka honeys have been confirmed to have cancer-reducing operations. A study on the impact of Manuka honey on the improvement of symptoms of esophagitis after radiation has shown its protective impact on heart disease (Rao *et al.*, 2016).

From the therapeutic investigation of Manuka honey on squamous cell carcinoma and oral injuries, there were notable protective impact and inflammation reduction respectively (Drain and Fleming, 2015).

## **2.8 Antimicrobial Activity of Honey**

### **2.8.1 Antibacterial activity**

Honey has been shown to be antimicrobial (Dureja *et al.*, 2003). Honey inhibits bacteria that are gram positive as well as gram negative. Mohapatra *et al.* (2011) reported that honey alcohol extracts had an inhibitory effect on all bacteria. Honey has potent antibacterial properties, including resistance to a wide variety of antibiotics, as well as pathogenic and non-pathogenic bacteria. Antimicrobial activities may be bactericidal or bacteriostatic, depending on the concentration used (Manyi-Loh *et al.*, 2011). Gram-positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis*, and *Micrococcus luteus*, as well as Gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*, are all susceptible to honey extracts of methanol, ethanol, and ethyl acetate in a laboratory environment as reported by Mohapatra *et al.* (2011).

Honey has bactericidal effect against *P. aeruginosa* (Henriques *et al.*, 2011). At Minimum Inhibitory Concentrations (MIC) of 9.5 % (w/v), and Minimum Bactericidal Concentration (MBC) of 12 % (w/v), honey exhibits bactericidal effect with a 5 log reduction estimated within 4 hours above. Honey with carbohydrate solutions  $\geq 15$  % (v/v) effectively inhibits *H. pylori*. In-vitro tests of isolates of *Campylobacter spp.* are highly susceptible to honey solution (Lin *et al.*, 2009).

According to Hassanein *et al.* (2010), bacterial isolates from wounds of hospital patients: *Aeromonas schubertii*, *Haemophilus paraphrohaemlyticus*, *Micrococcus luteus*, *Cellulosimicrobium cellulans*, *Listonella anguillarum* and *Acinetobacter baumannii*, exhibit sensitivity to solutions of honey at various concentrations ranging between 25 % and 40 %.

In many bacterial infections, resistance to clinically administered antibiotics has been established. Wound infections were eliminated by 4.0-14.8 percent honey using a medical-grade honey, even with the clinically acquired antibiotics present in the wound environment. Honey resistance as typical to other antimicrobials was barely induced by bacteria (Blair *et al.*, 2009).

Honey sensitivity was demonstrated against vancomycin-sensitive *enterococci* and methicillin-resistant *Staphylococcus aureus* isolated from the wound at varying concentrations according to Cooper *et al.* (2002). Honey exhibits a synergistic activity against MRSA (Jenkins and Cooper, 2012), where sub-lethal a concentration (i.e 6 percent w/v) of manuka honey was able to reverse the drug tolerance to oxacilin of MRSA. By combining with other ingredients, progress was made to boost the antibacterial efficacy of honey. Medical-grade honey containing antimicrobial peptides like synthetic bactericidal peptide 2 (BP2) has faster antimicrobial property against

*Pseudomonas aeruginosa* and *Staphylococcus epidermidis* according to Kwakman *et al.* (2011a).

### **2.8.2 Antiviral effect**

Honey's antimicrobial activity against a wide range of microorganisms, including multi-antibiotic-resistant strains, has been demonstrated in various reviews and clinical trials. (Kumarasamy and Mahendran, 2015). Antiviral activity against Rubella virus has been documented in honey and is used topically to treat persistent herpes simplex lesions (Al-Waili and Haq, 2004).

According to numerous sources, manuka honey has a strong inhibition effect against influenza virus (Watanabe *et al.*, 2014). As an anti-infective (especially antiviral) action, due to the hydrogen peroxide, pasture honey is also used. Haile *et al.* (2017) stated that raw honey, as opposed to acyclovir, can remove herpes.

### **2.8.3 Antifungal effects**

Honey's antifungal activity was studied, but only a few fungi species were examined. It has antifungal activity against dermatophytes and can activate human mycosis (Tinea) (Shariati *et al.*, 2015).

The traditional treatment of fungal diseases is limited and part of the reason for this is due to the limited range of currently available antifungal medicines and the costly treatment, especially due to the need for prolonged treatment (Haile *et al.*, 2017). Several studies have been performed in recent years on the *in vitro* sensitivity of superficial mycosis to honey antifungal agents (Jessup *et al.*, 2000). Several studies have looked into the therapeutic properties of natural honey as well as its antifungal activity (Ji *et al.*, 2009). Recently, multi-flora honey samples have all been tested for their ability to prevent

the growth of 40 yeast strains, including *Candida albicans*, *Candida krusei*, *Trichosporon*, and *Candida Glabrata* (Koc *et al.*, 2009). Uni-floral honey had activity against *Penicillium* species is often greater than 10 percent at concentration (Kacaniova *et al.*, 2011).

#### **2.8.4 Anti-parasite effects**

Earthworms (*Pheretimaposthuma*), tapeworms (*Raillietinaspiralis*) and roundworms (*Ascaridiagalli*) were used to test the anti-parasitic properties of honey (Haile *et al.*, 2017). For the anti-helminthics assay, different concentrations (100-300 mg/mL) of sweetener extract have been examined (Haile *et al.*, 2017). The determination of the worms' paralysis and death time has been confirmed by honey.

The action of natural honey against helminths is due to its acidic pH level (3.2-4.5); this hinders the growth of helminths and produces an uninhabitable atmosphere for their growth (Sajid and Kamran, 2012). The result has shown that water extract has vermifugal activity and as an anthelmintic it has been found to be effective. Higher extract concentrations have produced paralytic effects much earlier, and all worms have shortened the time to death. Aqueous extract has demonstrated dose-dependent anthelmintic activity, providing the shortest amount of time of paralysis and death with 300 mg/ml dilution for all three forms of worms (Prasad *et al.*, 2010).

### **2.9 Other Microbial Related Use of Honey in Treatment**

#### **2.9.1 Dental effect**

Oral honey use can have a beneficial impact on tooth decay and oral improvement and is necessary during teeth operations (Atwa *et al.*, 2014).

In an article published in the *Journal of Dental Science*, the patients undergoing convectional treatment with Asian polyfloral honey, as an apitherapeutic agent, reduces

tooth extraction pain while also preventing oral infections such as gingivitis and dental caries (Atwa *et al.*, 2014).

Honey, both unprocessed and refined, has a wide range of antibacterial activity and has a high potential for minimizing dental caries susceptibility according to Mohapatra *et al.* (2011). English *et al.* (2004) and Atwa *et al.* (2014) discovered that New Zealand manuka honey protects against dental plaque and gingivitis, as well as other oral problems, in addition to its carioprotective properties.

According to Steinberg and colleagues' research, raw honey is either non-cariogenic or less cariogenic than sugar (Ajibola, 2015). As Ahmadi-Motamayel *et al.* (2013) and his colleagues studied Iranian honey's antibacterial properties in apitherapy, they discovered that it is not only non-cariogenic but also anti-cariogenic (Ahmadi-Motamayel *et al.*, 2013).

Honey's antimicrobial properties help to prevent dental caries pathogenesis by inhibiting bacterial growth. Furthermore, raw honey consumption is healthy and does not cause oral diseases such as gingivitis and periodontal disease, according to one study (English *et al.*, 2004). Volunteers who chewed New Zealand manuk honey as “honey leather” had highly significant reductions in mean plaque scores (0.99 reduced to 0.65;  $P = 0.001$ ) compared to the control group, indicating a potential therapeutic role for honey in oral health (English *et al.*, 2004).

Honey's potential to be non-cariogenic may be attributed to the protective effect of its synergistic constituents (Eteraf-Oskouei and Najafi, 2013).

### **2.9.2 Gastroenterology**

Honey has been confirmed to have effects of preventing and treating gastrointestinal disorders such as peptic ulcers, gastritis, and gastroenteritis. Honey is a potent inhibitor

of the causing agent of peptic ulcers and gastritis, *Helicobacter pylori* (Osato *et al.*, 1999). According to Abeshu and Geleta (2016), Honey is natural and will not raise blood-sugar levels; honey mixed with water is a good curative agent for colic.

The consumption of honey raises the bacterial population of microflora, which is important for the health of the digestive tract. Honey intake increases the population of natural flora known as *bifidobacteria*, according to the paper published by Abeshu and Geleta (2016), where its constituents have been shown to have a prebiotic effect similar to fructooligosaccharide (FOS) effects.

### **2.9.3 Wound healing property of honey**

Wound, a body injury that can be caused by abuse, surgery or accident involving membrane breakage (such as skin) and typically underlying tissue damage. Abeshu and Geleta (2016) reported that the wound causes tissue damage, blood vessel disturbance, blood component extravasations and hypoxia. By causing greater pain, irritation and inconvenience, the production of wound infection has adverse impact on health may lead to serious sickness or death consequence.

Honey is an example of a naturally occurring remedy that has been used to treat wounds. Through its restorative tissue growth and epithelialization impacts, it helps promote faster healing process with little or no scarring (Al-Waili *et al.*, 2011).

According to Al-Wali *et al.* (2011), the physical effects of honey, such as acidity and osmotic effect, as well as its chemical impact, such as hydrogen peroxide antimicrobial property only at the site of injury, once taken orally, are the primary sources of tissue repair property. Prostaglandins and nitric oxide play an important part in the healing process (Abeshu and Geleta, 2016).

Honey has proved to be successful in a number of circumstances. Honey was used as a dressing and applied to the mucous layers of the body cavities with no harmful effects. Honey has been used without causing any allergic reactions or significant side effects. In addition, wound odour is easily eliminated, granulation and epithelialization improves, exudates are decreased in length, and microbe wounds are sterilized (Al-Waili *et al.*, 2011).

Cut skin grafting is a common technique for protecting skin defects. Infections, delays in healing, fluid and electrolyte inequalities, scar development, and pain may all occur during the revitalization of skin removal donor sites. Honey-impregnated gauzes with a faster epithelization time and a low feeling of pain are comfortable, safe, and easy to use (Misirlioglu *et al.*, 2003).

Medicated honey bandages are increasing growing recognition and medicinal applications due to honey's healing effect on different forms of wounds. The Food and Drug Administration (FDA) has approved Derma Sciences Medihoney® dressing with active manuka honey to help wound healing by providing a moist environment in 2007 (Al-Waili *et al.*, 2011).

Medihoney® bandages are intended for diabetes patients who have light to moderately exuding wounds, such as venous or arterial leg ulcers, full or minimum thickness pressure ulcers/sores, and first and second soft tissue bums (Abeshu and Geleta, 2016).

Tissue repair times were shortened following Medihoney® treatment as compared to conventional care, according to a study conducted in the United Kingdom (US), and although the results are not statistically significant, they are clinically significant. Honey can be used to treat wounds at any point of healing. According to Simon *et al.* (2006), antimicrobial honey is useful in treating malignant wounds of different etiologies.

This, combined with the ideal tissue regeneration characteristics of maintaining a moist wound environment (which is required for immediate healing), non-toxicity, anti-inflammatory activity, deriding procedure, scarring decrease, and re-epithelialization stimulation, has resurrected honey's use in wound care. Honey is most commonly used to treat burns on the skin (Al-Waili *et al.*, 2011).

In a total of 104 superficial burn injury cases reported Abeshu and Geleta (2016) compared the performance of honey as a dressing to silver sulfadiazine gauze dressing. In the 52 patients treated with honey, 91 percent of the injuries were sterile within 7 days, and 87 percent of the wounds healed within 15 days. Honey's medical advantages, according to the report, helped burn burns recover quicker and with less complication than conventional therapy. Honey's ability to speed up the healing process in wounded areas, ulcers, and skin burns is due to its hydrophobic nature, hypertonicity, lower pH, and complex chemical properties.

The stimulatory effects of oral honey administration suggest that a tissue growth factor may be involved, rather than wound acidification or improved tissue nutrition, which may promote growth (Al-Waili *et al.*, 2011). Because of its antibacterial properties, honey is thought to be the most important component in wound healing. According to many reports, honey, on the other hand, acts directly on skin cells involved in the recovery process (Majtan *et al.*, 2010; Ranzato *et al.*, 2012).

Numerous studies have been conducted on honey's immunomodulatory properties, which may explain, at least in part, its ability to speed wound healing. While other researchers hypothesized that bacterial lipopolysaccharide contamination of honey may cause immunostimulatory effects, immunoregulatory honey constituents were ruled out. A 5.8 kD a constituent from manuka honey stimulates TNF-a production by macrophages via toll-like receptors (Tonks *et al.*, 2007).

## 2.10 The Precaution of Using Honey

Honey can be poisoned by the environment, such as pesticides, antibiotics and poisonous plants, like any other natural food (Bogdanov, 2006). It is known that a few plants produce nectar that contains toxic substances. Two primary toxin groups relevant to nectar are diterpenoids and pyrazolidine alkaloids. According to Rao *et al.* (2016), toxic cyclic polyhydroxylated hydrocarbons or diterpenoids can be found in plants belonging to the *Ericaceae* family's *Rhododendron* subfamily, such as *Rhododendron ponticum*. Consumption of the above-mentioned tainted honey induces symptoms such as dizziness, nausea, vomiting, sweating, exhaustion, blurred vision, convulsions, and loss of consciousness, limb parenthesis, heavy perspiration, headache, stomach ache, delirium, vision weakness, and salivation. Since local beekeepers are aware of these poisonous plants, honey that may contain poisonous substances is not sold (Bostan *et al.*, 2010). Honey toxicity from other plants has also been documented (Islam *et al.*, 2014). The presence of *Clostridium botulinum* in honey is a health consequence for children (Rao *et al.*, 2017).

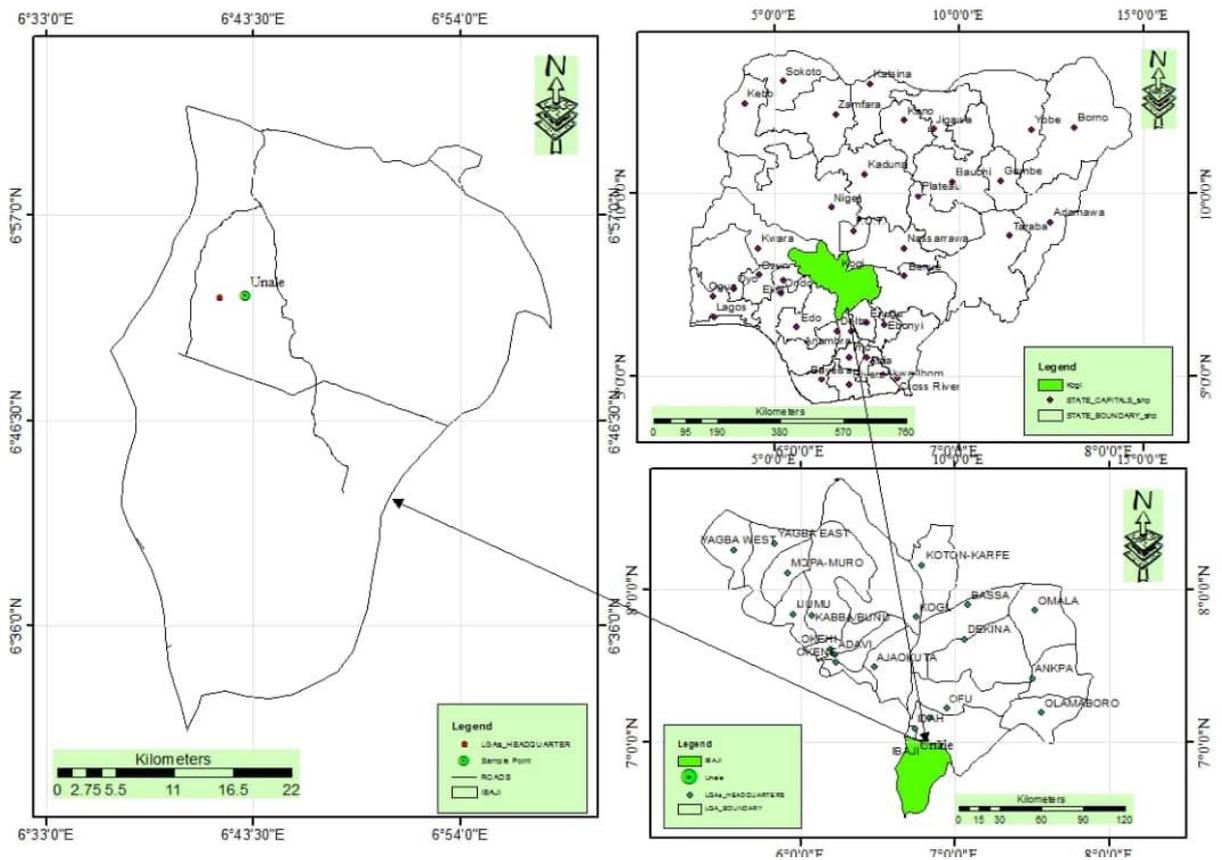
This bacterium's spores can live in honey, but they are incapable of developing toxins. Therefore, the bacteria spores from honey will live in the stomach of children less than a year and become toxic in the immature digestive tract leading to disease and even death (Tanzi and Gabay, 2002). To minimize the risk of honey-borne poisoning in areas where toxic nectar plants are present, it is recommended that honey be purchased from a market rather than from individual beekeepers (Edgar *et al.*, 2002).

## **CHAPTER THREE**

### **3.0 MATERIALS AND METHODS**

#### **3.1 Source of Honey**

Honey was collected from a local bee producer in Unale ( $6^{\circ} 53'13.11''$  N and  $6^{\circ} 44'22.83''$  E), Ibaji Local Government Area, Kogi State, Nigeria. The location of the collection area was determined with the aid of Geographical Positioning System (GPS) and the coordinates were used to generate the map shown in Figure 3.I. This was done in the Department of Geography Federal University of Technology, Minna, Nigeria. The sample was collected during the month of February, 2019 in a sterile container and transported to the Department of Microbiology Laboratory, Federal University of Technology, Minna, Niger State and stored at  $4^{\circ}\text{C}$  until use.



**Figure 3.1: Map of Honey Sourced Area.**

### **3.2 Characteristics of the Honey**

The quality of the honey was ascertained using the method described by Nwankwo *et al.* (2014) and Elijah *et al.* (2015): (i). Dropping some of the sample onto sand: if it is an undiluted honey, it will not sink immediately, (ii) Pouring a small quantity into a cup of water: if undiluted, it will go down to the bottom of the cup without mixing up with the water except when stirred (iii) Dipping a finger into the honey sample, dropping one or two drops on the ground: if it is undiluted, it will go down like a thread without breaking. (iv) Dipping a match stick unto the honey and sticking to it: the matches will be burn if it is an undiluted honey and the honey will even act as a fuel while the matches is burning.

### **3.3 Processing of the Honey Sample**

The honey sample was filtered through sterile musclin cloth to remove debris, sterilized at 121°C for 15 minutes, streaked on the nutrient agar plate and incubated at 37°C for 24hrs to verify the presence of microorganism. Thereafter, the honey sample was diluted into various concentrations with distilled water into the following: 100 (without water addition), 80 and 60 percent volume by volume for antibacterial susceptibility testing.

### **3.4 Preparation of Nutrient Agar Medium**

Twenty-eight grams of nutrient agar was dissolved in 1L of distilled water according to the manufacturer's instruction.

### **3.5 Test Organisms**

Test organisms used were: *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*. They were collected from the Microbiology Department, Federal University, Minna, Niger State, Nigeria. The organisms were confirmed using morphological and biochemical characteristics, and kept at 4°C until the test was conducted.

### **3.6 Gram Staining**

A smear of 24 hours old bacterial culture of all isolates was prepared on a grease free glass slide. The smear was first air dried and then heat fixed. The heat fixed smear was then flooded with crystal violet stain for I minute and then washed with distilled water for 5 seconds. The washed slides were then flooded with Gram's iodine solution for I minute and then again washed for 5 seconds with distilled water. The slides were then washed with 95 % ethanol in drop wise manner. After washing with distilled water again the smear was flooded with safranin for I minute. The stained slides were finally washed with distilled water, air dried and observed under microscope. All the observation was done under oil immersion objective.

### **3.7 Catalase Test**

The catalase test is biochemical test for aerobic organism that detects the production of catalase enzymes in an organism. Clean glass slide was used to conduct catalase test. Emulsion of the isolate was made on a clean glass slide using wire loop followed by addition of three percent H<sub>2</sub>O<sub>2</sub>. Catalase (enzymes) positive will be indicated by effervescence (Bashua and Oladunmoye, 2017)

### **3.8 Oxidase Test**

The oxidase test is used to analyze the ability of bacteria to produce the enzyme cytochrome oxidase. This enzyme catalyses the transfer of electrons from cytochrome c to molecular oxygen and is usually found in bacteria which uses oxygen as the terminal electron acceptor during respiration. An 18 hours' bacteria culture was transferred aseptically to the filter paper wet with three drops of sterile distilled water. one drop of oxidase reagent was added and observed. After ten second, the spot the sport for the development of red colour which indicated was observed and compared with the control, blue colour indicated oxidase positive (Bashua and Oladunmoye, 2017).

### **3.9 Starch Hydrolysis Test**

Starch agar was prepared by adding 1 % of soluble starch nutrient agar and autoclaved at 121°C for 15mins after which the plates were poured and allowed to gel. The organisms were then streaked once across the surface of the plates and incubated at 37°C for 24 hours. The plates were then flooded with some quantity of Gram's iodine. Unhydrolyzed starch formed blue black colour with the iodine while hydrolyzed starch appeared as a cleared zone which results from α-amylase activity. Reddish brown zones around the colony indicated partial hydrolysis of starch (Bashua and Oladunmoye, 2017).

### **3.10 Triple Sugar Iron Test (TSIA)**

TSI agar is a modified agar media. It comprises of three main sugars, glucose (0.1 %) sucrose (1 %) and lactose (1 %) (Kliger, 1917). Besides carbohydrate it contains beef extract, yeast extract, and peptone which are the source of nitrogen, vitamins and minerals. Phenol red is the pH indicator. During preparation the tubes containing the molten agar are angled. The slant is aerobic, while the butt is anaerobic. The tubes were inoculated by first stabbing at the butt, which was followed by streaking at the slant and were incubated at 37°C for 24 hours. If any of the carbohydrate is fermented the drop in pH will cause the medium to change from reddish orange to yellow. A deep red colour indicates alkalization of the peptones. Sodium thiosulphate in the medium is reduced by some bacteria to hydrogen sulphide, a colorless gas. The hydrogen sulphide thus upon reaction with ferric ions in the medium, produce iron sulphide, a black insoluble precipitate. The bacteria are said to be glucose fermenter if the reaction tube produce an alkaline slant (red) and an acid butt (yellow), signifying that only glucose is metabolized. Since, this substrate is present in minimal concentration; the small amount of acid produced on the slant surface is oxidized rapidly, whereas in the butt, acid reaction is maintained because of reduced oxygen tension and slower growth of the organism. When the reaction tube is overlaid with acid, over acid in both the slant (yellow) and the butt (yellow) then the bacteria are said to be glucose, lactose and / or sucrose fermenters. When there is no acid produced in both the slant and the tube the bacteria are said to be glucose, lactose and sucrose non fermenter.

### **3.11 Preparation of Potato Dextrose Agar Medium**

Potato dextrose agar was prepared by adding 39 g of the powder medium into 1000 mL of distilled water (Bashua and Oladunmoye, 2017). A suspension formed was heated on the heating mantle with occasional swirling of the flask dissolved. The conical flask was plugged with cotton wool and wrapped carefully with aluminum foil. This was autoclaved

at 121°C for 15 minutes. After sterilization in the autoclave, the medium was allowed to cool and 1 % of streptomycin was added to inhibit bacteria.

### **3.12 Identification of Fungi**

The identity of the fungi isolate was done by observation of certain morphological characteristics, macroscopic and microscopic (Bashua and Oladunmoye, 2017). as follows: On a grease-free slide, a drop of distill water was applied. The teasing needle was used to picked cultured fungi, teased on the slide and then viewed under the microscope, using X10 and X40 objective lenses.

### **3.13 Preparation of Standard Inoculums**

The turbidity organism suspension from 18 hours' bacterial culture were made in sterile distilled water and compared with the McFarland turbidity standard, until the cloudiness correspond with the scale number 0.5 standard by visual comparison, which corresponded to  $1.5 \times 10^8$  cfu/mL (Odiba *et al.*, 2014).

### **3.14 Antimicrobial Sensitivity Testing**

The following concentration: 100 %, 80 %, and 60 % (v/v) of honey samples were prepared and used to test against the organisms using agar well diffusion method (Abalaka *et al.*, 2016). Three well were bored with the aid of 5 mm diameter sterile cork borer; The well was made at various positions on the plates, equidistantly. Each well was sealed at the bottom with molten agar to prevent spillage. The sterile medium was seeded with 0.1 ml of the standard inoculum of the test microorganisms; the inoculum was spread evenly over the surface of the medium with a sterile swab. A standard cork borer of 5 mm in diameter was use to cut cups (well) at the center of each inoculated medium and 0.1 mL of the honey solutions were introduced separately into each well on the medium, the plates were incubated at 37°C for 24 hours for bacteria. The fungi were incubated for

four days at room temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) at 100 % honey concentration. The zones of inhibition were measured with meter rule and recorded.

### **3.15 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)**

The minimum inhibitory concentrations (MICs) were estimated using the tube dilution method; the lowest concentration of honey with no turbidity was used as the minimum inhibitory concentration (MIC) (Abalaka *et al.*, 2016). The MIC was determined by preparing honey concentrations (100 %, 80 %, 60 %, 40 %, 20 % and 10 %, (v/v) in nutrient broth. Nine mL of each of the concentration was introduced into 10 mL test tube. This mixture was then inoculated with 0.1mL culture of the test organism standardized to approximately  $1.5 \times 10^8$  cfu/mL and was incubated at  $37^{\circ}\text{C}$  for 24 hours. The tubes were observed to determine the lowest concentration of honey in the test tube with no turbidity or cloudiness compared with the control. This was taken as the minimum inhibitory concentration (MIC) (Abalaka *et al.*, 2016; Mba-Omeje *et al.*, 2017)

Minimum bacterial concentration was determined for each set of test tubes used for the MIC test. A loopful of the broth culture was collected from those test tubes which showed no growth and was inoculated onto sterile nutrient agar by streaking. MBC was determined when no visible growth was seen (Mba-Omeje *et al.*, 2017).

### **3.16 Phytochemical Screening of the Honey**

Qualitative tests were carried out on the honey sample using methods described by Jayashree (2013) and Abalaka *et al.* (2016).

#### **3.16.1 Test for flavonoids**

A portion of honey in each case was heated with 10 mL of ethyl acetate in a test tube over a steam bath for 3 minutes. The mixture was filtered and 4 mL of the filtrate was

shaken with 1 mL of dilute ammonia solution. Yellow coloration was observed thus indicating the presence of flavonoids.

#### **3.16.2 Test for phenols and tannins**

Honey was mixed with 2 mL of 0.1 % ferric chloride ( $\text{FeCl}_3$ ), blue-green or black colouration indicated the presence of phenol and tannins.

#### **3.16.3 Test for saponins**

Frothing test: Two mL of the honey was vigorously shaken in the test tube for 2 minutes. Frothing was observed indicating the presence of saponins.

#### **3.16.4 Test for alkaloid**

Alkaloid was tested by adding 2 mL of honey in the test tube contained 5 mL of 1% aqueous HCl on a steam bath and shaken, two to three drops of solution of picric acid was added. The preliminary evidence for the presence of alkaloids was formation of a reddish brown precipitate.

#### **3.16.5 Test for steroids**

Steroid test was performed by adding 2 mL of honey into test tube, mixed with 2 ml of acetic anhydride followed by 2 mL of sulphuric acid. No colour changed from violet to blue or green in the samples indicated the absence of steroids.

#### **3.16.6 Test phlobatannins**

The presence or absence of the phlobatanins was determined by boiling 1 % aqueous hydrochloric acid with small portion of honey sample; red precipitates indicate the presence of phlobactanins.

### **3.16.7 Test for cardiac glycosides**

Cardiac Glycosides was detected by adding 5 mL of honey into the test tube, mixed with 2 mL of glacial acetic acid and one drop of ferric chloride solution, followed by the addition of 1 mL concentrated sulphuric acid. Brown ring formed at the interface indicated deoxysugar characteristics of cardenolides. A violet ring may appear beneath the brown ring, while in the acetic acid, layer; a greenish ring may also form just gradually throughout the thin layer.

### **3.16.8 Test for anthraquinones**

Five milligram of honey was shaken with 5 mL chloroform, the chloroform layer was filtered and 0.5 cm<sup>3</sup> of 10 % ammonia was added to the filtrate. The mixture was shaken thoroughly, the formation of a pink/violet or red, yellow colour in the ammonical phase indicated the presence of anthraquinones.

### **3.16.9 Test for reducing sugar (benedict test)**

Five milligram of honey was mixed thoroughly with 3 cm<sup>3</sup> distilled water and filtered, 3 drops of the filtrate was added to 3 cm<sup>3</sup> Benedict reagents and placed in a boiling water bath for 5 minutes. The formation of a brick red precipitate indicates reducing sugar.

## **3.17 Specific Actions of Honey on Pathogenic Organisms**

### **3.17.1 Catalase inhibition**

Catalase is a widely distributed antioxidant enzyme that breaks down hydrogen peroxide into water and oxygen. Several pathogens produce catalase to defend themselves against oxidative stress as well as attacks by hydrogen peroxide, a common weapon used by the host's immune system.

In order to address these, an assay was developed to measure catalase activity. The assay uses simple and readily available reagents, namely hydrogen peroxide, Triton X-100, and

catalase (Iwase *et al.*, 2013). The underlying principle of this approach is that the oxygen bubbles generated from the decomposition of hydrogen peroxide by catalase are trapped by the surfactant Triton X-100. The trapped oxygen bubbles are then visualized as foam, the test-tube height of which is measured to quantify the catalase activity.

In the present research, 18 – 24 hours of actively growing bacteria was used according to Iwase *et al.* (2013) with little modification. One hundred micro milliliter of bacterial suspension were transferred into 4 mL of honey in the test tube at each respective MIC concentration (100 %, 80 % and 10 %) and incubated at room temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) for 3 hours. After the incubation, the cells were harvested by centrifugation at 4000 rpm for 10 minutes. The pellets were used for quantification of cellular catalase activity. One hundred micro milliliter bacterial pellet was added in Pyrex test tube 13 mm diameter  $\times$  100 mm height. Subsequently, 0.1 mL of 1 % Triton X-100 and 0.1 mL of undiluted hydrogen peroxide was added to the solutions and mixed thoroughly and sterile distilled water which contained the same cell suspension was used as control. Following the completion of the reaction, the height of oxygen forming foam that remained constant in the test tube was measured after 5 minutes using a meter rule (ruler) and recorded (Wang *et al.* 2015). The enzyme-generated oxygen bubbles trapped by Triton X-100 were visualized as foam, whose height was estimated.

### **3.17.2 Protein leakage**

An important characteristic of plant extracts is their hydrophobicity which enables them to attack the lipids of the bacterial cell membrane, disturbing the structures and inducing more permeability (Kusumaningrum *et al.*, 2014). Leakage of the cytoplasmic membrane has been analysed by determination of the absorbance of suspension containing cell materials including proteins that were absorbed at 595 nm (Kusumaningrum *et al.*, 2014).

In this study, bacterial cultures were transferred to 9 mL of fresh sterile nutrient broth in the test tubes and incubated at 37°C for 18 hours. After incubation, 0.1 mL of the actively growing bacterial cells was suspended in 4 mL of honey at each respective MIC concentration. These were incubated at room temperature for one hour and two hours. After incubation, cultures were centrifuged at 4000 rpm for 25 minutes and bacterial pellets were removed. One ml of Bradford reagent was added to 1 mL of supernatant and incubated at room temperature (25°C ±2°C) for 5 minutes. Then the absorbance of the supernatant was measured at 595 nm using UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) for protein leakage determination in each case and the protein estimation was done using Bovin Serum Albumin (BSA) as standard (Kusumaningrum *et al.*, 2014).

### **3.17.3 Preparation of Bradford reagents**

One hundred milligrams of Coomassie Brilliant Blue G-250 (Sigma-Aldrich) was dissolved in 50 milliliters of 95 percent ethanol, followed by 100 milliliters of 85 percent (w/v) phosphoric acid, dilution to 1000 milliliters, and filtration by Whatman paper number 1

### **3.18 Data Analysis**

The analysis of variance of the values obtained for antibacterial susceptibility, protein leakage and catalase inhibition analysis were carried out using IBM SPSS Statistic version 23. All data were expressed as Mean ± Standard Error Mean of triplicate determinations. Values with the same superscript on the same column are not significantly different at (P < 0.05). Values with the same subscript on the same row are not significantly different at (P < 0.05).

## **CHAPTER FOUR**

### **4.0 RESULTS AND DISCUSSION**

#### **4.1 Results**

##### **4.1.1: Phytochemical screening of honey**

The phytochemical screening of the bioactive component of the honey showed the presence of saponins, phenols, flavonoids, tannins, reducing sugars, anthraquinones cardiac glycosides and alkaloids while steroids and phlobactanins were not present as shown in Table 4.1

**Table 4.1: Bioactive Component of Honey**

<b>Bioactive components</b>	<b>Inference</b>
Saponins	+
Phenols	+
Akaloids	+
Flavonoids	+
Taninns	+
Reducing sugar	+
Cardiac glycosides	+
Phlobactanins	-
Steroids	-

**Key:** - = Absent and + = Present

#### **4.1.2: Antimicrobial susceptibility test of the honey**

The result of the antimicrobial susceptibility test showed *E. coli* had zone of inhibition of  $29.0 \pm 0.6$ mm,  $27.0 \pm 0.6$ mm and  $19.0 \pm 0.6$ mm at 100 %, 80 % and 60 % concentration of honey respectively, *B. subtilis* had  $15.0 \pm 0.6$ mm,  $11.0 \pm 0.6$ mm and  $8.0 \pm 0.6$ mm at 100, 80 and 60 % concentration of honey respectively while *P. aeruginosa* had  $13.7 \pm 0.3$ mm,  $11.0 \pm 0.6$ mm and  $6.3 \pm 0.3$ mm at 100, 80 and 60 % concentration of honey respectively. It showed that *E. coli* had highest zone of inhibition (29 mm) followed by *B. subtilis* while *P. aeruginosa* had the least zone of inhibition (6.3 mm) showed in Table 4.2.

**Table 4.2: Antimicrobial Susceptibility Test (mm) of Honey on the Isolates**

<b>Honey Concentration (% v/v) / mean zone of inhibitions (mm)</b>				
<b>Bacteria</b>	<b>100</b>	<b>80</b>	<b>60</b>	<b>Ciprofloxacin</b>
<i>E. coli</i>	29.0±0.58 <sup>c*</sup> <sup>b**</sup>	27.0 ±0.58 <sup>b*</sup> <sup>b**</sup>	19.0±0.58 <sup>a*</sup> <sup>b**</sup>	32.0±0.28 <sup>d*</sup> <sup>b**</sup>
<i>B. subtilis</i>	15.0±0.58 <sup>c*</sup> <sup>a**</sup>	11. 0±0.58 <sup>b*</sup> <sup>a**</sup>	8.0±0.58 <sup>a*</sup> <sup>a**</sup>	30.0±0.00 <sup>d*</sup> <sup>a**</sup>
<i>P. aeruginosa</i>	13.7±0.33 <sup>c*</sup> <sup>a**</sup>	11. 0±0.58 <sup>b*</sup> <sup>a**</sup>	6.3±0.33 <sup>a*</sup> <sup>a**</sup>	30.0±0.00 <sup>d*</sup> <sup>a**</sup>

Results represent Mean ± Standard Error Mean of triplicate determinations. Values with the same superscript on the same column are not significantly different at (P < 0.05). Values with the same subscript on the same row are not significantly different at (P < 0.05).

#### **4.1.3: Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)**

The result of MIC and MBC are shown in Table 4.3. *Escherichia coli* had the lowest MIC at 10 % and MBC at 20 % followed by *Bacillus subtilis* which had MIC at 80 % and MBC at 100 % while *Pseudomonas aeruginosa* had MIC at 100 % without the MBC.

**Table 4.3: The MIC and MBC of Honey on the Bacteria Isolates**

<b>Honey concentration (% v/v)</b>		
<b>Bacteria</b>	<b>MIC</b>	<b>MBC</b>
<i>Escherichia coli</i>	10	20
<i>Pseudomonas aeruginosa</i>	100	Nil
<i>Bacillus subtilis</i>	80	100

Results represent Mean ± Standard Error Mean of triplicate determinations. Values with the same superscript on the same column are not significantly different at (P < 0.05).

Values with the same subscript on the same row are not significantly different at ( $P < 0.05$ ).

#### 4.1.4: Catalase inhibition of honey on bacteria isolates

The result of catalase inhibition of honey on tested bacterial isolates after three hours of incubation at room temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) at respective MIC concentration of honey and control (untreated) were showed in Table 4. 4. The catalase activity of the untreated cells were higher than the treated. *E. coli* had  $11.0 \pm 0.6$  and  $14.0 \pm 0.6$  (mm); *B. subtilis* had  $30.0 \pm 1.1$  and  $40.0 \pm 1.1$  (mm) while *P. aeruginosa* had  $31.7 \pm 8.5$  and  $45.0 \pm 0.6$  (mm) for the treated and untreated respectively. *P. aeruginosa* had highest activity for both treated and untreated cells followed by *B. subtilis* while *E. coli* had the least. However, *B. subtilis* showed highest catalase inhibition upon treatment compared to other isolates.

**Table 4.4: Catalase Inhibition of honey on Bacterial Isolates**

Bacteria	Treated (mm)	Not treated (control) (mm)
<i>E. coli</i>	$11.0 \pm 0.58_{a^{*d}^{**}}$	$14.0 \pm 0.58_{b^{*d}^{**}}$
<i>B. subtilis</i>	$30.0 \pm 1.16_{a^{*b}^{**}}$	$40.0 \pm 1.16_{b^{*b}^{**}}$
<i>P. aeruginosa</i>	$31.7 \pm 8.51_{a^{*b}^{**}}$	$45.0 \pm 0.58_{a^{*c}^{**}}$

Results represent Mean  $\pm$  Standard Error Mean of triplicate determinations. Values with the same superscript on the same column are not significantly different at ( $P < 0.05$ ). Values with the same subscript on the same row are not significantly different at ( $P < 0.05$ ).

#### 4.1.5: The result of protein leakage activity of honey

The result of protein leaked by the test isolates after incubation at room temperature ( $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) at respective MIC concentration of honey were recorded as follows: *E. coli*

22.0±0.6 and 35.3±0.9 µg/ml; *B. subtilis* had 31.0±0.6 and 49.0±0.6 µg/ml while *P. aeruginosa* had 49.7±0.9 and 60.0±0.6 µg/ml at 1 hour and two hours' incubation respectively were showed in Table 4.5. The leakage of protein was time dependent for each organism and organism dependent.

**Table 4.5: The Protein Leakage (µg/ml) Activity of Honey**

<b>Bacteria</b>	<b>1 hour exposure</b>	<b>2 hours exposure</b>
<i>E. coli</i>	22.0±0.58 <sub>a</sub> <sup>*d*</sup>	35.3±0.88 <sub>b</sub> <sup>*d*</sup>
<i>B. subtilis</i>	31.0±0.58 <sub>a</sub> <sup>*b*</sup>	49.0±0.58 <sub>b</sub> <sup>*b*</sup>
<i>P. aeruginosa</i>	49.7±0.88 <sub>a</sub> <sup>*c*</sup>	60.0±0.58 <sub>b</sub> <sup>*c*</sup>

Results represent Mean ± Standard Error Mean of triplicate determinations. Values with the same superscript on the same column are not significantly different at (P < 0.05). Values with the same subscript on the same row are not significantly different at (P < 0.05)

## 4.2 Discussion

The result for phytochemical screening of the honey contained some bioactive compounds which possess good antimicrobial properties on the test isolates used in this study and therefore justified its aged long medicinal uses for curing purposes. These bioactive compounds; saponins, phenols, flavonoids, tannins, reducing sugars, anthraquinones cardiac glycosides and alkaloids were Similar to the results obtained by Elijah *et al.* (2015). Also, Nwankwo *et al.* (2014) in related study also confirmed the presence of alkaloids, flavonoids, saponins, steroids, reducing sugar and glycosides. Agbaje *et al.* (2006) equally attested to this fact that Alkaloids, anthraquinone, glycosides, cardiac glycosides, flavonoids, saponins, tannins, and reducing compounds may all be present in honey. The presence of these bioactive compounds were not exactly the same, which could be as result of nectar source, floral source, climatic factor and soil nutrients as reported by Elijah *et al.* (2015). Secondary metabolites like alkaloids, flavonoids, phenolic compounds may be responsible for various biological activities such as antioxidants and antimicrobials as reported by Mbah-omeje *et al.* (2017).

Ali *et al.* (2018) reported that flavonoids inhibit the activity of enzymes by forming complexes with bacterial cell walls, extracellular and soluble proteins, more lipophilic flavonoids disrupt cell wall integrity or microbial membranes at low concentrations. Saponins facilitated the entry of toxic material or leakage of vital constituents from the

cell (Ali *et al.*, 2018). Omojate *et al.* (2014) reported that phytochemicals have diverse antimicrobial mechanisms including damaging cell wall and cytoplasmic membrane.

The honey had activity on both Gram positive and Gram negative bacteria used in this analysis, according to the antimicrobial susceptibility test on the agar well diffusion. The zones of inhibitions were concentration dependent as reported by Olatunji *et al.* (2014). Among the bacterial tested, *Escherichia coli* had the highest zone of inhibition at all the various concentration used compared to *Pseudomonas aeruginosa* and *Bacillus subtilis*. Elijah *et al.* (2015), also recorded highest zone of inhibition for *E. coli* when compared to the *P. aeruginosa* at the same concentration (0.2 mL) of undiluted honey). Similar result was obtained by Raju and Goli (2013) for *E. coli*. In their study, *E. coli* is more sensitive to honey than *Pseudomonas*. Olatunji *et al.* (2015), Who worked on the in-vitro antimicrobial effect of different honey samples against selected microorganisms marketed in Abuja, Nigeria, reported that honey have activity against *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis* as reported in this presented study except one honey sample from one of the five markets that, *P. aeruginosa* showed resistant to. Kwakman *et al.* (2010) reported that honey had bactericidal activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* within 24 hours in the range of 10-40 percent honey concentration.

The result of the MICs and MBCs showed. *E. coli*, *B. subtilis* and *P. aeruginosa* had MIC at 10 %, 80 % and 100 % (v/v) concentration respectively. *E. coli* had the lowest MIC. Bunza *et al.* (2019) recorded similar MIC in their work on comparative evaluation of the antibacterial effects of honey with standard antibiotics on bacterial isolates from wound infection where *E. coli* had 20 % MIC and *P. aeruginosa* had 50 % MIC.

*E. coli* and *B. subtilis* had 20 % and 100 % MBC respectively while *P. aeruginosa* had no MBC. The honey sample used was bacteriostatic to *P. aeruginosa*. Bunza *et al.* (2019) had similar observation where *P. mirabilis*, which had 100 % MIC had no MBC as applicable to *P. aeruginosa* in this present research. Olatunji *et al.* (2015) also recorded resistance for *P. aeruginosa* in some of the honey he used. According to the investigation by Alemseged *et al.* (2018) on the antibacterial properties of mixture honey and garlic (*Allium sativum*) extracts against respiratory tract infection causing bacteria, they reported that honey was effective against all tested pathogenic organisms except *P. aeruginosa*.

The specific action of honey on enzymes, catalase of the bacterial isolates after 3 hours' treatment showed that honey inhibited exogenous enzymes, catalase for all the tested organisms. Wang *et al.* (2015) recorded similar result when they measured the catalase activity of *S. aureus* after treatment with antibiotics, vancomycin and ciprofloxacin.

The bacterial enzymes, catalase, the major weapon used by many aerobic bacteria to withstand oxidative stress by neutralize H<sub>2</sub>O<sub>2</sub> to water. H<sub>2</sub>O<sub>2</sub> had been the defense used by immune system to attack bacterial cell (Iwase *et al.*, 2013). For this investigated honey, the reduced catalase expression could help eliminate *P. aeruginosa*, *B. subtilis* and *E. coli* infection by reducing the organisms' ability to cope with oxidative stress. Catalase inhibition could be one of the specific action of honey against the bacteria.

Quantitatively, the amount of catalase released within the organisms differs. *P. aeruginosa* had highest catalase activity followed by *B. subtilis* while *E. coli* had the least. *P. aeruginosa*, which had higher catalase activity have been reported to have resistance to honey (Olatunji *et al.*, 2015). This might be the reason for its survival strategy. *B. subtilis* had been reported to cause nosocomial infection and opportunistic diseases as

reported by Saleh *et al.* (2014). *E. coli* which had lower catalase activity was more susceptible to honey.

The specific actions of honey on bacterial cells were determined by leakage of protein after bacterial cells have been exposed to honey at their respective MIC concentration over time. Protein leakages induced were time-dependent. The protein leaked after two hours were higher than the ones after one hour. The increment in protein leakage with time could be as result of more damage to the cell wall and membrane. This observation cuts across all the tested organisms used in this experiment. Kusumaningrum *et al.* (2014) made similar observation of different values of protein leaked by *E. coli* and *S. aureus* in their experiment. Nadial (2017) equally observed similar leakage difference in potassium leakage in *E. coli* and *S. aureus* where the value of potassium leaked increased with time. Also, Singh *et al.* (2016) made similar observation. In their study, they noticed a progressive protein leakage up to 5 hours after which the leakage decline as a result of protease, which led to protein degradation. *Pseudomonas aeruginosa* had higher protein leakage followed by *Bacillus subtilis* while *Escherichia coli* had the least. The observed lower protein leakage by *B. subtilis*, gram positive bacteria when compared to gram negative, *P. aeruginosa* could be due to the differences in the structural features of the cell wall, particularly the thickness of the peptidoglycan layer of Gram positive bacteria, which functions as a protective barrier against antibacterial agents (Yuan *et al.*, 2017). However, *E. coli*, which was the most sensitive organism in the study, had lower protein leakage. May be its sensitivity to honey may not be as a result of cellular leakage.

Protein leakage by all the isolates could be as a result of cytoplasmic membrane damage which led to cellular leakage of protein. Zakaria (2015) reported similar investigation when he checked the mechanism of action of honey on pathogenic wound bacterial strains. From this study, it could be deduced that honey can induce cellular leakage for

both gram negative and positive bacteria which may be possible specific actions of honey against bacterial pathogens.

The antimicrobial activity of honey was equally tested against fungi (*Trichophyton equinum* and *Trichophyton verricosum*) at 100 % concentration. *Trichophyton equinum* was more sensitive to honey than *Trichophyton verricosum* at the same concentration. Shariati *et al.* (2015) had similar report in their work on dermatophyte strains from 3 genera, *Trichophyton*, *Microsporum* and *Epidemophyton* by agar dilution technique method. These species of fungi are known to be causative agent of dermatophytes as reported by Shariati *et al.*, (2015). Dermatophytes are groups of hemogene keratinolytic fungi that have the ability to attack keratinized tissues of human and animals and cause dermatophytose infection that is a kind of colonization of fungi on skin (Shariati *et al.*, 2015).

## **CHAPTER FIVE**

### **5.0 CONCLUSION AND RECOMMENDATIONS**

#### **5.1 Conclusion**

It was observed that honey sampled from Kogi State, Nigeria had antimicrobial activity against the tested isolates. The presence of bioactive components like saponins, phenols, flavonoids, tannins, reducing sugars, anthraquinones cardiac glycosides and alkaloids may be responsible for its antimicrobial activity. Protein leakage and catalase inhibition were novel specific actions of honey on microbial pathogens. Once the bacterial cell wall structural integrity is compromised, the organism in question will be affected, likewise catalase inhibition. The organism's ability to withstand stress is impaired, hence it will either inhibited or died.

Therefore, it can be concluded that this honey could be used to treat diseases cause by these organisms.

#### **5.2 Recommendations**

The following are recommended for profitable use of honey and consideration:

- I. Honey should be considered as alternative antimicrobial agents for the treatment of skin diseases

- II. The antibacterial activity of honey should be tested in-vivo.
- III. Other mechanism of action of honey like protease inhibition and DNA leakage should be research into.
- IV. The shelf life of honey should be determined

## REFERENCES

- Abalaka, M.E., Daniyan, S.Y., Akpor, O.B. & Inyinbor, A.A. (2016). Antimicrobial, *In-vitro* Free Radical Scavenging, Antioxidant Properties of Leaf, Bark and Root Extracts from *Khaya senegalensis*. *UMYU Journal of Microbiology Research*, 1(1), 45-54.
- Abdallah, E. M. (2011), Plants: An alternative source for antimicrobials. *Journal of Applied Pharmaceutical Science*, 1 (6), 16-20.
- Abdulrhman, M., El-Hefnawy, M., Hussein, R. & El-Goud, A.A. (2011). The glyceimic and peak incremental indices of honey, sucrose and glucose in patients with type 1 diabetes mellitus: effects on C-peptide level-a pilot study. *Acta Diabetologica Journal*, 48(2), 89-94.
- Abeshu., M.A. & Geleta, B. (2016). Medicinal Uses of Honey. *Journal of Biology and Medicine*, 8(2), 1-7.
- Adams, C. J., Boulton, C. H., Deadman, B. J., Farr, J. M., Grainger, M. N., Manley-Harris, M., & Snow, M. J. (2008). Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, 343(4), 651-659.
- Adams, C. J., Manley-Harris, M., & Molan, P. C. (2009). The origin of methylglyoxal in New Zealand manuka (*Leptospermum scoparium*) honey. *Carbohydrate Research*, 344(8), 1050-1053.
- Adebiyi, F.M., Akpan, I., Obianjuwa, E.I. & Olaniyi, H.B. (2004). Chemical/physical characterization of Nigerian honey. *Pakistan Journal of Nutrition*, 3 (5), 278 - 81.
- Adesoji, F. & Oluwakemi, A. (2008). Differential effect of honey on selected variables in alloxan-induced and fructose-induced diabetic rats. *African Journal of Biomedicine Research*, 11(2), 191-196.

- Agbaje, E.O., Ogunsanya, T. & Aiwerioba, O.I.R. (2006). Conventional Use of Honey as Antibacterial Agent. *Annals of African Medicine*, 5, (2), 78 – 81.
- Agyare, C., Asase, A., Lechtenberg, M., Niehues, M., Deters, A., & Hensel, A. (2009). An ethnopharmacological survey and in vitro confirmation of ethnopharmacological use of medicinal plants used for wound healing in Bosomtwi-Atwima-Kwanwoma area, Ghana. *Journal of Ethnopharmacology*, 125(3), 393-403.
- Ahmadi-Motamayel, F., Hendi, S.S., Alikhani, M.Y. & Khamverdi, Z. (2013). Antibacterial activity of honey on cariogenic bacteria. *Journal of Dentistry, (Tehran)*. 10(1), 10–15.
- Ajibola, A. (2015). Novel Insights into the Health Importance of Natural Honey. *Malays Journal of Medical Science*, 22(5), 7-22.
- Ajibola, A., Idowu, G. O., Amballi, A. A., Oyefuga, O. H., & Iquot, I. S. (2007). Improvement of some haematological parameters in albino rats with pure natural honey. *Journal of Biological Sciences Resources*, 2(1), 67-69.
- Akujobi, C.O. & Njoku, H.O. (2010). Bioassay for the determination of microbial sensitivity to Nigerian honey. *Global Journal of Pharmacology*, 4 (1), 36-40.
- Alemseged, M., Adugna, S. & Bayu, E. (2018). Antibacterial Properties of Mixture Honey and Garlic (*Allium sativum*) Extracts Against Respiratory Tract Infection Causing Bacteria. *Journal of Pharmacovigilance*, 6(4), 1-4.
- Ali, M., Umar, A. & Nas. F.S. (2018). Antibacterial Efficacy of *Carica Papaya* Leaves and Stem Bark Extracts on Clinical Isolates of Methicillin Resistant *Staphylococcus Aureus* (MRSA). *Bioequivalence & Bioavailability International Journal*, 2(2), 1-8.
- Allen, K.L., Hutchison, G. & Molan, P.C. (2000). The potential for using honey to treat wounds infected with MRSA and VRE. *First World Healing Congress, Melbourne, Australia, and Medical Research*, 3(2), 113-118.
- Almeida-Muradian, L.B., Stramm, K.M. & Estevinho, L.M. (2014). Efficiency of the FT-IR ATR spectrometry for the prediction of the physiochemical characteristics of *M. elipona subnitida* honey and study of the temperature's effect on those properties. *International Journal of Food Sciences & Technology*, 49(1), 188-195.
- Al-Naama, R. T. (2009). Evaluation of in-vitro inhibitory effect of honey on some microbial isolate. *African Journal of Bacteriology Research*, 1 (6), 64-67.
- Al-Waili, N. S. (2003). Effects of daily consumption of honey solution on hematological indices and blood levels of minerals and enzymes in normal individuals. *Journal of Medicinal Food*, 6 (2), 135-140.

- Al-Waili, N. S., & Haq, A. (2004). Effect of honey on antibody production against thymus-dependent and thymus-independent antigens in primary and secondary immune responses. *Journal of medicinal food*, 7(4), 491-494.
- Al-Waili, N., Salom, K., & Al-Ghamdi, A. A. (2011). Honey for wound healing, ulcers, and burns; data supporting its use in clinical practice. *Journal of Scientific World*, 11, 766–787.
- Ang-Lee, M. K., Moss, J., & Yuan, C. S. (2001). Herbal medicines and perioperative care. *Journal of the American Medical Association*, 286(2), 208-216
- Anyanwu, C.U. (2012). Investigation of *in vitro* antifungal activity of honey. *Journal of Medicinal Plants Research*, 6(18), 3512-3516.
- Astal E.L., Ashour, Z.Y. & Kerrit, A.A.M. (2005). Antimicrobial activity of some medicinal plant extracts in Palestine. *Pakistan Journal of Medical Sciences*, 21(2), 187-193.
- Astin, J. A. (1998). Why patients use alternative medicine: results of a national study. *Journal of the American Medical Association*, 279 (19), 1548-1553.
- Attah, F., Otalù, O., Cletus, A.U, Otuoze, V.O & Garba D.E. (2020). Use of Medicinal Plants as Antimicrobial Agents: A Review of the Successes Story. *International Journal of Medicinal Plants and Natural Products (IJMPNP)*, 6(3), 28-34.
- Attah F., Abalaka M.E., Adobu U.S., Mamman G.P., and Apeh T.A. (2021). A Review of All-Inclusive Therapeutic Potency of Pure Honey: A New Hope for Antibiotics Alternative. *European Journal of Pharmaceutical and Medical Research*, 8(1), 18-25.
- Atwa, A.A., AbuShahba, R.Y., Mostafa, M. & Mohamed, I.H. (2014). Effect of honey in preventing gingivitis and dental caries in patients undergoing orthodontic treatment. *Saudi Dental Journal*, 26(3), 108–114.
- Baig, S. & Attique, H. (2014). Cytotoxic activity of honey in hepatoma cells: in vitro evaluation. *Pakistan Journal of Medicine and Dentistry*, 3, 12-16.
- Bang, L. M., Bunting, C., & Molan, P. (2003). The effect of dilution on the rate of hydrogen peroxide production in honey and its implications for wound healing. *The Journal of Alternative & Complementary Medicine*, 9(2), 267-273.
- Bashkaran, K., Zunaina, E., Bakiah, S., Sulaiman, S.A., Sirajudeen, K. & Naik, V. (2011). Anti-inflammatory and antioxidant effects of Tualang honey in alkali injury on the eyes of rabbits: experimental animal study. *BMC Complement. Alternative Medicine*, 11(1), 90.
- Bashua, O.C.& Oladunmoye, M.K. (2017). Antimicrobial activities of the indigenous black soap fortified with honey on some selected skin pathogens. *American Journal of Research Communication*, 5(10), 28-64.
- Bertrams, J., Kunz, N., Müller, M., Kammerer, D., & Stintzing, F. C. (2013). Phenolic compounds as marker compounds for botanical origin determination of German

- propolis samples based on TLC and TLC-MS. *Journal of Applied Botany and Food Quality*, 86(1), 143-153.
- Blair, S.E., Cokcetin, N.N., Perry, E.J. & Carter, D.A. (2009). The unusual antibacterial activity of medical-grade *Leptospermum* honey: antibacterial spectrum, resistance and transcriptome analysis. *European Journal of Microbiology and Infectious Diseases*, 28, 1199-1208.
- Bogdanov, S. (2006). Contaminants of bee products. *Apidologie*, 37(1), 1-18.
- Boorn, K L., Khor, Y.Y., Sweetman, E., Tan, F. Heard, T.A. & Hammer, K.A. (2010). Antimicrobial activity of honey from the stingless bee *Trigonacarbonaria* determined by agar diffusion, agar dilution, broth microdilution and time-kill methodology. *Journal of Applied Microbiology*, 108 (5), 1534-1543.
- Borsato, D. M., Prudente, A. S., Döll-Boscardin, P. M., Borsato, A. V., Luz, C. F., Maia, B. H. & Miguel, O. G. (2014). Topical anti-inflammatory activity of a monofloral honey of *Mimosa scabrella* provided by *Melipona marginata* during winter in Southern Brazil. *Journal of Medicinal Food*, 17(7), 817-825.
- Bostan, M., Bostan, H., Kaya, A. O., Bilir. O., Satiroglu, O., Kazdal, H., Karadag, Z. & Bozkurt, E. (2010). Clinical Events in Mad Honey Poisoning: A Single Centre Experience. *Bulletin of Environmental Contamination and Toxicology*, 84 (1), 19-22.
- Brudzynski, K. (2006). Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. *Canadian Journal of Microbiology*, 52, 1228-1237.
- Bruni, I. Galimberti, A., Caridi, L., Scaccabarozzi, D., De Mattia, F., Casiraghi, M., & Labra, M. (2015). A DNA barcoding approach to identify plant species in multiflower honey. *Food Chemistry*, 170, 308-315.
- Buba, F., Gidado, A. & Shugaba, A. (2013). Physicochemical and microbiological properties of honey from North East Nigeria. *Biochemistry and Analytical Biochemistry*, 2(142), 61-67.
- Bunza, N.M., Kumurya, A.S., Isah, A.A, AbdulAzeez, B.O, Nafiu, F. (2019). Comparative Evaluation of the Antibacterial Effects of Honey with standard antibiotics on bacterial isolates from wound infection. *Asian Journal of Research in Medical and Pharmaceutical Sciences*, 7(1), 1-7.
- Carter, C., & Thornburg, R. W. (2004). Is the nectar redox cycle a floral defence against microbial attack? *Trends in plant science*, 9(7), 320-324.
- Chepulis, L. M. (2007). The effects of honey compared with sucrose and a sugar-free diet on neutrophil phagocytosis and lymphocyte numbers after long-term feeding in rats. *Journal of Complementary and Integrative Medicine*, 4(1), 1-9.

- Cooper, R.A., Molan, P.C. & Harding, K.G. (2002). The sensitivity to honey of Gram-positive *cocci* of clinical significance isolated from wounds. *Journal of Applied Microbiology*, 93, 857-863.
- Cornara, L., Biagi, M., Xiao, J. & Burlando, J. (2017). Therapeutic Properties of Bioactive Compounds from Different Honeybee Products. *Journal of Frontiers in Pharmacology*, 8(142), 1-20.
- Cowan, M.M. (1999). Plant products as antimicrobial agents, *Clinical Microbiology Review*. 12(4), 564-582.
- Deshpande, A. R., Mohd, S. P., Banole, A. M., & Patil, D. P. (2005). Screening of some plant extracts for antibacterial activity. *Asian Journal of Microbiology Biotechnology and Environmental Sciences*, 7(4), 755-758.
- Dixon, R. A., & Pasinetti, G. M. (2010). Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience. *Plant Physiology*, 154(2), 453-457
- Drain, J. & Fleming, M.O. (2015). Palliative management of malodorous squamous cell carcinoma of the oral cavity with Manuka honey. *Journal of Wound, Ostomy and Continence Nursing*, 42(2), 190–192.
- Dureja, H., Kaushik, H. & Kumar, V. (2003). Development in Nutraceuticals. *Indian Journal of Pharmacology*, 35, 363-372.
- Edgar, J. A., Roeder, E. L. & Molyneux, R. J. (2002). Honey from Plants Containing Pyrrolizidine Alkaloids: A Potential Threat to Health. *Journal of Agricultural and Food Chemistry*, 50 (10), 2719-2730.
- Eisenberg, D. M., Davis, R. B., Ettner, S. L., Appel, S., Wilkey, S., Van Rompay, M., & Kessler, R. C. (1998). Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *Journal of the American Medical Association*, 280 (18), 1569-1575
- Eleazu, C.O., Iroaganachi, M.A., Eleazu, K.C. & Okoronkwo, J.O. (2013). Determination of the physico-chemical composition, microbial quality and free radical scavenging activities of some commercially sold honey samples in Aba, Nigeria: The effect of varying colours. *International Journal of Biomedical Research*, 4 (1), 32-41.
- Elijah, M.I., Imohiosen, O., Lamidi, B.T., & Umar, M.S. (2015). Biochemical screening of pure honey and its antibacterial activity on some bacterial isolates compared with common antibiotics. *International Journal of Pharmaceutical Science Invention*, 4 (5), 15-20.
- English, H.K., Pack, A.R. & Molan, P.C. (2004). The effects of manuka honey on plaque and gingivitis: A pilot study. *Journal of International Academy of Periodontology*, 6(2), 63–67.

- Erstad, B. L. (2003). Osmolality and osmolarity: narrowing the terminology gap. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 23(9), 1085-1086.
- Eteraf-Oskouei, T. & Najafi, M. (2013). Traditional and modern uses of natural honey in human diseases: A review. *Iran Journal of Basic Medical Science*, 16(6), 731–742.
- Fahim, H., Dasti, J.I., Ali, I., Ahmed, S. & Nadeem, M. (2014). Physico-chemical analysis and antimicrobial potential of *Apis dorsata*, *Apis mellifera* and *Ziziphus jujube* honey samples from Pakistan. *Asian Pacific Journal of Tropical Biomedicine*, 4(8), 633-641.
- Fauzi, A.N., Norazmi, M.N. & Yaacob, N.S. (2011). Tualang honey induces apoptosis and disrupts the mitochondrial membrane potential of human breast and cervical cancer cell lines. *Journal of Food Chemistry and Toxicology*, 49(4), 871–878.
- Galimberti, A., De Mattia, F., Bruni, I., Scaccabarozzi, D., Sandionigi, A., Barbuto, M. & Labra, M. (2014). A DNA barcoding approach to characterize pollen collected by honeybees. *PloS one*, 9(10), e109363.
- Genersch, E. (2010) American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology*, 103, S10–S19.
- Ghosh, S., & Playford, R. J. (2003). Bioactive natural compounds for the treatment of gastrointestinal disorders. *Clinical Science*, 104(6), 547-556.
- Gomes, S., Dias, L.G., Moreira, L.L., Rodrigues, P., & Estevinho, L. (2010). Physicochemical, microbiological, and antimicrobial properties of commercial honeys from Portugal. *Food and Chemical Toxicology*, 48, 2, 544-548.
- González-Teuber, M., Eilmus, S., Muck, A., Svatos, A., & Heil, M. (2009). Pathogenesis-related proteins protect extrafloral nectar from microbial infestation. *The Plant Journal*, 58(3), 464-473.
- Grover, J. K., Yadav, S., & Vats, V. (2002). Medicinal plants of India with anti-diabetic potential. *Journal of Ethnopharmacology*, 81 (1), 81-100.
- Gupta, S. S. (1994). Prospects and perspectives of natural plant products in medicine. *Indian Journal of Pharmacology*, 26 (1), 1-12.
- Hadjmohammadi, M.R. & Nazari, S.S. (2010). Separation optimization of quercetin, hesperetin and chrysin in honey by micellar liquid chromatography and experimental design. *Journal of Separation Science*, 33(20), 3144-3151.
- Haile, G., Tola, N., Mekonnen, N. & Furgassa, W. (2017). Review on Medicinal and Nutritional Value of Bee's Honey: Senior Seminar on Animal Health. *Journal of Biomedicine and Nursing*, 3(1), 58-67.

- Haron, M.N., Rahman, W.F.W.A., Sulaiman, S.A. & Mohamed, M. (2014). Tualang honey ameliorates restraint stress-induced impaired pregnancy outcomes in rats. *Euroepian Journal of Integrated Medicine*, 6, 657–663.
- Hassanein, S.M., Gebreel, H.M. & Hassan, A.A. (2010). Honey Compared with Some Antibiotics against Bacteria Isolated from Burn-wound Infections of Patients in Ain Shams University Hospital. *Journal of American Science*, 6(10), 301-320.
- Hawley, P., Hovan, A., McGahan, C.E. & Saunders, D. (2014). A randomized placebo-controlled trial of manuka honey for radiation-induced oral mucositis. *Journal of Supportive Care in Cancer*, 22(3), 751–761.
- Henriques, A.F., Jenkins, R.E., Burton, N.F. & Cooper, R.A. (2011). The effect of manuka honey on the structure of *Pseudomonas aeruginosa*. *European Journal of Clinical Microbiology & Infectious Diseases*, 30, 167-171.
- Hillwig, M. S., Liu, X., Liu, G., Thornburg, R. W. & MacIntosh, G. C. (2010). Petunia nectar proteins have ribonuclease activity. *Journal of Experimental Botany*, 61(11), 2951-2965.
- Ibrahim, H., & Aliyu, A. (2015). The antimicrobial activity of honey on bacterial isolates from burns/wounds of patients attending General Hospital, Ankpa, Kogi, Nigeria. *Advances in Life Science and Technology*, 38(6), 234-240.
- Irish, J., Carter, D. A., Blair, S. E., & Heard, T. A. (2008). Antibacterial activity of honey from the Australian stingless bee *Trigonacarbonaria*. *International Journal of Antimicrobial Agents*, 32 (1), 89-90.
- Islam, M. N., Khalil, M. I., Islam, M. A. & Gan, S. H. (2014). Toxic Compounds in Honey. *Journal of Applied Toxicology*, 34 (7), 733-742.
- Iwase, T., Tajima, A., Sugimoto, S., Okuda, K. I., Hironaka, I., Kamata, Y. & Mizunoe, Y. (2013). A simple assay for measuring catalase activity: a visual approach. *Scientific Reports*, 3, 3081.
- Jayashree, D. (2013). Phytochemical analysis and TLC fingerprinting of methanolic extracts three medicinal plants. *International Research Journal of Pharmacy*, 4(6), 1-4.
- Jenkins, R.E. & Cooper, R. (2012). Synergy between oxacillin and manuka honey sensitizes methicillin-resistant *Staphylococcus aureus* to oxacillin. *Journal of Antimicrobial Chemotherapy*, 67, 1405-1407.
- Jerković, I., Mastelić, J., & Marijanović, Z. (2011). A variety of volatile compounds as markers in unifloral honey from Dalmatian sage (*Salvia officinalis* L.). *Chemistry & Biodiversity*, 3 (12), 1307-1316.
- Jessup, C.J., Warner, J, Isham, N., Hasan, I. & Ghannoum, M.A. (2000), Antifungal Susceptibility Testing of Dermatophytes: Establishing a Medium for Inducing Conidial Growth and Evaluation of Susceptibility of Clinical Isolates. *Journal of Clinical Microbiology*, 38(1), 341–344.

- Ji, H. F, Li, X.J. & Zhang, H.Y. (2009). Natural Products and Drug Discovery. *EMBO Reports*. 10(3), 194-200.
- Jindal A., & Vashist H. (2013). Antimicrobial Activities of Medicinal Plants-Review. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 3(1), 222-230.
- Kacaniova, M., Fatrcova-Sramkova, K., Nozkova, J., Melich, M., Kadasi-Horakova, M., Knazovicka, V., Felsociova, S., Kunova, S. & Mariassyova, M. (2011). Antiradical Activity of Natural Honeys and Activity of Turkish Honey Against *Candida Spp.* And *Trichosporon Spp.*: An In Vitro Evaluation. *Medical Mycology*, 47, (7), 707-712.
- Kaškonienė, V., & Venskutonis, P. R. (2010). Floral markers in honey of various botanical and geographic origins: A Review. *Comprehensive Reviews in Food Science and Food Safety*, 9(6), 620-634.
- Kaur, S., Mirza, A. & Singh, J. (2017). Recent advances of honey in modern medicines: A review. *Journal of Pharmacognosy and Phytochemistry*, 6(4), 2063-2067.
- Khandal, R. K., Chauhan, A., Pandey, V. & Chacko, K.M. (2010). Antibacterial Activity of Raw and Processed Honey. *Electronic Journal of Biology*, 5(3), 58-66.
- Kliger I.J. (1917). A Simple Medium for the Differentiation of Members of the Typhoid Paratyphoid Group. *American Journal of Public Health*, 7, 1042-1044
- Kingsley, A. (2001). The use of honey in the treatment of infected wound. *British Journal of Nursing*, 10(22Suppl.), S13-6, S18 and S20.
- Klaudiny, J., Albert, Š., Bachanová, K., Kopernický, J. & Šimúth, J. (2005). Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera*. *Insect Biochemistry and Molecular Biology*, 35 (1), 11-22.
- Koc, A.N., Silici, S., Ercal, B.D., Kasap, F., Hormet-Oz, H.T. & Mavus-Buldu, H. (2009). Antifungal Activity of Turkish Honey Against *Candida Spp.* And *Trichosporon Spp.*: An In Vitro Evaluation. *Medical Mycology*, 47(7), 707-712.
- Krishnan, S.S. (2018). Traditional herbal medicines – A review. *International Journal of Research and Analytical Reviews (IJRAR)*, 5(4), 610-614.
- Kumarasamy, D. & Mahendran, S. (2015). Antimicrobial activity of some honey samples against pathogenic bacteria. *International Letters of Natural Sciences*, 7, 15-20.
- Kustiawan, P.M., Puthong, S., Arung, E. & Chanchao, C. (2014). In vitro cytotoxicity of Indonesian stingless bee products against human cancer cell lines. *Asian Pacific Journal of Tropical Medicine*, 4(7), 549–556.
- Kusumaningrum, H. D., Imelda, F. & Faridah, D. N. (2014). Bacterial inhibition and cell leakage by extract of *Polygonum minus Huds.* Leaves. *International Food Research Journal*, 21(2), 553-560.

- Kwakman, P. H. S., Te Velde, A. A., de Boer, L., Vandenbroucke-Grauls, C. M. J. E. & Zaat, S. A. J. (2011a). Two major medicinal honeys have different mechanisms of bactericidal activity. *PLoS One*, 6(3), e17709.
- Kwakman, P. H., de Boer, L., Ruyter-Spira, C. P., Creemers-Molenaar, T., Helsper, J. P. F. G., Vandenbroucke-Grauls, C. M. J. E. & TeVelde, A. A. (2011b). Medical-grade honey enriched with antimicrobial peptides has enhanced activity against antibiotic-resistant pathogens. *European Journal of Clinical Microbiology & Infectious Diseases*, 30(2), 251-257.
- Kwakman, P. H., teVelde, A. A., de Boer, L., Speijer, D., Vandenbroucke-Grauls, C. M., & Zaat, S. A. (2010). How honey kills bacteria. *The Federation of America Societies for Experimental Biology Journal*, 24(7), 2576-2582.
- Li, X. A., Huang, C., & Xue, Y. (2013). Contribution of lipids in honeybee (*Apis mellifera*) royal jelly to health. *Journal of Medicinal Food*, 16(2), 96-102.
- Lin, S.M., Molan, P.C. & Cursons, R.T. (2009). The in vitro susceptibility of *Campylobacter* spp to the antibacterial effect of manuka honey. *European Journal of Clinical Microbiology and Infectious Diseases*, 28(4), 339-344.
- Mahato T.K., & Sharma K. (2018). Study of Medicinal Herbs and Its Antibacterial Activity: A Review. *Journal of Drug Delivery & Therapeutics*. 8(5), 47-54.
- Majtan, J., Kumar, P., Majtan, T., Walls, A. F. & Kludiny, J. (2010). Effect of honey and its major royal jelly protein 1 on cytokine and MMP-9 mRNA transcripts in human keratinocyt. *Experimental Journal of Dermatology*. 19(8), 73-79.
- Malik, K.I., Malik, M.A.N., & Aslam, A. (2010). Honey compared with silver sulphadiazine in partial-thickness burns. *International Wound Journal*, 7, 413-417.
- Mandal, M.D. & Mandal, S. (2011). Honey: its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine*, 1, 154-160.
- Mandal, S., Roy, S., Mandal, M., Pal, N.K., Das, M.K., Halder, D. & Sircar, B. (2016). Exploration of Antibacterial and Antioxidative Property of Two Natural Honey Samples from Malda District, India. *Open-access Journal*, 6(4), 1-5.
- Manyi-Loh, C. E., Clarke, A. M., & Ndip, N. (2011). An overview of honey: therapeutic properties and contribution in nutrition and human health. *African Journal of Microbiology Research*, 5 (8), 844-852.
- Mato, I., Huidobro, J.F., Simal Lozano, J. & Sancho, M.T. (2003). Significance of non-aromatic organic acids in honey. *Journal of Food Protection*, 66(12), 2371-2376.
- Mbah-omeje, K. N., Ugwu, C. C., Ezugwu, R. I. & Iloputaife E. J. (2017). Antimicrobial and Phytochemical Studies of *Carica Papaya* Leaves Against *Escherichia Coli* and *Staphylococcus aureus*. *World Journal of Pharmacy and Pharmaceutical Sciences*, 7 (1), 180-189.

- McChesney, J.D., Venkataraman, S.K. & Henri, J.T. (2007). Plant natural products: Back to the future or into extinction? *Journal of Phytochemistry*, 68, 2015-2022.
- Misirlioglu, A., Eroglu, S., Karacaoglan, N., Akan, M., Akoz, T. & Yildirim, S. (2003). The use of honey as an adjunct in the healing of split-thickness skin graft donor site. *Journal of Dermatologic Surgery*, 29 (2), 168-172.
- Mohammed, M., Sulaiman, S.A. & Sirajudeen, K.N.S. (2013). Protective effect of honey against cigarette smoke induced-impaired sexual behavior and fertility of male rats. *Journal of Toxicology and Industrial Health*, 29 (3), 264–271.
- Mohammed, S.E. & Babiker, E.E. (2009). Protein Structure, Physicochemical Properties and Mineral Composition of *Apis Mellifera* Honey Samples of Different Floral Origin. *Australian Journal of Basic and Applied Sciences*, 3, 2477-2483.
- Mohapatra, D.P., Thakur, V. & Brar, S.K. (2011). Antibacterial efficacy of raw and processed honey. *Biotechnology Research International Journal*, 6, 1-6.
- Mohd, M., Taqi A.K. & Firoz, M. (2012). Medicinal Plants of Rural India: A Review of Use by Indian Folks. *Indo Global Journal of Pharmaceutical Sciences*, 2(3), 286-304
- Molan, P. (2001). Why honey is effective as a medicine: The scientific explanation of its effects. *Bee world*, 82 (1), 22-40.
- Molan, P. C. (1999). Why honey is effective as a medicine. I. Its use in modern medicine. *Journal of Bee World*, 80(2), 80–92.
- Morais, M., Moreira, L., Feás, X., & Estevinho, L. M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity. *Food and Chemical Toxicology*, 49(5), 1096-1101.
- Mosavat, M., Ooi, F.K. & Mohamed, M. (2014). Effects of honey supplementation combined with different jumping exercise intensities on bone mass, serum bone metabolism markers and gonadotropins in female rats. *BMC Complement. Alternative Medicine*, 14(126), 1-8.
- Mundo, M. A., Padilla-Zakour, O. I., & Worobo, R. W. (2004). Growth inhibition of foodborne pathogens and food spoilage organisms by select raw honeys. *International Journal of Food Microbiology*, 97 (1), 1-8.
- Nadia, H.E. (2017). Antimicrobial activities and mode of action of the selected novel thienopyrimidines derivatives, 2-[2-(diphenylmethylene) hydrazino]-5-isopropyl-3-methylthieno [2, 3-d] pyrimidin-4-one. *Periodicum Biologorum*, 119 (1), 27–36.
- Nair, R., Kalariya, T. & Chanda, S. (2005). Antibacterial activity of some selected Indian medicinal flora. *Turkish Journal of Biology*, 29(1), 41-47.

- Nautiyal, C. S., Chauhan, P. S., & Nene, Y. L. (2007). Medicinal smoke reduces airborne bacteria. *Journal of Ethnopharmacology*, 114 (3), 446-451.
- Nayik, G.A., Shah, T.R., Muzaffar, K., Wani, S.A., Gull, A., Majid, I. & Bhat, F.M. (2014). Honey: its history and religious significance: A Review. *Universal Journal of Pharmacy*, 3(1), 1-5.
- Newman, D. J., Cragg, G. M., & Snader, K. M. (2000). The influence of natural products upon drug discovery. *Natural Product Reports*, 17(3), 215-234.
- Nwankwo, C.M., Ezekoye, C.C. & Igbokwe, S.O. (2014). Phytochemical screening and antimicrobial activity of Apiary honey produced by honey (*Apis mellifera*) onclinical strains of *Staphylococcus aureus*, *Escherichal Coli* and *Candidas Albican*. *Journal of Biotechnology*, 13(23), 2367-2372.
- Odiba, J.O., Musa, A.M. Hassan, H.S. & Yahaya, S.M. (2014). *In-vitro* free radical scavenging and antimicrobial activity of honeybee propolis extracts from North-Western, Nigeria. *Journal of Natural Products Plant Resources*, 4 (6), 19-24.
- Olajuyigbe, O.O., Adeoye-Isijola, O.M., Osopale, A.B., Nwankwo, A. & Adedayo, O. (2017). Antibacterial Activities of Selected Samples of Honey in South-Western Nigeria against Bacteria in Wound Infections. *International Journal of Sciences*, 6(9), 1-8
- Olakunle, T.P., Akinro, E.B., Agbolade, J.O., Yoyinoye, A.S. Ghazali, M.A. & Oladeji, S.S. (2013). Inhibitory Effect of Pure Honey on Microorganisms Isolated from Wound. *Journal of Environmental Science, Toxicology and Food Technology*, 3(3), 80-84.
- Olatunji, K. T., Aboh, M. & Oladosu, P. (2018). In-Vitro Antimicrobial Effect of Different Honey Samples against Selected Microorganisms Marketed in Abuja Nigeria, *Journal of Applied Life Sciences International*, 19(1), 1-7.
- Omafuvbe, B.O. & Akanbi, O.O. (2009). Microbiological and physico-chemical properties of some commercial Nigerian honey. *African Journal of Microbiology Research*, 3(12), 891-896.
- Omojate, G.C., Enwa, F.O., Jewo, A.O. & Eze, C.O. (2014). Mechanisms of antimicrobial actions of phytochemicals against enteric pathogens a review. *Journal of Pharmaceutical, Chemical and Biological Sciences*, 2, 77-85.
- Opal, S.M., Mayer, K. & Medeiros, A. (2000). Mechanisms of Bacterial Antibiotic Resistance. *Principles and Practice of Infectious ieseases 5th ed. Ch. 16 (eds. Mandell, G. L., Bennett, J. & Dolin, R.)*. Churchill Livingstone, Philadelphia, USA, 236–253.
- Ortega-Ramirez, L.A., Rodriguez., G.I, Leyva, J.M., Cruz-Valenzuela, M.R., Silva-Espinoza, B.A. & Gonzalez-Aguilar, G.A, (2014). Potential of medicinal plants as antimicrobial and antioxidant agents in food industry: a hypothesis. *Journal of Food Sciences*, 79(2),129-37.

- Osato, M.S., Reddy, S.G. & Graham, D.Y. (1999). Osmotic effect of honey on growth and viability of *Helicobacter pylori*. *Journal of Digestive Disease and Science*, 44(3), 462-464.
- Parkash, J., Prasad, D.N., Shahnaz M. & Dev. D. (2018). Herbs as Traditional Medicines: a Review. *Journal of Drug Delivery & Therapeutics*, 8(5), 146-150.
- Parmar N. & Rawat M. (2012). Medicinal plants used as antimicrobial agents: a review. *International Research Journal of Pharmacy*, 3(1), 31-40.
- Paulus. H. S., Kwakman & Sebastian, A. J. Zaat. (2012). Antibacterial Components of Honey: Critical Review. *International Union of Biochemistry and Molecular Biology Life*, 64(1), 48–55
- Peter. B.O, Olufemi. E.A. & Iyabo. O.O. (2007). African Health Sciences; Honey; a reservoir of microorganisms and inhibitory agent for microbes. *Journal of Africa Health Science*, 7(3), 159 – 165.
- Pinho, E., Ferreira, I. C., Barros, L., Carvalho, A. M., Soares, G., & Henriques, M. (2014). Antibacterial potential of northeastern Portugal wild plant extracts and respective phenolic compounds. *Biomedicine Research International*, 81,45-90.
- Poonkothai, M.S., HemaIswarya, D., Kavitha. & Vallikannu. (2005). Antibacterial activity of the leaves of *Galinsoga ciliate*. *Asian Journal of Microbiology, Biotechnology and Environmental Science*, 7, 747- 748.
- Prasad, P., Kumar, P.P., Satyavathi, K. & Prabhakar, M.C. (2010). Comparative studies onanthelminthic activity of natural sweeteners. *Research. Journal of Pharmaceutical Biological and Chemical Sciences*, 1(3), 510–514.
- Raju, E.V. N. & Goli, D. (2013). *In-vitro* comparative antimicrobial activity of commercial and raw honey against various bacteria isolated from ear discharge. *Scholars Academic Journal of Pharmacy (SAJP)*, 2(1), 5-11.
- Rakhi, K., Chute, N.G. & Deogade, M.K. (2010). Antimicrobial activity of Indian honey against clinical isolates. *Asiatic Journal of Biotechnology Resources*, 1, 35-38.
- Ramadan, M. F., & Al-Ghamdi, A. (2012). Bioactive compounds and health-promoting properties of royal jelly: A review. *Journal of functional foods*, 4 (1), 39-52.
- Ramasamy. S. & Manoharan, C. (2004). Antibacterial effect of volatile components of selected medicinal plants against human pathogens. *Asian Journal of Microbiology, Biotechnology and Environmental Science*, 6, 209-210.
- Ranzato, E., Martinotti, S. & Burlando, B. (2012). Epithelial mesenchymal transition traits in honey-driven keratinocyte wound healing: comparison among different honeys. *Wound and Repair Regeneration*, 20(5), 778–785.

- Rao, P. V., Krishnan, K. T., Salleh, N., & Praveen, Gan, S. H. (2016). Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review. *Revista Brasileira de Farmacognosia*, 26(5), 657-664.
- Ratnieks, F. L., & Carreck, N. L. (2010). Clarity on honey bee collapse? *Science*, 327 (5962), 152-153.
- Rojas, R., Bustamante, B., Bauer, J., Fernández, I., Albán, J., & Lock, O. (2003). Antimicrobial activity of selected Peruvian medicinal plants. *Journal of Ethnopharmacology*, 88 (2-3), 199-204.
- Rout, S. P., Chowdary, K. A., Kar, D. M., & Das, L. (2009). Plants as source of novel anti-diabetic drug: present scenario and future perspectives. *Current Trends Biotechnology and Pharmacy*, 3(1), 37-55.
- Saad, B., Azaizeh, H., & Said, O. (2005). Tradition and perspectives of Arab herbal medicine: a review. *Evidence-Based Complementary and Alternative Medicine*, 2(4), 475-479.
- Saleh, F. Kheirandish, F., & Azizi, H. M., (2014). Molecular Characterisation of *Bacillus subtilis* Isolated from Burn and Wound in Iran. *Journal of Research Molecular Medicine*, 2(2), 40-44.
- Sajid, M. & Kamran, M. (2012). Characterization of the Nematicidal Activity of Natural Honey. J. Anthelmintic Activity of Natural Sweeteners. *Research Journal of Pharmaceutical, Biological and Agric. Food Chemistry*, 60(30), 7428–7434.
- Saranraj, P. & Sivasakthi. S. (2018). Comprehensive Review on Honey: Biochemical and Medicinal Properties. *Journal of Academia and Industrial Research (JAIR)*, 6(10), 165-178.
- Savant. C.V, Mannasaheb, B.A. & Joshi, H. (2014). Importance of Antimicrobial Agents from Plants in Present Scenario: A Review. *International Journal of Pharmacognosy*, 1(8). 472-484.
- Schley, P. D., & Field, C. J. (2002). The immune-enhancing effects of dietary fibres and prebiotics. *British Journal of Nutrition*, 87(S2), S221-S230.
- Schramm, D. D., Karim, M., Schrader, H. R., Holt, R. R., Cardetti, M., & Keen, C. L. (2003). Honey with high levels of antioxidants can provide protection to healthy human subjects. *Journal of Agricultural and Food Chemistry*, 51(6), 1732-1735.
- Seow, Y. X., Yeo, C. R., Chung, H. L., & Yuk, H. G. (2014). Plant essential oils as active antimicrobial agents. *Critical Reviews in Food Science and Nutrition*, 54 (5), 625-644.
- Shariati, A., Khademian, A., Pordeli, H., Ebrahimi, N., Tajari, M.E., Aleaghil, S.A. Yazarloo, E. Badeli, Z. & Babakordi, B. (2015). Anti-fungal activity analysis of natural honey samples in Golestan Province against some common dermatophyte strains In-vitro. *International Journal of Molecular and Clinical Microbiology*, 5(1), 516-522.

- Simon, A., Sofka, K., Wiszniewsky, G., Baser, G, Bode U. & Fleischhack, G. (2006). Wound care with antibacterial honey (Medihoney) in pediatric hematologyoncology. *Journal of Supportive Care Cancer*, 14(1), 91-97.
- Singh, A., Malhotra, S., & Subban, R. (2008). Anti-inflammatory and analgesic agents from Indian medicinal plants. *International Journal of Integrative Biology*, 3(1), 57-72.
- Singh, M Mallick. A.k., Banerjee, M. & Kumar, R. (2016), Loss of outer membrane integrity in Gram-negative bacteria by silver nanoparticles loaded with *Camellia sinensis* leaf phytochemicals: plausible mechanism of bacterial cell disintegration. *Bulletin Materials Science*, 39(7), 1871–1878.
- Singh, P., Chourasia, H.R., Agarwal, M., Malhotra, A., Sharma, M., Sharma, D. & Khan, S. (2012). Honey as Complementary Medicine: - A review. *International Journal of Pharmaceutical and Biological Sciences*, 3(2), 12-31.
- Subrahmanyam, M. (2007). Topical application of honey for burn wound treatment-an overview. *Annals Journal of Fires and Burn Disaster*, 20(3), 137-9.
- Süerdem, T.B & Akyalçin, H. (2017). In vitro screening of antibacterial activity of honey samples collected from Kosovo. *Journal of Scientific Perspectives*, 1(2), 31-39.
- Sushila, N., Neha, S., Ajay K., Sandip P. & Amit, K. (2012). Comparative Antimicrobial potential of Raw and Commercial Honey Against Various Bacteria Isolated from Wound and Samples. *Asian Journal of Biochemical and Pharmaceutical Research*, 2(2), 31-39.
- Swellam, T., Miyanaga, N., Onozawa, M., Hattori, K., Kawai, K., Shimazui, T. & Akaza, H. (2003). Antineoplastic activity of honey in an experimental bladder cancer implantation model: in vivo and in vitro studies. *International Journal of Urology*. 10(4), 213–219.
- Szabadi, E. (2006). St. John's Wort and its Active Principles in Depression and anxiety. *British Journal of Clinical Pharmacology*, 62(3), 377-378.
- Tambekatr, D.H. & Kharate, M.A. (2005). Studies on antimicrobial properties of leaves extract of some edible plants. *Asian Journal of Molecular Biology and Environmental Science*, 7, 867- 872.
- Tanzi, M. G. & Gabay, M. P. (2002). Association between Honey Consumption and Infant Botulism *Pharmacotherapy*, 22 (11), 1479-1483.
- Taormina, P. J., Niemira, B. A., & Beuchat, L. R. (2001). Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. *International Journal of Food Microbiology*, 69(3), 217-225.
- Tenore, G.C., Ritieni, A., Campiglia, P. & Novellino, E. (2012). Nutraceutical potential of mono.oral honeys produced by the Sicilian black honeybees (*Apis mellifera ssp. sicula*). *Journal of food and Chemical Toxicology*, 50 (6), 1955-1961.

- Tonks, A. J., Dudley, E., Porter, N. G., Parton, J., Brazier, J., Smith, E. L. & Tonks, A.A. (2007). A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. *Journal of Leukocyte Biology*, 82(5), 1147–1155.
- Tonks, A., Cooper, R., Jones, K., Blair, S., Parton, J. & Tonks, A. (2003). Honey stimulates inflammatory cytokine production from monocytes. *Cytokine*, 21(5), 242–247.
- Usman, Y., Nasir, I. A., & Ahmed, N. (2015). In-vitro antimicrobial activities of crude and diluted Nigerian honey against some bacteria isolated from infected wound. *New York Science Journal*, 8(7), 1-6.
- Vickers, A., & Zollman, C. (1999). Herbal medicine. *British Medical Journal British Medical Journal*, 319(7216), 1050-1053.
- Viuda-Martos, M., Ruiz-Navajas, Y., Fernandez-Lopez, J. & Perez-Alvarez, J.A. (2008). Functional properties of honey, propolis, and royal jelly. *Journal of Food Sciences*, 73(9), 117–124.
- Wang, Y., Hougaard, A. B., Paulander, W., Skibsted, L. H., Ingmer, H., & Andersen, M. L. (2015). Catalase expression is modulated by vancomycin and ciprofloxacin and influences the formation of free radicals in *Staphylococcus aureus* cultures. *Applied Environmental Microbiology*, 81(18), 6393-6398.
- Wang, Yu-hua; Pei, S.J. & Xu, J.C. 2002: Sustainable Management of Medicinal Plant Resources in China: *Literature Review and Implications*, in *Resources Science*, 24(4), 81-88.
- Watanabe, K., Rahmasari, R., Matsunaga, A., Haruyama, T. & Kobayashi, N. (2014). Antiinfluenza Viral Effects of Honey In Vitro: Potent High Activity Of Manuka Honey. *Archives Journal of Medical Research*, 45(5), 359-365.
- Weigel, K. U., Opitz, T., & Henle, T. (2004). Studies on the occurrence and formation of 1, 2-dicarbonyls in honey. *European Food Research and Technology*, 218 (2), 147-151.
- Won, S. R., Li, C. Y., Kim, J. W., & Rhee, H. I. (2009). Immunological characterization of honey major protein and its application. *Food Chemistry*, 113 (4), 1334-1338.
- Xu, H. X., & Lee, S. F. (2001). Activity of plant flavonoids against antibiotic resistant bacteria. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, 15(1), 39-43.
- Yuan, Y, Peng, Q. & Gurunathan, S. (2017). Effects of Silver Nanoparticles on Multiple Drug-Resistant Strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from Mastitis-Infected Goats: An Alternative Approach for Antimicrobial Therapy, *International Journal of Molecular Science*, 18, 569
- Zakaria, A.S. (2015). Mechanism of antibacterial action of honey on pathogenic wound bacterial strains: a proteomic analysis. *International Research Journal of Pharmacy*, 6(11), 1-11.

Zappala, M., Fallico, B., Arena, E., & Verzera, A. (2005). Methods for the determination of HMF in honey: a comparison. *Food control*, 16(3), 273-277

## APPENDIX

### Appendix A: Antifungal activity of honey

The results of Antimicrobial activity of honey at 100 % concentration on the selected fungi were showed in Appendix A. *T. equinum* is more sensitive than *T. verrucosum* at the same concentration

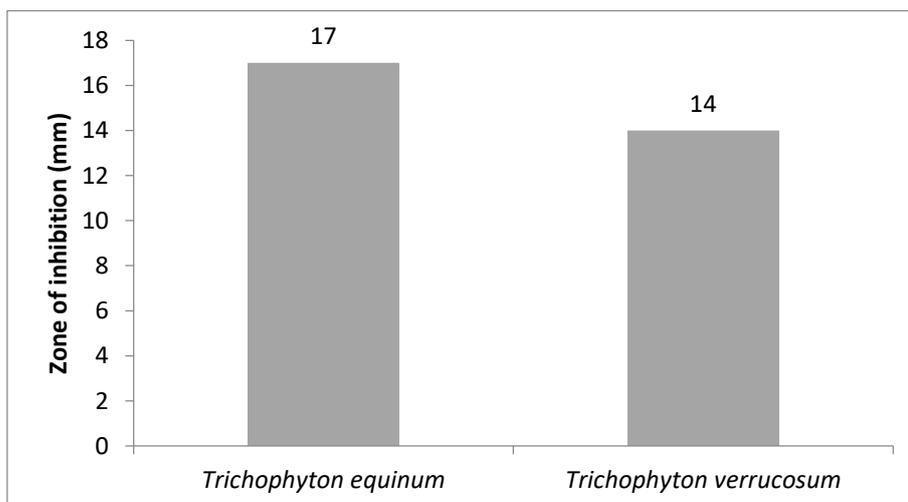


Figure 3: Anti- fungal activity of honey

## Appendix B: Biochemical characteristics of bacterial isolates

The confirmatory tests were carried out on bacterial isolates and the following bacteria: *Escherichia Coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* were confirmed in Table 5

**Table 5: Biochemical characteristics of bacterial isolates.**

Isolates	Shape	Gram reaction	Catalase	Citrate	Indole	Starch Hydrolysis	Oxidase	Suspected orgaisms
A	Rod	Negative	+	-	+	-	-	<i>Escherichia coli</i>
B	Rod	Negative	+	+	-	-	+	<i>Pseudomonas aeruginosa</i>
C	Bacilli	Positive	+	+	-	+	-	<i>Bacillus subtilis</i>

