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THE ANTIMICROBIAL ACTIVITY OF SUDANESE HONEYS ALONE AND IN COMBINATION WITH PLANT EXTRACTS AND ETHYLENEDIAMINETERAACETIC ACID (EDTA)

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**THE ANTIMICROBIAL ACTIVITY OF SUDANESE HONEYS ALONE AND IN COMBINATION
WITH PLANT EXTRACTS AND ETHYLENEDIAMINETERAACETIC ACID (EDTA)**

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DECLARATION

Statement 1

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged by footnote giving explicit references. A bibliography is appended.

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DEDICATION

This thesis is dedicated to my parents for their love, kindness, and support throughout my life especially when I needed them the most. Even when they are not with me, their prayers and love always kept me alive, inspired and guided me.

This thesis is dedicated to my lovely patient wife for her love, patience and support throughout the years not to mention my continuous headaches throughout my PhD.

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ABSTRACT

Honey and plant extracts have a long history of medicinal use in Sudan that continues to the present day. The antimicrobial properties of Manuka honey and its use as a topical wound treatment is now widely recognised but of limited availability in developing countries. This study examines the physico-chemical properties and antimicrobial activity of Sudanese honeys and synergistic interactions between selected honeys and plant extracts. A sample of 60 floral honeys from Sudan were collected direct from honey producers and 15 plant extracts were obtained from Sudan National Centre for Research. These were characterised and the antimicrobial activity against *Escherichia coli* (NCTC 10418) and *Staphylococcus aureus* (NCTC 6571) determined using bioassay and minimum inhibitory concentration methods. Selected honeys, Ethylenediaminetetracetic acid (EDTA) and plant extracts were then studied for synergistic interactions. Although the honeys had similar physico-chemical characteristics as honeys from other published studies, none exhibited the non-peroxidal activity associated with Manuka honey. A number exhibited strong peroxidal activity. The type of hive used, the site of collection and producer (beekeeper) had a statistically significant impact on the antimicrobial activity. *Acacia nilotica* and *Tamarindus indica* plant extracts showed marked antimicrobial activity before and after autoclaving and were chosen for synergistic interaction with a selected honey. Synergy was screened using an agar diffusion bioassay using Canvas software to measure the change in area of zone of growth inhibition to determine bacteriostatic synergy. This was then confirmed using the time-kill curve method. Synergistic interaction was noted between honey and EDTA against *Staphylococcus aureus* and Acacia and EDTA against *Escherichia coli*. The results are discussed in relation to the medical use of honey and plant extracts in Sudan and concludes that there is a need to further explore the potential for local production of bioactive honeys for use in conjunction with plant extracts. The Sudanese government should conduct further translational research in this area.

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Abbreviations

DHA- Dihydroxyacetone

DPPH- 2, 2-diphenyl-1-picrylhydrazyl

DMSO- Dimethylsulfoxide

E. coli- Escherichia coli

EDTA-Ethylenediaminetetraacetic acid

MGO-Methylglyoxal

MIC- Minimum inhibitory concentration

MBC- Minimum bactericidal concentration

NCR- National Centre for Research

S. aureus-Staphylococcus aureus

VRE- Vancomycin resistant enterococci

MRSA- Methicillin resistant *Staphylococcus aureus*

ORAC (Oxygen-Radical Absorbance Capacity) assay

CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Humans have known honey and plants for many centuries and used them as sources for nutrients as well as medicine. Today there is a growing body of literature demonstrating the efficacy of honey in various health aspects and particularly as a novel agent for wound management. The majority of the literature in the last two decades has been focused on honeys from particular sources abundant in New Zealand and Australia. The floral source of the honeys belongs to the family of *Leptospermum* trees which gives nectar from manuka and Jelly bush flowers (Allen *et al.*, 1991).

The potential effects of selected honeys for the treatment of particular diseases has been known for centuries as certain honeys were selected for the treatment of particular ailments; however, it was not until recently that the research has proved that certain honeys possess unusual antimicrobial properties (Blair *et al.*, 2009; Allen *et al.*, 1991) and hence have been the choice for wound management. The extensive research into the antibacterial properties of *Leptospermum* honeys has led to the approval of licensed products for wound management in regions including Canada, USA, Australasia, Europe and Hong Kong (Henriques, 2006)

The selection of those honeys to be the main source for medical grade honey used for topical application was based on the fact that the activity of the majority of honeys other than manuka and jelly bush honeys depends largely on the levels of hydrogen peroxide which could be reduced or nullified by the catalase from the human body, while the activity of *Leptospermum* honeys remains unaffected.

The success of *Leptospermum* honeys and their global acceptance for topical application in modern medicine should encourage less developed countries throughout the world to

explore locally produced honeys as well as medicinal plants in an attempt to identify potential natural products with distinctive bioactive properties. Production of natural antimicrobials and adoption of new strategies are important measures particularly for the less developed countries like Sudan where medicinal prescriptions are sold over the counter without microbiological testing and many natural products are used based on their perceived efficacy and empirical knowledge rather than any scientific base.

Moreover, the search for local honeys or honey combinations with distinct antibacterial activity is necessary due to the high demand for manuka honey which has led to sharp increase in the prices of manuka honey in recent years (Stephens *et al.*, 2010) which makes manuka honey out of reach for much of the population of Sudan as well as third world countries.

Medical grade honeys are usually sterilised by irradiation (Molan and Allen, 1996) which is an expensive approach compared to autoclaving which could be more convenient if any plant extract showed antimicrobial activity that could persist after autoclaving; hence a natural and inexpensive sterile remedy would be effective and ideal alternative in third world countries like Sudan.

Although a number of plant extracts have a long history of use in folk medicine in Africa and the Arabic Peninsula alone and in combinations with honey both for topical application or in the form of oral preparations for treatment of fever and gastrointestinal disorders, there have been limited studies validating the use of herbal extracts alone and in combination with honey.

Screening and identifying distinctive local honeys and plant extracts is an essential step for their acceptance and application in modern conventional medicine. The scientific evaluation of the bioactive properties of natural products could be a solution not only for the spread of antimicrobial resistance but as natural preservatives with additional value in the food industry as a possible preservative against food spoilage as well as an antioxidant activity that might help in the retardation of the harmful effects which could be caused by oxidative stress due to free radicals present in foods and the human body.

In recent years the increased demands for safer, less processed foods that are free from chemical additives accompanied with the ban on selected preservatives such as butylated hydroxy toluene (BHT) in some countries has led to the incorporation of plant essential oils in food packaging as well as introduction of honey in some fresh salads (Rasmussen *et al.*, 2008). Therefore, it is necessary to add to the available knowledge of the benefits of honey and plants extracts and look for beneficial combinations that could be used for prophylaxis, preservation and therapy. The *in vitro* screening and evaluation of the bioactivity of natural products and looking into whether such natural products could be enhanced in the presence of certain preservatives such as Ethylenediaminetetraacetic acid (EDTA) could yet open wide an area of crucial knowledge in the future keeping in mind the increased demands for safer, more natural and less processed foods, the continuous emergence of antibiotic resistant strains of bacterial pathogens and limited options for treatment as well as the other benefits from natural products and rich antioxidants which reduces the risk of oxidative stress.

1.2 Honey nomenclature and classification

Honey is a saturated or supersaturated sugar solution produced by social bees and some other social insects. Bees and insects gather nectar or honeydew from the flower of living plants and process by the addition of enzymes into honey, then store as a food for use in dearth periods (Crane and Visscher, 2009).

Despite the contributions of few other insects honey is chiefly produced by the bees which are social insects with a perennial life cycle. The bees are mainly classified into different groups which include all honey bees (*Apis spp.*), stingless bees (*Melipona* and *Trigona spp.*) as well as *Nectarina* wasps in South America and several species of honey ants, especially *Melophorus inflatus* in Australia. There are other social wasps and bumblebees (*Bombus spp.*) with annual life cycles which produce honey, but only very little (Crane, 1999).

Honey is classified in different ways based on floral source (Louveaux *et al.*, 1978), production method or extraction method as well as the source of food, i.e., whether it is collected by the bees from nectar of plants or from secretion of insects fed on plants which is explained here in 1.2.1 and 1.2.2

1.2.1 Nectar honey

The European Commission Council Directive 2001/110/CE (EU 110, 2001) defines nectar honey as natural sweet substance produced by *Apis mellifera* bees from the nectar of plants which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (EU 110, 2001).

1.2.2 Honeydew honey

European Commission Council Directive 2001/110/CE defines honeydew honey as a food obtained from secretions of living parts of plants or from excretions of plant-sucking insects.

The plant-sucking insects (Hemiptera) pierce the foliage or other plant covering parts, feed on the sap, and excrete the surplus as droplets of honeydew, which are gathered by the bees (EU 110, 2001).

Although the differentiation of honeydew honeys and nectar honeys could be done by pollen analysis they are far better distinguished through their physicochemical profiles since the honeydew honeys have higher pH, acidity, ash, electrical conductivity, and darker colour, as well as lower monosaccharide and a higher di- and trisaccharide content (Mateo *et al.*, 1992; Mateo and Bosch-Reig, 1997, 1998). In addition, honeydew contains cells of algae and fungi; however, they are not specific for its origin (Bogdanov *et al.*, 1997).

1.3 Native and imported Sudanese honeybees

Although the findings of Farouk and his colleagues were among the early publications on the application of honeys for wound management (Farouk *et al.*, 1988), since then the majority of research in Sudan was mainly concentrated on the bee species in Sudan and bees' behaviour rather than the honey itself or other aspects of Apitherapy. The studies on the bee species of Sudan has revealed that the native Sudanese honey bee could belong to one of two main lineages as classified by molecular approaches i.e., O which includes the Near and Middle Eastern subspecies and another lineage called Y including the *A. m. yemenitica* subspecies from Ethiopia (Franck *et al.*, 2001).

Ruttner has first classified the Sudanese native honey bees as *Apis mellifera nubica* and then reclassified it as *Apis mellifera jemenitica* (Ruttner, 1975). However, morphometrical studies carried out by El- Sarrag on the honeybee population in Sudan showed more than one subspecies (El-Sarrag *et al.*, 1992). El- Sarrag investigations have revealed two subspecies of honey bee in Sudan; a yellow banded group *Apis mellifera sudanensis* nov subsp and a

mixed group of *Apis mellifera nubica* Ruttner. *Apis mellifera sudanensis* is a small honeybee distributed all over the Sudan between latitudes 3°N and 16°20'N whereas *Apis mellifera nubica* is distributed along the international borders of Sudan with Ethiopia. Moreover, morphometrical studies carried by Mogga has divided Sudanese bee clusters into three sub-clusters; the smallest bee, *Apis mellifera jemenitica*, The medium bee *Apis mellifera sudanensis* and the largest bee *Apis mellifera bandasii* (Mogga and Ruttner, 1988).

The introduction of modern beekeeping into Sudan in 1928 saw vast imports of European honeybee stock including *A. m. ligustica*, *A. m. carnica*, *A. m. cypria* and *A. m. lamarkii* crosses. Since then there have been continued and repeated imports of foreign honeybees into the country (El-Sarrag and Nagi 1989). The dwarf honeybee, *A. florea*, was introduced to Sudan in 1985 (Lord and Nagi 1987; Mogga and Ruttner 1988). Beekeeping in Sudan has expanded through the efforts of a small number of beekeeping companies and there were about 200,000 honey hives and 50,000 beekeepers (El-Sarrag *et al.*, 1992). During 1998–2003 a total of 16,953 commercial colonies have been introduced to further develop apiculture and implement modern techniques including migratory beekeeping in Sudan (El-Niwieri and Moritz, 2008).

1.4 Beekeeping

Beekeeping is the art by which beekeepers gets honey and other bee products from the hive. It is possible that beekeeping started as early as 5000 BC and it was well established by the first Dynasty, around 3100 BC (Crane, 1999). Ancient Egyptians were reported among the first beekeepers. Around 3500 BC Upper and Lower Egypt were united under one ruler and the hieroglyph of a bee first appeared, denoting the king of Lower Egypt (Ransome, 1986).

1.4.1 Honey hunting (Bee-killing)

Bee-killing, or honey hunting, is a traditional activity in many regions of Africa and Asia. In most other regions of the world it is an incidental activity. Collecting honey from wild colonies is an easy and simple method by which people get nutrition at absolutely no financial cost. A variation on bee killing is traditional in some regions of Africa. Straw containers or clay pots are hung in trees to attract wild colonies. After the colony has been in the container for sufficient time to have built up honey stores, the container is lowered, the bees killed, and the -hive products taken. Even though the bees are attracted to a man-made container, this is still bee-killing (Gentry, 1982).

1.4.2 Beekeeping in Sudan

Beekeeping in Sudan as well as other places around the globe has started with honey hunting, followed by traditional beekeeping and top bar hives. The literature didn't report the time when beekeeping first came into Sudan but, same as with honey it could have started as early as ancient Egyptians as a result of the joint history of the Upper and Lower Nubian Kingdoms. Beekeeping was established in Egypt by 2400 BC. This was evidenced by stone base from the Sun Temple of Ne-Woser-Re, not far from Cairo, which clearly depicts an apicultural scene (Darby *et al.*, 1977). By 1200 BC King Ramses 11 gifted the Nile god some tonnes of honey (Crane, 1975).

1.4.2.1 Honey hunting in Sudan

Throughout the year wild bee colonies are found in some parts of Sudan. Local farmers around the area collect the honey when trees containing the colonies are felled upon cleaning and preparation for cultivation of crops. Although honey hunters usually regret killing the colony, they know of no other way of obtaining the honey (Hussein, 2000).

1.4.2.2 Traditional beekeeping in Sudan

Until recently traditional beekeeping was the sole source of honey production in Sudan. This practice has continued where the *Acacia spp*, *Ziziphus Spina Christi* (sidr) and other melliferous trees are found (Hussein, 2000) and still remain the main source of honey in Sudan despite the introduction of top bar hives accompanied by Carniolan bees from Egypt (*Apis carnica*) as many people prefer to have local honeys from endogenous Sudanese *Apis mellifera* rather than those from *Apis carnica*.

1.4.2.3 Modern beekeeping in Sudan

Modern beekeeping was initiated by the joint efforts of the Faculty of Agriculture, University of Khartoum, Sudan National Council for Research and "Near East Foundation. The joint efforts started by forming a body known as National Beekeeping Project (NBP) and introducing seminars about modern beekeeping. In 1987, a group of apiculturists and agriculturists formed the Sudan - Bee and Agriculture Voluntary Association" (Eisa and Roth, 2008).

Modern beekeeping involves the use of top bar hives which needs financial resources but, in turn yields high quality honey. Since the majority of the beekeepers do not have sufficient funds to improve their hives they continue to use woven baskets and clay pots. Professional beekeepers introduced the use of modern low-technology, Kenya tops bar hives, Omdurman clay hives, Guffa basket hives and modern hives (Hussein, 2000). Modern apiculture in Sudan today depends mainly on imported honeybee stock. However, apiculture is still restricted to very few locations only and the vast majority of honeybee colonies still exist in the wild (El-Niwieri and Moritz 2008).

1.5 Beekeeping and socioeconomic conditions in Sudan

Beekeeping is an activity which could undertaken in small as well as large-scale production (Gentry, 1982). This fact means individuals, small groups or even large governmental projects could undertake beekeeping. A successful beekeeping project only needs the right choice of apiary which should fulfill the following requirements: (i) a plentiful food resources for the bees, that is, nectar and pollen in an area which is rich with diverse indigenous fruit trees for the bees foraging throughout the year, (ii) a close source of water, and (iii) abundant vegetation to provide shade for the hives (Fadare *et al.*, 2008).

Sudan is one of the largest countries in Africa and the Middle East, even after the separation of South Sudan there are plenty of lands and water resources including the river Nile as well as plenty of rain and ground water, plenty of various wild honey bees with variable vegetations and variable climatic conditions all which make Sudan a potential place for small and large scale beekeeping projects.

Agriculture and animal production represent the main stay of the economy of Sudan. The cultivated crops include wheat, gum arabic, cotton, barley, sorghum, sesame and peanuts (Saied *et al.*, 2008). The major exporters of honey in Africa are Ethiopia and Tanzania (Hussein, 2000). Although some honey from Sudan is exported to countries such as Saudi Arabia, the production level in Sudan is poor as compared to the available resources.

The bees are well known pollinators that use sources such as pollen and nectar and add to the productivity of the cultivated crops. Beekeeping could be a secondary source of income for the framers as well as a source of hard currency for Sudan. A study in Adamawa state, Nigeria reported that beekeeping was by all means more profitable and cost effective than

farming as demonstrated by participants and demanded adoption of apiculture by people with low income such as low income farmers (Ja'afar-Furo, 2007).

Moreover, Sudan is rich with plenty of trees which produce gum arabic such as *Acacia* trees and *Ziziphus Spina Christi* which are also a major source of honeys from Sudan and the production of honey and gum arabic can be undertaken by the same people even if they are not full time farmers, i.e., they harvest the honey and come only when they expect the honey to be ready. Another option could be giving women the chance to run this business.

1.6 Native bees contribute to biodiversity and enhance crop productivity

Native bees (*Apis mellifera*) are usually a good vehicle for pollination and could possibly fully pollinate the crops, particularly when they are close to the natural habitat (Klein *et al.*, 2003; Klein *et al.*, 2007). The demonstration that native bees can provide sufficient crop pollination throughout a region of intensive human land use presents an example of the important role which could be played by the bees in biodiversity (Naeem, 1998). In Sudan there is excessive use of lands without any control measures which could lead to under productivity as well as erosion in the region. Recently studies performed within areas that use the same farming practices and cultivar reveal that isolation of crop fields from the pollinator diversity that natural habitat harbours is leading to negative impacts on yields of pollinator-dependent crops (Kremen *et al.*, 2002; Carnevalheiro *et al.*, 2010).

1.7 Honey applications: a historical perspective

Honey has been a valuable food, medicine and sweetener throughout the ages. Although it is difficult to follow exactly when the relationship between humans and the bees started and all the efforts by which ancient people have tried to domesticate the bees and how

exactly humans have learned to get the best out of them this section summarises some of the literature citing the uses of honey.

1.7.1 Honey in ancient times

The use of honey for therapeutical purposes is well established in ancient prescriptions as well as modern wound management. The earliest records of the application of honey in medicine could be traced back to the Egyptian Papyri as well as Sumerian clay tablets dated from 1900 to 1250 BC where honey was in almost one third of the prescriptions (Molan, 1992a). Other uses of honey by ancient Egyptians also included treatments for the eyes and skin as well as in embalming and wounds. "Hippocrates (460-357 BC) found that honey cleaned sores and ulcers of the lips and healed buncles and running sores. Aristotle (384-322 BC) referred to pale honey being a good salve for sore eyes" (Al Waili, 2003). The ancient Greeks were reported to have used honey to treat fatigue: athletes drank a mixture of honey and water before major athletic events (Crane, 1975).

The Babylonian used honey for the treatment of ear infections, eye infections and an ointment for the skin (Apimondia 2001 cited in Henriques, 2006). The medicinal uses of honey alone and in combination with other components including herbs and essential oils to treat various ailments including burns, wounds, eye infections as well as gastrointestinal disorders might be traced back to ancient civilizations of the Egyptians, Assyrians, Greeks and Romans (Zumla and Lulat, 1989). In 50 AD, Dioscorides described honey as being "good for all rotten and hollow ulcers" and "good for sunburn and spots on the face" (Gunther, 1934 cited in Molan, 2006). Many African tribes use honey to treat snakebites, fever and as a laxative. Moreover, the Masai warriors have used honey to gain more power and enhance

their strength which is probably due to the high sugar content of honey (Apimondia 2001 cited in Henriques, 2006).

It has been reported that the Egyptians used honey in their spiced breads, cakes and pastries, and for priming beer and wine (Tannahill, 1975). In Ancient Rome honey was used in a wider range of culinary dishes. Honey has been used in salad dressings in order to balance the acidity of the vinegar as well as an essential ingredient of many sauces (Crane, 1975). Reports have mentioned that the wines drunk at the beginning and end of meals were sweetened with honey; and meat, while fruit and vegetables were sometimes preserved by immersion in honey (Free, 1982). Refined sugar which is used in cooking today has been known and used in medicines, but had no place in cooking (Wilson, 1973). Almost half of one late Roman cookery book included honey as an ingredient in almost 500 recipes (Style, 1992).

1.7.2 Honey use in the Middle Ages

During the Middle Ages honey was used for sweetening all type of dishes from appetizers, soups, cheese to fish dishes, roast meats as well as vegetables. However, it is difficult to predict whether a dish is savoury or sweet from the title assigned to it in a recipe. Today it is easy to predict a meat or cheese dish will usually be savoury; however, it was not the same in the Middle Ages, where meat, fish dishes and the pastry lids of 'savoury' pies might often be sweetened (Wilson, 1973).

Daude de Pradas has reported the application of honey in folk medicine in approximately 1200 AD (Crane, 1975). In a text book about honey Beck and Smedley (1997) have mentioned that honey has been used as a remedy for gastric and intestinal complaints, the diuretic effect of honey were recorded as a favoured remedy for kidney inflammations and

stones. In addition Hindu people had great faith in the medical virtues of honey, mainly for the treatment of coughs, pulmonary issues and gastric disorders. Moreover, they have reported the use of particular honeys for the treatment of specific disorders and the general application of honeys for treatment of skin diseases and smallpox, as well as in surgical dressings. Furthermore, they also reported the use of a mixture of honey and crushed bees by German women for the regulation of the menstrual flow as well as the energetic and cosmetic benefits (Beck and Smedley, 1997).

1.7.3 Honey in modern medicine

In more recent times, honey has played a relatively minor role in medicine in the developed countries mostly due to it not being accepted by Western practitioners who preferred to use antibiotics since they were not sure of the honeys' mode of action (Molan, 1992a). However; the applications of honey continued in the Middle East, China, Africa and Indian nations since they consider honey as a valuable source for the treatment of internal as well as external ailments (Beck and Smedley, 1997).

Populations in rural communities from almost all nations have documented the use of honey for wounds management as well as other ailments through time (Henriques *et al.*, 2010). Honey has been shown in one clinical trial to be effective against bacterial diarrhoea, (Haffejee and Moosa, 1985), and to aid in the treatment of eye infections (Fotidar and Fotidar, 1945, cited in Molan, 2001a; Al Waili, 2004).

Although honey has played a minor role in Western medicine since the development of Penicillin and other antibiotics which were considered as miracle drugs they were forced to rediscover the antibacterial potential of honeys; probably due to the emergence of multi-

drug resistant pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) and *Pseudomonas aeruginosa* (Molan, 1992a).

Throughout the last two decades much research has been carried out in order to explore the mysterious role played by honey in the management of wounds and burns, which has led to scientific evidence that demonstrated honey is an effective antibacterial agent (Molan and Allen, 1996; Cooper *et al.*, 2002a). The *in vitro* findings that honey is an effective antibacterial agent and proved to be even superior to many antiseptics and antibiotics are matching with the clinical trials as well as the *in vivo* and *in vitro* experiments that was demonstrated by many cases in which honey successfully eradicated antibiotic resistant and sensitive strains that conventional therapy has failed to eradicate (Subrahmanyam, 1991; Farouk *et al.*, 1988; Efem, 1988)

Medicinally, honey is used to enhance wound- healing in humans (Aysan *et al.*, 2002; Cooper *et al.*, 2001), treatment of gastric ulcer (Kandil *et al.*, 1987) and shortening of the duration of diarrhoea (Salem, 1981; Haffejee and Moosa, 1985). The use of honey was based on empirical knowledge rather than scientific knowledge. People didn't know how honeys cured infections but, knew it worked, this fact led to the use of antibiotics instead of honey (Molan, 2001b).

Only now researchers are beginning to understand why honey has such therapeutic and beneficial potential; honey indeed could be the elixir (Molan and Allen 1996) that the ancient people believed. Research is showing a number of other health-related benefits, including a laxative effect, beneficial effects on blood glucose levels (Cortes *et al.*, 2011), anti-inflammatory and immune stimulating properties and potentially a cancer-preventative action (Manyi-Loh *et al.*, 2011).

1.7.4 Bees honey in Sudan

In the recent years Sudanese researcher S.A. Mohammed (Mohammed and Babiker, 2010) conducted research into properties of Sudanese local honeys in attempts to identify some of the physicochemical properties including the electrical conductivity, ash content, protein and amino acid content for selected Sudanese honeys. Moreover, Makawi tested the HMF levels of a range of food products including honey in order to provide basis for legislation of Sudanese honey as well as sugar containing products. The study demonstrated the importance of testing the HMF concentration and the implications of elevated levels of HMF as a barrier to the marketing of Sudanese food products including honey, jams and juices (Makawi *et al.*, 2009a,b). Locally produced Sudanese honeys are widely used for treatment of wounds, eye infections as well as gastrointestinal disorders; however, little or no research has been published into the antimicrobial activity of these honeys since the work of Farouk and Wadi (Farouk *et al.*, 1988; Wadi *et al.*, 1987).

Although the study by Farouk proved that locally produced Sudanese honeys could be a genuine source for antibacterial therapy they were never quantified. The application of locally produced honeys alone and in combination with other medicinal plant extracts for medicinal use as well as food preservation is well established based on empirical knowledge as well as religious beliefs and ancient reports.

1.8 The composition of natural honey

Although honey is a unique saturated complex solution all honeys are not the same since they vary depending on the variation in their botanical source, geographical location, bee species, storage condition, beekeeping as well as the year and time of collection during the

year all could affect the chemical profile of the honey (White, Jr. and Ruda, 1978; White, 1980). Table 1.1 shows average composition of honey.

Table 1.1 Average composition of honeys and ranges of values (from White, 1975)

Component (% except pH and diastase value)	Average	Standard deviation	Range
Water	17.2	1.5	13.4 - 22.9
Glucose	38.2	2.1	27.2 - 44.3
Fructose	31.3	3.0	22.0 - 40.7
Sucrose	1.3	0.9	0.2 - 7.6
Maltose	7.3	2.1	2.7 - 16.0
Higher sugars	1.5	1.0	0.1 - 8.5
Free acids (as gluconic acid)	0.43	0.16	0.13 – 0.92
Lactone (as glucolactone)	0.14	0.07	0.0 - 0.37
Total acid (as gluconic acid)	0.57	0.20	0.17 - 1.17
Ash	0.169	0.15	0.020 - 1.028
Nitrogen	0.041	0.026	0.000 - 0.133
pH	3.91	-	3.42 - 6.10
Diastase value	20.8	9.8	2.1 - 61.2

1.8.1 Sugar content

Honey is a complex saturated or super saturated solution which mainly made up of two components sugars and water. The sugars or carbohydrates make up more than 90% of the honey's total dry matter (White, 1979; Anklam, 1998). It has been reported that honey is

made up of more than 180 substances (Jones, 2009; White *et al.* 1963); which Bogdanov has estimated to be actually even closer to 600 substances (Bogdanov *et al.*, 2004). The carbohydrates content of honeys includes a variety of sugars such as the monosaccharides fructose (levulose) as well as glucose (dextrose), sucrose and maltose and disaccharides oligosaccharides which seem to differ according to the floral source of the honey (Molan and Allen, 1996).

1.8.2 Osmolarity

Due to the high sugar content of honey, the osmotic pressure of honey is usually high leading to low water activity (*aw*) reported range = 0.562–0.62 (Tysset and Rousseau, 1981; Bogdanov *et al.*, 1997), which gives the osmolarity an essential role in the antimicrobial activity of undiluted honeys; since, the growth of many bacterial species, for example, is completely inhibited when the (*aw*) is in the range of 0.94-0.99 (Molan, 1992b).

1.8.3 Water content

Honey water content is an important quality parameter, which must be determined in order to prevent the spoilage of honey due to fermentation. The honey moisture content is not like other parameters which are optionally accepted, since it affects the quality as well as the shelf life of the honey (Bogdanov *et al.*, 2004). The International Honey Commission (IHC) has set a maximum limit of 20g water/100g of honey for any honey sample to be accepted for honey trade. The moisture content has a direct effect on other honey properties such as glucose crystallization and viscosity of the honey (Bogdanov *et al.*, 2004). The honey moisture content is evaluated by either refractive index, gravimetric technique or Karl Fischer titration (Sanchez *et al.*, 2010).

1.8.4 Acidity

Acidity is another factor which contributes to the antimicrobial activity of honey. Although it was thought to have a major role, more recent studies have demonstrated that acidity actually plays a minor role in the antibacterial activity of honey (Pothmann, 1950, cited in Molan, 1992a, b). There are about 30 organic acids in honey (Mato *et al.*, 2003); however, gluconic acid which is produced due to the activity the enzyme glucose oxidase is main organic acid present in the honey in the range of 0.23-0.98% (White, 1975).

1.8.5 Hydroxymethylfurfuraldehyde (HMF)

Hydroxymethylfurfuraldehyde (HMF) is also present in minor quantities. HMF which could be formed in the presence of acid due the breakdown of fructose has been considered as evidence for the adulteration of honey; however, it has been proved that even fresh honeys do contain minor amounts of HMF (Zappala *et al.*, 2005) which could easily be elevated if the honey is stored in moderate or high temperatures; hence, it is necessary to store honey in a refrigerator or a cool place (White, 1975; White and Subers, 1964a,b) so as to keep the levels of HMF to the minimum since, HMF is one of the main factors which are considered for the quality and marketing of honey.

1.8.6 Enzymes

Moreover, honey contains a number of enzymes including glucose oxidase, invertase, and amylase, which appear to originate from honeybees (Molan, 1992a). Glucose oxidase plays an essential role in the antibacterial activity of honeys as well as the generation of gluconic acid. The enzyme Invertase catalyses the conversion of sucrose obtained from the nectar and into the monosaccharides fructose and glucose in a ratio of 1.2:1 between glucose and fructose (Anklam, 1998). There are other enzymes such as catalase and acid phosphatase

(White, 1975) which are also present in some honeys but these are likely to be derived from the pollens and nectar of plants.

1.8.7 Minor components

Furthermore, honey is rich with other components although in minor amount. These include amino acids (mainly proline), vitamins including vitamin A , B-vitamins (riboflavin, niacin, pyridoxine, panthothenic acid, and folic acid),vitamin-C (ascorbic acid), vitamin D, and vitamin E, Honey also contains a significant number of minerals, including calcium, phosphorous, iron, zinc, selenium, chromium, potassium, magnesium, and manganese, and organic acids (Bobis *et al*, 2008). Other components present in honeys also include flavonoids, antioxidant substances and unidentified plant-derived elements (phytochemical components) (Sato and Miyata 2000).

1.9 The bioactivity of honey

The complex composition of the bees' honey which contains high concentrations of sugars leading to low water activity as well as the presence hydrogen peroxide, flavonoids and other phytochemicals from the botanical source all together gives the honey potential bioactive properties. Although the bioactive properties of honey include antibacterial, antioxidant, anti-inflammatory as well as many other properties this thesis will mainly focus on the antibacterial activity and to a lesser extent on the antioxidant potential of honey.

1.9.1 Antibacterial Properties of Honey

The antibacterial activity of honey *in vivo* has been noticed (Aristotle, circa 350 B.C from a 1910 translation). Van Ketel, a Dutch scientist was the first to demonstrate the antibacterial activity of honey for the first time *in vitro* (Dustmann, 1879). *In vitro* studies have identified different aspects of the antibacterial properties of honey including those attributable to low

pH (acidity), high sugar content and high osmolarity (Sakett, 1919), the presence of hydrogen peroxide (White *et al.*, 1963; White and Subers 1964a,b) and compounds derived from floral sources (Bogdanov, 1997).

Allen *et al.*, (1991) has demonstrated certain honeys were still showing significant antibacterial activity even after the removal of hydrogen peroxide by the addition of catalase. This type of antibacterial activity is referred to as non-peroxidal activity (Allen *et al.*, 1991). These findings have later led researchers to be aware of two main types of antibacterial activity in honeys; a heat and light sensitive activity as well as a heat and light stable activity. Bogdanov has suggested that the non-peroxide activity might be mainly derived from a honeybee origin, while the floral source could play a role as well (Bogdanov *et al.*, 1997). Other reports have mentioned flavonoids and phenolic acids as possible sources for the antibacterial activity of honey (Wahdan, 1998; Taormina *et al.*, 2001). The antibacterial activity of honey is dependent on various factors including botanical origin, geographical location as well as bee species and the contribution of the beekeeper (Henriques, 2006). All these factors lead to variation in antibacterial potency of honeys; however, storage of the honey as well as the levels of HMF and Maillard reaction products could play a role as well.

1.9.2 Antioxidants in honey

Honey has been reported as a potential antioxidant which has the ability to reduce the oxidative reactions within the food system as well as the human body. Oxidative reactions might cause deleterious reactions in food products such as lipid oxidation in meat, enzymic browning in fruits and adverse health problems including chronic diseases and cancers (Gheldof and Engesth, 2002). The antioxidant activity of honey could be attributed to

phenolic acids, flavonoids, enzymes, ascorbic acid, Maillard reaction products, proteins, amino acids and organic acids (Gheldof *et al.*, 2002). The antioxidant capacity varies greatly which could be justified by variation of the botanical source and the total phenolic contents (Gheldof *et al.*, 2003). Other reasons for the variation antioxidant activity might be due to the difference in the enzymatic activity as well as the difference in plant secondary metabolites (Frankel *et al.*, 1998).

1.10 Honey and glycemic regulation

The research into the positive and negative impacts that honey could bring to healthy as well as impaired consumers has spread into fields other than wound management (Cortes *et al.*, 2011). Research into the potential application of honey as a supplement in the diet of healthy individuals as well those with impaired glucose tolerance, diabetes, and their related comorbidities has found that compared to glucose and sucrose, the consumption of honey decreases glycemic levels and blood lipids in healthy, diabetic and hyperlipidemic individuals (Cortes *et al.*, 2011; Ahmad *et al.*, 2008). Interestingly in addition to reducing the levels of glycemic index it was found that prolonged honey intake seem to reduce fasting glucose levels in humans, suggesting that honey consumption influences plasma glucose regulation. These findings means that honey could be anticipated as a nutritional dietary supplement for healthy individuals as well as for those suffering from alterations in glycemic regulation (Cortés *et al.*, 2011). Despite being largely made of a variety of carbohydrates honey as well as plants contain amounts of phytochemicals which contribute to the regulation of insulin and there are fascinating reappraisals of traditional treatment of diabetes (Sato and Miyata, 2000).

1.11 Honey and herbs as food and medicine in Sudan

Honey and herbs are widely used in Sudan for nutrition, healing as well as food preservation particularly in rural areas where nomadic people do not have refrigerators to keep their food. The motivation behind the excessive use of honey and plant extracts in Sudan varies from religious beliefs to the relative expense of antibiotics as well as preference of many people who rely only on honey and medicinal plants and do not go to physicians or hospitals except in emergencies which need immediate care.

Honey and plant extracts have been praised in Islam more than 1400 hundred years ago. Among the plants which have been appreciated in Islam are *Nigella sativa*, *Cucurbita maxima*, ginger, clove as well as many others. Honey and selected plants were praised in the Qur'an and Sunnah which are the sources followed by Muslims. Due to the religious perspective of Sudanese population probably as well as effectiveness, honey is usually found in every Sudanese house since the majority of the population (more than 97%) are Muslims.

This study will explore some of the honey and plant extract combinations used in Sudanese folk medicine. The plant extracts and the honeys will be screened for antibacterial activity and plants showing good antibacterial activity will be selected for combination studies, particularly those which still show good antibacterial activity after autoclaving as identifying inexpensive natural products that can be rendered sterile by heat is important if they are to be applied in modern medicine as well as food industries following extensive research.

1.11.1 Honey and bees in Islam

Honey has been praised in Islam as well as other religions and cultures including the Ayverda and ancient Chinese cultures for many centuries. The importance of honey for

Muslims nutrition and therapy is evidenced in their two main references Qura'n (see the Ayat and the translation in Figure 1.1) and Sunnah. Al-Bukhari and Muslim recorded in their Sahihs from Qatadah from Abu Al-Mutawakkil `Ali bin Dawud An-Naji from Abu Sa'id Al-Khudri that a man came to the Messenger of Allah and said, "My brother is suffering from diarrhea". He said, "Give him honey to drink". The man went and gave him honey, and then he came back and said, "O Messenger of Allah! I gave him honey to drink, and he only got worse." The Prophet said, "Go and give him honey to drink".

So he went and gave him honey, then he came back and said, "O Messenger of Allah! It only made him worse. " The Prophet said, "Allah speaks the truth and your brother's stomach is lying. Go and give him honey to drink". So, he went again and gave him honey, and he recovered.

Al-Bukhari, also reported in his Sahih from Ibn `Abbas that the Messenger of Allah said:

"Healing is to be found in three things: the cut made by the cupper, or drinking honey, or in branding with fire (cauterizing), but I have forbidden my Ummah (followers) to use branding". (Al-Bukhari, 1976).

وَأَوْحَىٰ رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ

وَمِمَّا يَعْرِشُونَ (68) ثُمَّ كُلِي مِنْ كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا ۚ يَخْرُجُ مِنْ بُطُونِهَا

شَرَابٌ مُخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِلنَّاسِ ۚ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَتَفَكَّرُونَ (69)

Figure 1.1 Bees and honey in Quran

Translation

And your Lord revealed to the bees: "Build dwellings in the mountains and the trees, and also in the structures which men erect. Then eat from every kind of fruit and travel the paths of your Lord, which have been made easy for you to follow." From their bellies comes a drink of varying colours, in which there is healing for mankind. Indeed in that is a sign for a people who give thought/who reflect. (Qur'an, 16:68, 69)

The bees and the making of honey has been mentioned and praised in Quran more than fourteen hundred years ago (Figure 1.1). The Quran has stated that the honey is produced by the workers and female bees, a fact which has only been known recently. However; 1400 years ago the Quran refers to bees that generate the honey as females (Quran 16.68-69). The Arabic grammar is in the female mode. And your Lord (Allah) revealed to the bees: Build your hives in mountains, trees and in what people build. Then eat (for females) from every fruit and follow (for females) your Lord's enslaved paths, from their bellies (for females) exits drink of different colours, in it healing for man. These are signs for those who contemplate. Since, modern science has only confirmed this fact recently this means that the holy books contain valuable information that need to be verified by scientific research for the benefit of the mankind.

1.11.2 Honey and herbs as nutraceuticals

A nutraceutical is defined as any substance that might be considered a food or part of a food and provides medical or health benefits including the prevention and treatment of disease (Andlauer and Forts, 2002). Nutraceuticals are food or food supplements which could again make the fine line between food and medicine as it has been throughout the last 2000 years, from the time of Hippocrates (460–377 BC) to the dawn of modern medicine where there was little distinction made between food and drugs. Hippocrates has pointed that the practice of medicine itself consisted largely of the wise choice of natural food products (Andlauer and Forts, 2002). Honey and phytochemicals are examples of nutraceuticals which could be used for nutrition and give plenty of healthy effects as both has been proved to be rich with antioxidants (Gheldof *et al.*, 2002; Schramm *et al.*, 2003), antibacterial (Molan, 1992a;b; Cooper *et al.*, 2002a,b) due to the high sugar content combined with the

osmolarity, acidic pH, presence of phytochemicals and enzymes (White *et al.*, 1963). Table 1.2 shows the average nutrients in honey in relation to human requirements (Crane, 1980). Sudan is one of the third world countries where the people believe in honey and other plant extracts used in folkloric medicine and rely on them for their nutrition as well as healing.

Table 1.2 Nutrients in honey in relation to human requirements (From Crane, 1980)

Nutrient	Unit	Average amount in 100 g honey	Recommended daily intake
Energy equivalent	Kcal	304	2800
<u>Vitamins</u>			
A	I.U.	-	5000
B1 (Thiamin)	mg.	0.004 - 0.006	1.5
B2 (Riboflavin)	mg.	0.002- 0.06	1.7
Nicotinic acid (niacin)	mg.	0.11.- 0.36	20
B6 (Pyridoxine)	mg.	0.008 - 0.32	2.0
Pantothenic acid	mg.	0.02 - 0.11	10
Bc (Folic acid)	mg.	-	0.4
B12 (Cyanocobaltamine)	mg.	-	6
C (Ascorbic acid)	μg	2.2 - 2.4	60
D	mg.	-	400
E (Tocopherol)	I.U.	-	30
H (Biotin)	I.U.	-	0.3
<u>Minerals</u>	mg.		
Calcium	mg.	4 - 30	1000

Chlorine	mg.	2 - 20	
Copper	mg.	0.01 - 0.1	2.0
Iodine	mg.	-	0.15
Iron	mg.	1. - 3.4	18
Magnesium	mg.	0.7 - 13	400
Phosphorous	mg.	2 - 60	1000
Potassium	mg.	10 - 470	-
Sodium	mg.	0.6 - 40	-
Zinc	mg.	0.2 0.5	15

1.12 Antibacterial agents in humans life

The application of antimicrobial agents has been established to combat infectious diseases caused by various microorganisms. The history of human kind since ancient time from a medical perspective could be thought of as an ongoing battle against infectious diseases (Bockstael *et al.*, 2009). The continuous struggle of humans is divided between fighting a wide range of microorganisms and certain metabolites from the humans own body such as free radicals which could contribute in the establishment of diseases (Holmes *et al.*, 1992; Ames *et al.*, 1993).

During ancient times humans relied largely on honey and plant extracts as sources for antimicrobial agents. The application of honey and medicinal plants for the treatment of wound infections continued until the mid 1940s when antibiotics were introduced. The discovery of penicillin by Alexander Fleming in 1929 has marked the beginning of the antibiotic era which was further established by Dogmak's introduction of Sulpha drugs in

1932. The number of new antibiotics widely increased between the years 1940 and 1960 and this has led to a reduction in the mortality rate (Spellberg *et al.*, 2008). With the spread of antibiotics, honey and plant extracts were no longer parts of conventional medicine in developed countries; however, many third world countries have continued using them to the present day. The success of antibiotics in rapidly reducing high mortality and morbidity rates has encouraged excessive use of those antibiotics and led to the application of antibiotics in other fields including treating animal infections, in agriculture poultry and aquaculture (Banerjee, 1998). The wide application of antibiotics in various aspects of life exposed humans to different antibiotics and led to the development of antibiotic resistant bacteria (Malhotra-Kumar *et al.*, 2007).

The post antibiotic era has continued until the early 1970s , where infectious diseases were controlled and thought off as something of the past and that there is no place for infectious diseases in modern medicine. In 1967 and 1969, the US Surgeon General, William H. Stewart, was reported to have commented: "that we had essentially defeated infectious diseases and could close the book on them and that the focus should be shifted to other disorders where there is still need for therapy such as cancer, diabetes and cardiovascular therapies" (Spellberg *et al.*, 2008).

1.13 Antibacterial agents and applications in foods

Antimicrobials are used in food for two main reasons: (1) to control natural spoilage processes (food preservation), and (2) to prevent/control growth of micro-organisms, including pathogenic micro-organisms (food safety). Reports have shown that supplementing animal feeds with antibiotics came at the same time of the isolation, identification and characterization of vitamin B12 in 1948; however their approval for the

use as animal feed additives came later in 1950 (Gersema and Helling, 1986). Food animals were often exposed to antimicrobial compounds to treat or prevent infectious diseases and/or to promote growth (McEwen and Fedorka-Cray, 2002). Eleven compounds are listed as growth promoters (AGP), fifteen are listed to treat coccidiosis and six are listed for other purposes. Seven of these compounds, including bacitracin, chlortetracycline, erythromycin, lincomycin, novobiocin, oxytetracycline, and penicillin are also used in human medicine (Jones and Ricke, 2003).

1.14 Antibacterial agents and increased incidence of resistance

Bacterial resistance was observed immediately after the introduction of penicillin which kept on increasing until it has reached a point where some hospitals have only a single antibiotic for treatment of certain bacterial infections which are caused by multi resistant pathogens such as MRSA (Methicillin Resistant *Staphylococcus aureus*) an endemic pathogen in hospitals worldwide (Boyce *et al.*, 2005).

1.14.1 Factors that led to the spread antibacterial resistance

The frequent exposure of patients to antibiotics tends to induce selective pressure leading to an increased chance of bacteria acquiring resistant genes (Malhotra-Kumar *et al.*, 2007). Although bacteria have the ability to acquire resistance against antibiotics naturally either via mutation or gene transfer (Livermore, 2003), the increased resistance of bacteria to antibiotics is thought to be induced by human due to the miss use of antimicrobial agents (Cristino, 1999) in cases such as the use of antibiotics for prophylaxis during pregnancy or stress relief. This means that the existing lifestyle contributes to the development of resistant micro-organisms by exposing people to antibiotics while they are in a healthy condition (Deasy, 2009; Džidić *et al.*, 2008).

Moreover, the abuse of antibiotics in the health sector where the incorrect diagnosis, incorrect drug prescriptions, in addition to the unnecessary use of third generation antibiotics for treating irrelevant microbial infections has contributed as well. Furthermore, there are other factors which could have led to the continuous development of microbial resistance such as multiple exposure to products containing antimicrobial agents (e.g., toothpaste and cosmetic soaps), the use of poor quality antibiotics, patients poor adherence to treatment regimens, poor hospital hygiene as well as the spread of antimicrobial resistant strains associated with the increase ratios of international travel between different countries (Coia *et al.*, 2006; Deasy, 2009).

1.14.2 Mechanisms of antibacterial resistance

In order to be effective against given bacteria, antibiotics should have a vital target susceptible to a low concentration of the antibiotic in the bacteria. Moreover, the antibiotic should have the ability to directly contact to and bind to its intended bacterial target site in sufficient quantity (Finch, 2003). Whether antibiotic resistance is intrinsic or acquired, bacteria use their genetic determinants of resistance to encode different resistance mechanisms to neutralise the action of antibiotics, these resistance mechanisms can be classified into the following three basic types: (i) enzymatic inactivation by either destruction or chemical modification of the antibiotics; (ii) prevention of antibiotic access to the target site (iii) alteration of the antibiotic target site (Schwarz and Chaslus-Dancla, 2001; Mulvey and Simor, 2009).

1.14.3 Measures against the spread of antibacterial resistance

The means which have been applied to combat bacterial resistance to antibiotics include introducing new antibiotics to replace those with diminished activity since effective

treatment is essential in infection-control programmes. Moreover, combined therapy have been developed which is based on the use of two antibiotics with different mode of action in order to combat the infection in short time and minimise the chances new resistant strains emerging (Michel and Gutman, 1997). Combination therapy has been used for many years to treat cancer and tuberculosis, as well other infections particularly in immunocompromised patients.

Researchers have proposed high-tech solutions including the application of molecular vaccines or gene therapy as novel tools, which could solve the problem of antibiotic resistance. Although some research has been conducted and money expended in studying these approaches, the applications of these methods in medicine is limited (Alnaimat, 2011) and therefore new alternatives are required for bacterial control. The clinical use of novel agents or natural substances could be one option in overcoming the continuous increase of resistant micro-organisms and minimising reliance on antibiotic therapy.

1.15 Manuka honey: a licensed wound management product

Manuka honey is recognized worldwide for its distinct non-peroxidal activity. Manuka honey native to New Zealand and Jelly bush honeys produced from nectar from the *Leptospermum* family which represents the main source for medical grade honey. Although the non-peroxide antibacterial activity of honey has been demonstrated for many years (Molan and Russell, 1988; Allen *et al.*, 1991), it was not until recently that the component(s) responsible for this activity were identified. Mavric identified methylglyoxal (MGO) as the source of the unusual antibacterial activity of manuka honey, which was confirmed and isolated by Adams (Adams *et al.*, 2008; 2009; Mavric *et al.*, 2008). Methylglyoxal is formed in organisms as a side-product of a number of metabolic pathways. The most important source of these

pathways is glycolysis (Lin, 2010). An *in vitro* investigation has demonstrated that although MGO plays an essential role in the non-peroxidal activity of honeys, other unknown factors play a synergistic role (Jenkins *et al.*, 2011) and enhance the antibacterial activity of manuka honey.

The non-peroxidal activity of manuka honey is rated by using an agar diffusion assay (Allen *et al.*, 1991) and is registered by UMF trade mark (Unique Manuka Factor). Manuka medical grade honey is marketed by companies including Comvita which produces *Medi honey*. Revamil honey which is produced in green houses in the Netherlands is another licensed wound product which has a distinct activity due to the presence of B-defensin (Kwakman *et al.*, 2008) and low amounts of MGO.

1.16 Herb honeys

Herb honey is a honey-like product obtained from bees fed on a saccharose-based food containing herbal extracts or fruit juices. Herb honeys could be useful source for medicine as well as nutrition supplements since they have the medicinal properties of the herbs or other raw materials present in the food fed to the bees (Socha *et al.*, 2009).

The basic chemical composition of herb honeys is similar to that of natural honeys and most of them meet the quality criteria concerning acidity, water content, hydroxymethylfurfural content and diastase number, applied to natural honeys (Juszczak *et al.*, 2009). However; herb honeys might be different from natural honeys in the profile of flavonoids and phenolic acids which could be due to the variation in the production method as well as availability of biologically active substances such as antioxidants in the food fed to the bees. Since herb honeys are rich in natural antioxidants, they could play a major role in human nutrition. Although herb honeys are rich with phenolic compounds it should be taken into account,

however, that the levels of individual phenolic compounds are also closely connected with the climatic conditions of an area (Kenjeric' *et al.*, 2007; Čeksterytė *et al.*, 2006).

1.17 The need for alternative antibacterial agents

There is an urgent need for new novel antibacterial agents that could be used against antibiotic resistant bacteria as well as antioxidants that could protect the humans as well as foods from the destructive effects of oxidative stress. An immediate attention is required in order to backup the current available antibiotics as multi drug resistant bacteria become a real threat. Despite the spread of multi- resistant (or pan-resistant) pathogens large pharmaceutical companies continue to decrease their support for antibacterial and antibiotic research and continue with chronic disease therapy (e.g. cardiovascular, CNS, pain, arthritis and cholesterol-lowering agents), which means that with increased spread and emergence of resistant strains the mortality and morbidity could rise to a maximum level (Barker, 1999; Guerrant and Blackwood, 1999).

In addition to spread of pan-resistant pathogenic bacteria there is an increased incidence of food borne diseases which have negative socio-economic implications that leads to huge loss of money and lives. Therefore, new control measures are required to produce quality safe food and develop natural antimicrobial agents (Bajpai *et al.*, 2008). Moreover, the wide spread of resistance pathogens has led to control measures including the ban on antibiotic growth promoters (AGP) which will largely will largely affect the poultry and animal industry (Thakare, 2004). To minimize the loss in growth, there is a need to find alternatives to AGP. There are a number of non-therapeutic alternatives including enzymes, probiotics, prebiotics, and herbs (Banerjee, 1998 cited in Thakare, 2004).

1.17.1 Honey and medicinal plants as antibacterial agents against microbial pathogens

The antimicrobial application of honey has been demonstrated by several studies. In fact honey has been used in food preservation since ancient times (Taormina *et al.*, 2001; Mundo *et al.*, 2004). Moreover, the increased resistance of bacteria to antimicrobial agents is deriving researchers and industrialists to look for a mean of control of bacterial resistance. Effective control of resistance could be brought by innovating a preservative system, which is built upon several factors such as the introduction of combinations of preservatives that might broaden the spectrum of activity and decreases resistance and reduce toxic effects (Russell, 1991). However, increased consumer awareness about synthetic chemical additives raised the demands for consumption of foods preserved with natural additives. This high demand for natural food preservatives tempted the researchers as well as food (processors) industrialists to look for food additives with a broad spectrum of antimicrobial activity (Marino *et al.*, 2001). The increasing demands for healthy and high quality foods could be justified by the wide spread of quality health data, the unease regarding the use of artificial and chemical preservatives which also affects the organoleptic and sensory properties of foods and the numerous outbreaks caused by foodborne pathogens (Tajkarimi *et al.*, 2010). A wide range of food grade chemicals have been added during food manufacture to extend the shelf-life by stabilizing chemical change or by preventing or inhibiting microbial growth. Traditional and natural antimicrobial agents with potential or current value for use in foods as “secondary preservatives” were recently reviewed (Davidson and Brannen, 2005). Plant-origin antimicrobials are present in a variety of plants, spices and herbs. Spices and herbs are used for both flavouring and preservation purposes. Spices and herbs, which were originally added for improving taste, can also naturally and safely improve shelf life of food products (Holley and Patel, 2005).

1.17.2 Honey and plant extracts as natural food additives

Food processing technologies such as chemical preservatives cannot eliminate food pathogens such as *Listeria monocytogenes* or delay microbial spoilage totally (Gutierrez, *et al.*, 2009). Cold distribution of perishable food can help, but it cannot guarantee the overall safety and quality of the product. Fruits and vegetables have been followed by increasing reports of food-borne pathogenic microorganisms because of the presence of pathogens in raw materials (Lanciotti *et al.*, 2004; Zhu *et al.*, 2008).

Food additives are added into food for different purposes among those are two main purposes where honey and plant extracts could play essential role and favour other chemical additives. Honey could play a role in the preservation of food, add to the nutritional food value and be used as replacement for sugars where sweetening is required (Branen *et al.*, 2002). Food additives are usually investigated for their toxicity and honey is natural product which is safe and does not have toxic effects especially if the honey was irradiated (Molan and Allen, 1996). In order to be approved the additives risks are balanced and if the risk is minimal then it is accepted. Certain additives could reduce the cases of food poisoning and work as antimicrobials in addition to adding to other functions such as the flavour, colour or addition to the nutritional value (Davidson and Branen 2005). As the public demand for quality safer food without chemical additives required for the inhibition of food pathogens there is a new challenge to provide food preserved with natural preservatives and honey and herbal extracts might play a role in the future of food industry. Humans have used honey in food preservation since ancient civilisations (Molan, 1992a,b). Many plant extracts and spices have been used for seasoning and preservation of foods (Holley and Patel, 2005) and they could play important role in food if potential plant extracts or any effective plant extract combinations are determined. Natural antimicrobials could

play a part in this post antibiotic era by reducing the need for antibiotics, controlling microbial contamination in food, improving shelf-life extension technologies to eliminate undesirable pathogens and/or delay microbial spoilage, decreasing the development of antibiotic resistance by pathogenic microorganisms or enhancing the immune system in humans are some of the benefits (Tiwari *et al.*, 2009).

In the recent years natural foods and additives from natural sources including bees honey as well as a number of spices and plant extracts has gained increased interests from researchers, food and pharmaceutical industries as well as consumers (Tajkarimi *et al.*, 2010).

The increased interest in the benefits of natural foods and additives as well as the increased awareness about the harmful effects arising from synthetic additives and preservatives led to high demands for natural functional foods that will boost the human health rather than products containing substances with deleterious effects. An example of harmful additives includes synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Both antioxidants (BHA) and (BHT) are reported to be carcinogenic; hence, they have been restricted in foods, pharmaceuticals and cosmetics. Some European countries as well as Japan have banned the use of tertiary butyl hydroquinone (TBHQ), the most potent synthetic food antioxidant and other countries may ban it as well in the future (Ibrahim *et al.*, 2009).

Although it is believed that foodstuffs with high protein and fat content can protect bacteria from the antibacterial effect of herbal extracts, essential oils, and other natural antimicrobials these natural antimicrobials could be used in combinations with each other or in combination with some of the existing preservatives such as EDTA to prevent the resistance. Combination of natural antimicrobials should be the choice in order to avoid

using higher concentrations of antimicrobials which could largely affect the organoleptic properties of the food and undesirable by the consumers (Tserennadmid *et al.*, 2010).

1.18 Ethylenediaminetetraacetic acid (EDTA)

EDTA is a chelating agent which has been found useful in increasing the permeability of gram negative bacteria to antibacterial agents; hence, acting synergistically by making the complex wall of many gram negative bacteria more permeable to and sensitive to the action of antibacterial agents. EDTA is an approved preservative agent in different foods such as cooked canned crab meat, mayonnaise, sausage, and salad dressings (Rasmussen *et al.*, 2008). EDTA forms metal complexes by reacting with alkaline earth and heavy metals and there by removes reactive multivalent cations from solution; which results in prevention of chemical or biochemical adverse reactions depending on metals. Moreover, EDTA is known to have the potential to inhibit the growth of food pathogens by the disruption of the bacterial membrane through the chelation with cations, which has been proved against Gram negative bacteria such as *Pseudomonas aeruginosa*. Furthermore, EDTA might act as a potentiator for other lethal agents either by increasing the permeability and giving access into the cell or by chelation of cations essential for the repair of injured cells (Davidson *et al.*, 1983).

Although there are many studies which involve the use of combination of two antibiotics, other studies involved a combination between antibiotic and non antibiotic such as essential oils, honey or even chelating agents such as EDTA. To our knowledge; there are no reports which include a combination of honey with herbal extracts or honey with EDTA, hence, in this research we aim to study the potentiation of honey with EDTA and plant extracts, should any synergistic interactions occur that could pave the way for potential use of this

combination in therapeutics as well as the food industry since honey is a food and sweetener and EDTA is an approved food preservative as well as potentiator and chelating agent (Rasmussen *et al.*, 2008).

1.19 Combination therapy

Clinicians usually apply combination antibacterial therapy to manage difficult infections particularly those caused by multidrug resistant bacteria. Combination therapy is also approached in other cases to prevent the emergence of multi drug resistant strains (Urban *et al.*, 2003).

Combination therapy is justified by the possibility of enhanced pharmacodynamic effect of the antibacterial combination that could be attained particularly against problematic strains of certain bacteria such as those of MRSA, VRE, ESBL or *Acinetobacter baumannii* it is also favourable to prevent the emergence of resistant strains as well for the treatment when the causative agent is unknown. However, not all antibacterial combinations are favourable as certain combinations could have a negative effect as one antibacterial agent might negate the other leading to antagonistic combination (Singh *et al.*, 2000). In Figure 1.2 the Arabic translations describe medicines from combinations of honey and plants.

The fact that an antibacterial combination could lead to an antagonistic or reduced effect rather than synergistic or enhanced effect necessitates the evaluation of antibacterial combinations particularly those used in folk medicine since, those combinations are used without scientific quantification and they could lead to antagonistic combinations which could cause harmful effects and complicate the conditions of the patients rather than curing them. In addition it is also necessary to verify antibacterial combinations particularly when

they are applied for prevention of emergence of resistant strains (Eliopoulos G.M. and Eliopoulos C.T. (1988).



Figure 1.2 From an Arabic translation of De Materia Medica of Dioscorides ("Preparation of Medicine from Honey").

1. 20 Aims and objectives

The aim of this study was the evaluation of antibacterial activity of selected Sudanese honeys, the search for any honeys with unusual antibacterial activity and the validation of selected honey/ plant extracts combinations currently used in folk medicine in Sudan.

Objectives of the study are expressed as aims in individual chapters as follows:

Chapter 2: To investigate the antimicrobial and physicochemical characteristics of selected Sudanese honeys

Chapter 3: Screening a range of plant medicinal extracts for their antimicrobial & antioxidant potential

Chapter 4: To investigate the bioactivity of selected Sudanese honey against *Escherichia coli* and *Staphylococcus aureus*

Chapter 5: To determine Synergistic interaction between combination of honey and plant extracts as well as honey and Ethylenediaminetetraacetic acid (EDTA)

CHAPTER TWO

ANTIMICROBIAL AND PHYSICO-CHEMICAL CHARACTERISATION OF SUDANESE HONEYS

1. Introduction

The physicochemical, antioxidant and antimicrobial properties of various honeys from different countries around the globe has been reported (Singh and Kumar 1997; Molan and Russell, 1988; Anupama *et al.*, 2003, Iurlina and Fritz, 2005).

Sudanese have used honeys as food as well as medicine since, as long as the ancient Egyptians where there was a joint history between Upper Egypt and Lower Egypt. Upper Egypt included large parts of the Northern states of Sudan today and this has continued until the Dam and regional borders has separated people from the same tribe (*Nuba*) in the North of Sudan and the South of Egypt. Probably the use of honey has continued since until it was established in other parts of Sudan especially with the introduction of Islam and Prophetic medicine.

Honey is an ancient remedy which has been used throughout the globe for centuries. Despite the relegation of honey from conventional medicine to folk medicine in Europe and other developed countries in the 1940's after the discovery of antibiotics, third world countries in Africa, Arabic Peninsula as well as many other countries including Sudan has never stopped using honey in medicine. The emergence of antimicrobial resistant strains has led to the re-evaluation of many ancient medicines including honey which was found to possess potential antibacterial activity against multi drug resistant pathogens including methicillin resistant *Staphylococcus aureus* (MRSA) (Natarajan *et al.*, 2001), vancomycin resistant *enterococci* (VRE) (Cooper *et al.*, 2002a) as well as many other problematic organisms including *Burkholderia cepacia* (Cooper *et al.*, 2000) and *Pseudomonas aeruginosa* (Cooper *et al.*, 2002b).

Although honey has various applications in Sudanese culture and folk medicine, the re-discovery of honey in modern medicine has been largely focused on the successful application of honey for wound management (Cooper *et al.*, 2001) and this was encouraged by the eradication and clearing of wounds by using Australasian honeys which were shown to have distinct antibacterial activity which was not found in many other honeys (Blair *et al.*, 2009).

The extensive research in Manuka honeys led by Prof. Peter Molan in University of Waikato in New Zealand and Prof. Rose Cooper in Cardiff Metropolitan University (previously University of Wales Institute, Cardiff) has led to the production of a range of licensed honey and honey dressings which are widely accepted for medicinal use in many countries including New Zealand (Molan and Betts 2004), Australia (Johnson *et al.*, 2005), UK (Dunford and Hanano 2004; Stephen-Haynes 2004), Netherlands (Kwakman *et al.*, 2008), USA and Canada (Henriques *et al.*, 2010) .

Since the composition of honey depends on bee species, botanical species and geographical region as well as the contribution of the beekeepers (Kücük *et al.*, 2007; Henriques *et al.*, 2005), it was necessary to evaluate the physicochemical characteristics of all the honeys involved in the study. The determination of the physicochemical parameters of the honeys also aids in the identification and authentication of the tested honeys and could lead to the selection of the tested honey for the continuous use for treatment of certain ailments or food additive for sweetening and preservation.

The application of specific types of honeys for the treatment of certain ailments has been practiced since ancient times. Dioscorides recommended the application of pale yellow honey from Attica for the treatment for "rotten and hollow ulcers", while Aristoles

preferred to apply pale honey in the preparation of salves for sore eyes and wounds (Molan, 2006). Even in the recent years certain honeys in folk medicine were claimed to have better values compared to others such as lotus honey in India and strawberry honey in Sardinia (Molan, 2006). The honey which is to be used for therapeutic purposes must be irradiated or sterilized before use since, natural honey contains microorganisms and spores (Snowdon and Cliver, 1996).

The marketing of honey is governed by standards from bodies such as the International Honey Commission (IHC), the EU Council Directive, and the Codex Alimentarius. The standards of Codex Alimentarius and EU directive are relatively similar with minor differences; however, the Codex Alimentarius is more detailed (Bogdanov, 2002). Depending on these standards the honey sample might be accepted or rejected for marketing.

Several studies attributed the therapeutic properties of honeys to the high sugar content, low water content, acidic pH, phytochemicals and hydrogen peroxide (Dold *et al.*, 1937 in White *et al.*, 1963) (Wakhle and Desai, 1991; Molan, 1992a; Wilix *et al.*, 1992; Postmes *et al.*, 1993; and McCarthy, 1995). However, extensive research in the field has led to the identification of selected honeys possessing an unusual non peroxide antibacterial activity (Molan and Russell 1988), which (Mavric *et al.*, 2008) has attributed to Methylglyoxal (MGO). Kwakman and his colleagues have extensively studied the antibacterial properties of a Revamil Source honey from the Netherlands and claimed that they were able to identify all the factors associated with the antibacterial activity of that honey, which they concluded as sugar content, hydrogen peroxide, MGO, and a bee defensin-1 which is also available in royal jelly (Kwakman *et al.*, 2010).

In Sudan there is very limited research in the physicochemical and antimicrobial properties of Sudanese honeys in spite of the wide application of honeys in the Sudanese medicine and culture (Mohammed and Babiker, 2010; Makawi *et al.*, 2009a; 2009b; Farouk *et al.*, 1988; Wadi *et al.*, 1987).

There is paucity of information on the antimicrobial potential of local Sudanese honeys, although recently some research has started in the National Centre for Research, Khartoum, Sudan most of the research is merely focused on the determination of physicochemical parameters of a range of Sudanese honeys from randomly selected beekeepers.

Aim

The aim of this study was to quantify the antibacterial activity of 60 Sudanese honeys against a phenol standard and search for any unusual antibacterial activity.

Objectives

1. To collect and determine physicochemical characteristics of selected Sudanese honeys
2. To determine the antimicrobial potential of these Sudanese honeys
3. To assess the presence of non-hydrogen peroxide component(s) in Sudanese honeys

2.2 Honey sample collection

With the assistance of the National Centre for Research (NCR), Khartoum, Sudan. Sixty honey samples were collected from beekeepers from distinct sites across Sudan Figure 2.1, Table 2.1. A questionnaire was used to evaluate the sampling procedure. The honey samples were collected during 2009. The samples were collected from different areas of Sudan, however, the samples from the Southern and Western regions didn't cover all the honey producing areas and this was particularly due to the crisis in the areas during collection

seasons. The Northern region is desert except around the path of the river Nile, hence, there were no honeys from the North of Sudan. All the honeys were raw honeys which were neither heated nor irradiated, packed in plastic containers of 250g then transported to Khartoum (NCR), once received the honeys were stored below 20°C until they were shipped to Cardiff Met where they were stored at 4°C until tested. So as not to allow any bias samples were randomly labeled with a combination of numbers and letters.

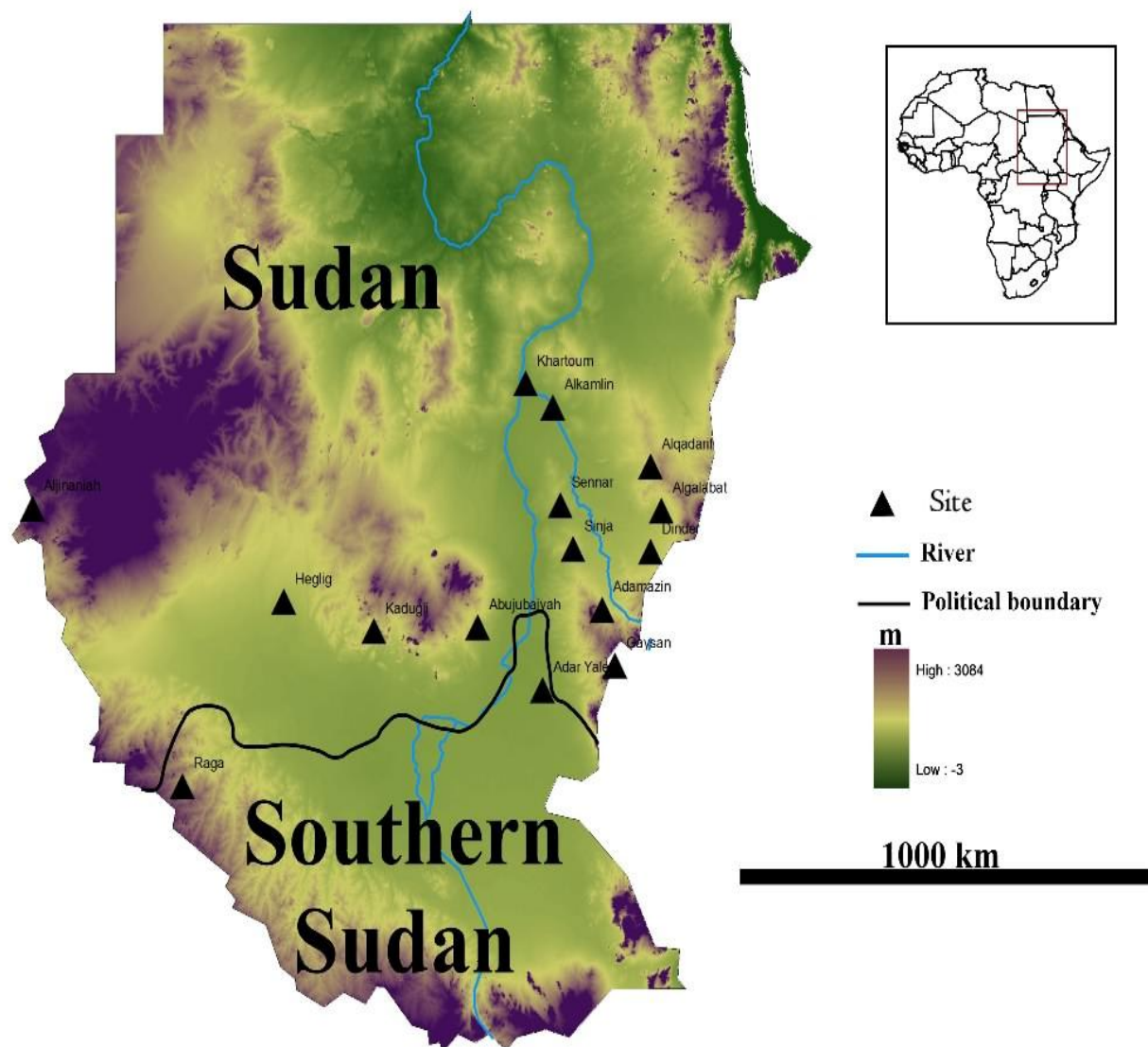


Figure 2.1 A thematic map illustrating honey collection sites

Table 2.1 Honey sample sites with their respective latitude and longitude locations and the region of Sudan.

Site	Location	Region
Aljinaniah	13°27`N 22°26`E	Western Region
Adar Yale	496354 1107248	Southern Region
Gaysan	10°44`N 34°47`E	Eastern Region
Khartoum	15° 52N` 32° 6`E	Central Region
Dinder	12°49`N 35°22`E	Central Region
Sinja	12°54`N 33°59`E	Central Region
Sennar	13°33`N 33°33`E	Central Region
Alqadarif	14°02`N 35°22`E	Eastern Region
Raga	08°27`N 25°40`E	Southern Region
Algalabat	13°23`N 35°44`E	Eastern Region
Alkamlin	15 °09`N 33° 17`E	Central Region
Kadugli	11°06`N 29°42`E	Western Region
Abujubaiyah	11°13`N 31°59`E	Western Region
Heglig	11°59`N 27°53`E	Western Region
Adamazin	11°45`N 34°20`E	Western Region

2.3. Physicochemical characterisation

The PH, moisture content, sugar content and hydroxymethylfurfural was determined for all the honey samples after equilibrating to 20 °C for 24 hours.

2.3.1 Moisture content

Using (0-25%) Atago HHR-2N refractometer (Japan), the moisture content was evaluated as described by the International Honey Commission (Bogdanov, 2002). After thoroughly mixing the honey a drop of each sample was placed on the lens of the refractometer which was held towards the light and the measurement was recorded (Henriques *et al.*, 2005).

2.3.2 Sugar Content

Using a Bellingham and Stanley (0-85%) refractometer (England) the sugar content was evaluated by placing a drop from each honey sample on the lens of the refractometer which was held towards the light and the measurement was recorded (Henriques *et al.*, 2005).

2.3.3 pH

The pH was determined following the method described by International Honey Commission (Bogdanov, 2002), using a magnetic stirrer 10g of honey was dissolved in deionised water and the PH was evaluated by a calibrated Jenway 3010 pH meter.

2.4 Hydroxymethylfurfural concentration

Five grams of each honey sample were dissolved in 25ml of deionised water, transferred to a 50ml volumetric flask after the addition of 0.5ml of carrez solution I (Potassium ferrocyanide in 100 ml deionised water) as well as 0.5mL of carrez solution II (Zinc acetate in 100 ml deionised water) then mixed and made up to 50ml by adding deionised water. The mixture was filtered through filter paper and the first 10ml of the filtrate were rejected and the remainder divided into two tubes. Five ml of deionised water were added to the first tube and 5ml of sodium bisulphite were added to the second tube. The readings were measured spectrophotometrically at wave lengths 284 and 336. The HMF concentration was

determined by subtracting the difference divided by weigh of the honey sample and multiplied by 149.7 to get the concentration in term of mg/kg (White, 1979).

2.5 Melissopalynology (Pollen analysis)

Melissopalynology was performed according to Louveaux *et al.* 1978, using the non-acetolytic method with some modifications. Reference pollens were not collected. The floral source of the honey samples was determined by dissolving 10g of each honey sample in 20 ml of deionised water, then centrifugation at 2500 rpm for 10 minutes. The supernatant was decanted and the sediment was resuspended in liquefied glycerine-gelatine, then transferred to a glass slide and mounted with cover slip. Using a Nikon microscope (model number YS-T 166073, Japan) a minimum of 100 pollen grains were counted in each sample for estimation of the relative frequencies of pollen types. The floral source was identified by the presence of more than 45% of the dominant pollen grain (Loveaux *et al.*, 1978). Morphological types of pollen grains were determined using photographs of selected pollen grains provided by Dr. Seif Eldin Abdurrahman Mohamed from the National Centre for Research, Khartoum, Sudan.

2.6 Antibacterial activity

The antibacterial activity of the honeys was assayed by the agar well diffusion method of Allen which was based on the punch plate assay for inhibitory substances described in the microbiology standard laboratory methods manual for the New Zealand dairy industry with some modifications (Allen *et al.*, 1991)

2.6.1 Preparation of honey samples for the assay

All samples were prepared aseptically by weighing 10g and were handled away from direct sunlight. The samples were added to sterile 10ml deionised water and placed in a 37°C

incubator for 30 min. From the 50% (w/v) solutions of each of the prepared honey samples were diluted to 25% (w/v) by taking 1 ml of each and adding it to either 1 ml of sterile deionised water (for total antibacterial activity) or 1 ml of catalase solution (for non-peroxidal activity). The catalase solution was prepared by dissolving 0.02 g catalase (Sigma) in 10ml of deionised water in order to determine the non peroxide activity of the honey samples compared to phenol standards with known concentrations.

2.6.2 Preparation of phenol Standard for the assay

A series of concentrations of phenol standard solution (2%, 3%, 4%, 5%, 6% and 7% w/v) was made by dissolving phenol in sterile deionised water. The set was stored in a dark refrigerator.

2.6.3 Preparation of bacterial culture for the assay

A reference culture of *Staphylococcus aureus* NCTC 6571 was used for the study. The inoculum was prepared by inoculating the bacteria in 100 ml TSB which was incubated at 37 ° C for 18-24 hours then the optical density was adjusted to 0.5 at 540 nm using a Jenway 6310 spectrophotometer by addition of further Tryptic Soy broth (TSB).

2.6.4 Assessing the total and non peroxidal activity

The antimicrobial activity of the honey samples was determined following an agar diffusion method (Allen, 1991). This method allows the differentiation between peroxide and non peroxide activity by the addition of either deionised water to determine the total activity or catalase solution 0.02 g (Sigma) in 10ml of deionised water in order to determine the non peroxide activity of the honey samples compared to phenol standards with known concentrations. This bioassay plate enables each honey sample to be tested in a quadruplicate.

Large square plates (Nunc Bioassay Dishes, 243 mm x 243 mm x 18 mm) seeded with *Staphylococcus aureus* (NCTC 6571) were prepared by adding 100 µl of an 18-h culture of *S. aureus* bacteria in Trypticase Soy Broth (Oxoid) to 150 ml of sterilized nutrient agar which was autoclaved and cooled to 45°C. Then immediately after mixing the agar was poured in the plates on a level surface and the plates were stored for 24 h at 4°C before being used.

Sixty-four wells were cut in the agar with a cooled flamed 8- mm cork borer, using a quasi-Latin square as a template. The template was prepared on a black card, 243 mm x 243 mm. A 25-mm grid was drawn on the card, 34 mm away from the sides, and the wells were centered on the intersections of the grid. The wells were numbered just above the intersections using a quasi-Latin square which enabled the samples to be placed randomly on the plate (Lin, 2010).

The honey samples were tested by adding 100 µl to each well. The plates were incubated for 18 hrs at 37°C and were then placed over the template. Using a digital calliper, the diameter of the clear zones was measured along the horizontal and vertical lines on the template and recorded in mm. All measurements were recorded without reference to the identity of the samples in the wells (Lin, 2010).

2.7 Statistical analysis

Samples were tested in triplicate, and each assay was repeated at least 3 times. Data were expressed as mean. Statistical analysis was done through Graph Pad Prism version 5.00 for Windows, Graph Pad Software, San Diego California USA (www.graphpad.com). Error bars represent means \pm SD. Differences between means were considered to be significant at $p < 0.05$.

2.8 Results and Discussion

Physicochemical parameters of honey could be a useful tool for the characterisation and identification of honeys from different areas of Sudan. Therefore, this chapter investigates the physicochemical and antibacterial properties of natural honey samples obtained from different places in Sudan. The results for the physicochemical characterisation and antimicrobial activity of the rested honeys are presented in Tables 2.2, 2.3, 2.4 and Figures 2.2, 2.3, 2.4, 2.5.

Table 2.2 The range and average values obtained from all honeys tested for moisture and sugar content, pH, and HMF.

Parameter	Range	Average
Moisture content	13.9 - 20.7%	17.3%
Sugar content	76 - 83%	80 %
pH	3 - 6	4.0
HMF	1.8 – 89 mg/Kg	30.5 mg/Kg

Table 2.3 The pH, sugar, water content and HMF results for all the honeys tested in this study

Code	pH	Sugar content%	Water content	HMF mg/kg
KDIII	3.05	81	16.1	11.83
NS1	3.02	77	21	16.62
KDV	4.57	82	15	3.3
MS4	3.62	82	15.8	62.72
SP5	4.13	82	15.5	19.61
AR1	3.9	79	18.7	31
HC8	6.23	81	16.2	3.44
HC17	4.67	81	17	9.3
HC9	4.87	82	15.6	72.16
XV8	3.15	82	15	59.58
MS3	3.84	81	16	15.12
HC5	3.87	79	18.9	30.4
XV6	3.71	82	15.2	22.16
SH4	3.35	78	21	24.9
NS3	4.96	77	20	45.06
AF3	4.65	80	17.8	19.01
AR2	3.89	76	19	4.34
SH5	4.0	77	20.5	84.28
SA1	3.19	78	19	45.5
KBv	3.5	80	16.5	38.2
SP6	4.05	79	18.7	59.28
NS10	4.17	79	18.8	11.23
KBIII	3.56	81	16.7	5.89
KBIV	3.6	82	15	89.07
KDI	3.87	82	14.8	26.4
HC3	5.11	79	17.9	78.59
SP3	4.38	83	13.9	7.04
XV4	3.98	82	15.3	47.5
AF1	4.91	80	18.9	4.34
XVL	4.23	79	18	21.56
XV3	3.78	82	15	56.7
XV1	2.98	82	15	60.7
XV2	3.21	81	15	52.25
MS6	3.83	79	18	60.33
SP2	4.35	80	17	3.44
HC6	4.75	79	18.2	6.59
SH1	3.44	78	19	12.3
AF2	4.73	79	18	9.28
NS5	5.19	83	14.2	78
HC7	4.0	78	19.2	47.46
NS4	3.06	83	13.7	6.3

NS8	3.92	79	15.8	50.9
BWX	3.77	82	15.6	54.34
KDII	3.19	80	18	1.8
NS9	4.19	77	20.7	15.7
HC10	3.64	81	17	9.3
NS2	4.83	79	19	4.9
SP1	4.19	82	15.5	30.4
HC2	3.53	81	16.7	25.3
HC1	4.3	82	15.8	17.07
XV7	4.03	79	18.8	42.7
XV5	3.57	83	15.1	20.8
SP4	3.28	77	20	45.7
HC4	3.21	76	21	4.8
AR3	5.15	79	19	27.8
KBI	3.11	83	14.8	8.8
KBII	3.61	79	19	46.71
KDIV	3.48	80	18	4.3
MS5	4	82	17.9	10.5
OII	5.01	81	20	42.81

Table 2.4 Total antibacterial activity and predominant floral source for all the honeys tested in this study

Code	Total activity equi Phenol %†	Pollen analysis
KDIII	ND*	Acacia spp.
NS1	16.2	Polyfloral
KDV	10.7	Ziziphus Spina christi
MS4	5.5	Polyfloral
SP5	4.5	Polyfloral
AR1	18.7	Polyfloral
HC8	16.2	Polyfloral
HC17	13.2	Polyfloral
HC9	23.7	Polyfloral
XV8	ND	Balanites aegyptiaca
MS3	18.3	Ziziphus Spina christi
HC5	11.2	Acacia spp
XV6	15.4	Polyfloral
SH4	ND	Ziziphus Spina christi
NS3	10.6	Acacia spp
AF3	10.5	Balanites aegyptiaca
AR2	7.3	Balanites aegyptiaca
SH5	5.2	Ziziphus Spina christi
SA1	ND	Ziziphus Spina christi

KBv		2.5	Polyfloral
SP6	ND		Acacia spp.
NS10		10.38	Polyfloral
KBIII	ND		Acacia spp.
KBIV	ND		Ziziphus Spina christi
KDI		7.9	Acacia spp.
HC3	ND		Ziziphus Spina christi
SP3	ND		Polyfloral
XV4	ND		Acacia spp.
AF1		6.6	Acacia spp.
XVL		23.4	Polyfloral
XV3		2.7	Ziziphus Spina christi
XV1	ND		Polyfloral
XV2		5.9	Polyfloral
MS6	ND		Polyfloral
SP2	ND		Acacia spp.
HC6		17.7	Ziziphus Spina christi
SH1		7.2	Ziziphus Spina christi
AF2		3.4	Balanites aegyptiaca
NS5		20.5	Ziziphus Spina christi
HC7		12.9	Ziziphus Spina christi
NS4		14.3	Ziziphus Spina christi
NS8		16.3	Polyfloral
BWX	ND		Ziziphus Spina christi
KDII		17.3	Polyfloral
NS9	ND		Polyfloral
HC10		20.3	Polyfloral
NS2	ND		Polyfloral
SP1		26.5	Polyfloral
HC2		13.9	Ziziphus Spina christi
HC1	ND		Sunflower
XV7		12.97	Polyfloral
XV5		16.8	Polyfloral
SP4	ND		Ziziphus Spina christi
HC4		5.5	Polyfloral
AR3		16.3	Polyfloral
KBI	ND		Acacia spp.
KBII		6.9	Acacia spp.
KDIV	ND		Polyfloral
MS5		2.1	Polyfloral
OII		5.2	Sunflower

†None of the Honeys tested exhibited non peroxidal activity so these data are not shown.

* ND = Not defined

The water content for all honey samples was in the range accepted by the EU directive as well as for the Codex Alimentarius except for three samples which met the old standard which was 21/100g of honey. The PH of all the honey samples was acceptable except for only one sample. The sugar content was in the normal range. While two samples exceeded the maximum acceptable level of HMF which is 80g/kg for tropical countries set by the Codex Alimentarius (Bogdanov, 2002).

The water content of the honey samples falls within the reported levels for floral honeys (Mincione and leuzzi, 1993; Anupama *et al.*, 2003; Malika *et al.*, 2005). The variations in the moisture content of honey could be attributed to the variations in the composition and botanical sources of honey (Cavia *et al.*, 2004; Malika *et al.*, 2005). The increase in the honey water content could also be indicator of adulteration (Tysset and Rousseau, 1981).

The water content of natural honeys is mostly in the range of 15-21% (White, 1975). The water content of honey is considered as the most important parameter because it has great effect on the honeys' shelf life. Due to the major role played by the levels of water present in the honey most of the other parameters are optional, while it is essential for the honey water content to be below 20g/100g of honey in order to be acceptable for global marketing (Codex Alimentarius 2001).

Chirife demonstrated the importance of the determination of water activity of honey to predict moisture exchange with environment because water activity drives the water from/to honey (Chirife *et al.*, 2006). The water activity of honey rarely exceeds 0.60 due to the high sugar and low moisture content which is enough to inhibit the growth of osmotolerant yeasts (Zamora and Chirife, 2006).

Although natural honeys have acidic pH that could be low enough to affect the growth of bacteria, this acidity could be changed by the effects of buffering fluids in the human body.

Gluconic acid which mainly originates as a result of the action of the enzyme glucose oxidase added by the bees at ripening is the predominant acid found in natural honeys (Naman *et al.*, 2005). Honey is a solution with high sugar content, high osmolarity and low water content all these combined together limits the growth of microorganisms (Efem, 1988).

The physicochemical results of this chapter indicates that the physicochemical parameters of local Sudanese honeys are fairly comparable with the local honeys from many parts of the world as shown in the available literature (Meda *et al.*, 2005; Henriques *et al.*, 2005; Singh and Kumar Bath, 1997; Molan and Russell, 1988; Anupama *et al.*, 2003, Iurlina and Fritz, 2005).

2.9 HMF (Hydroxymethylfurfural)

In this study only two honey samples has exceeded the 80 mg/kg which is highest acceptable for tropical countries and this could be due to bad storage, before they reached NCR ambient temperature over 35°C in Sudan or possible adulteration with invert sugars (White, 1980).

HMF which is formed during the decomposition of food products that contains sugars or carbohydrates is used as a quality indicator for the adulteration or excessive heat treatment during production of foods such as honey, tomatoes paste, syrup and jams (Makawi, 2009a). The National Institute of Environmental Health Science nominated testing HMF due to the fact that other members of the family are considered carcinogenic; however, the only reports for toxicity tests are mostly confined to mice and rats (Ulbricht *et al.*, 1984).

In a recent study Makwai *et al.*, 2009a reported a range between 5 mg/kg to 922 mg/kg for commercial honey samples. While, Cushman reported that it is common for honeys from

tropical countries to have content over 100mg/kg due the exposure of honeys to elevated temperatures during production and distribution (Cushman, 2007).

Quantification of HMF in honeys and other sugar products is necessary not only due to the concerns about toxicity which is limited to research in mice and rats but, due to the fact that HMF is a quality parameter which indicates heat treatment or adulteration with inverted sugars(White, 1980).One of the objectives of this research as well as the previous studies (Makawi *et al.*, 2009a) is to raise the attention of Sudanese authorities towards the importance of legislative and standards of food products to ensure the maintenance of good quality of food products in the Sudanese market which is focused on physical changes rather than chemical decomposition.

This study has shown the necessity of storing honey as well as other sugar containing food at temperatures below 30°C (White and Subers, 1964a;b; Makkawi *et al.*, 2009a). Many people in Sudan believes the darker the honey the better the quality which could be true in terms of antioxidant capacity (Gheldof *et al.*, 2002) but, the problem is that they also believe honey never expires and they do not pay attention to any Maillard reaction or any other enzymatic reaction which could have changed the original colour of the honey over time; hence, it is essential that the government must agree legislation and try to educate society about reactions in food that could directly or in indirectly harm the human body. This is important in Sudan and most of the third world countries because people there rarely care about the quality of the food.

2.10. Pollen Analysis

In spite of the extensive attempts to establish new methods for the clarification of botanical sources based on specific physicochemical characters with the help of advanced techniques

and equipments pollen analysis remains widely the most accepted and applied method for the determination of the honey floral source (Louveaux *et al.*, 1978; Maurizio, 1975).

Based on the results of melissopalynology honey is classified into two main types' monofloral and polyfloral. Honey is regarded as mono or unifloral when the represented pollens in the count have more than 45% of the pollen grains derived from one plant species since honey usually contain other pollens visited by the bee (Maurizio, 1975). Although many plants have enough pollen some of them represent low levels of pollens which give pollen counts that are only 10-20 %. Hence; those honeys from plants which are known to have low pollen counts are usually identified as mono floral honeys even if their count was below 20%. Examples of plants with very low pollen content include citrus, rosemary and lavender honeys. (Pérez-Arquillué *et al.*, 1995; Serra Bonvehí and Ventura Coll, 1995). Unlike floral honeys, honeydew honeys are usually identified microscopically by the presence of honeydew elements (HDE), such as microalgae, fungal mycelia and spores. A ratio of HDE/P higher than 3 is generally required to establish a honey sample as honeydew honey (Louveaux *et al.*, 1978).

Researchers are still trying to agree a better and accurate method for the determination of the honey floral source since melissopalynology have many disadvantages including the difficulty in counting, identification and interpretation of pollen slides which needs highly qualified and skilful personnel (Molan, 1998). Moreover some honeydew honeys could not be identified by pollen analysis since they do not fulfill the ratio of HDE/P higher than 3 which is generally recommended to establish a honey sample as honeydew honey such as those from *Quercus sp.* (Serra Bonvehí, *et al.*, 1987 in Soria *et al.*, 2004).

The results of melissopalynology slides in this study have shown that differences between the pollen slides of different honeys are mainly attributable to the variation among the

honeys botanical source. Although all the honeys were classified as blossom honeys two of the honeys were found to have some honeydew elements which could be justified by the source of the honeys (Raga) as the area is relatively high and the literature has demonstrated the influence of the beekeeping zone as honeydew honeys were found to originate from mountain zones (Soria *et al.*, 2004) and with exception of Raga, Gaysan and Aljinaniah all the other Sudanese honeys were collected from low areas as could be seen in the map (Figure 2.1). The interpretation of the pollen slides was very difficult as the identification was mainly based on the images of pollen grains found in the literature as in this study it was not possible to collect and prepare reference pollen slides for each site or beekeeping area which has resulted in the inaccurate interpretation of some slides as images for some pollens of some plants such as *Balanites aegyptiaca* could not be found and hence, when pollens were seen to be different from known pollens the study records the presumed pollen source given by the beekeepers which could turn to be false. Another problem was the determination of the Acacia pollens which were impossible to determine as there are very minor differences between different Acacia grains which needs very skilled personnel as well as other physicochemical tests for the confirmation of the Acacia species. Figure 2.6 shows some *Acacia* grains which was mounted after staining with Safranin. While melissopalynological analysis remains nowadays as the only technique which allows a direct botanical source characterization, physicochemical parameters afford quantitative results and allow an approximate estimation of the presence of honey blends (Soria *et al.*, 2004). When compared to nectar honeys, honeydew honeys are generally distinguished on the basis of their higher values of pH, acidity, ash, electrical conductivity and darker colour, as well as by a lower monosaccharide and a higher di- and trisaccharide content (Mateo and Bosch-Reig, 1997, 1998; Mateo *et al.*, 1992).

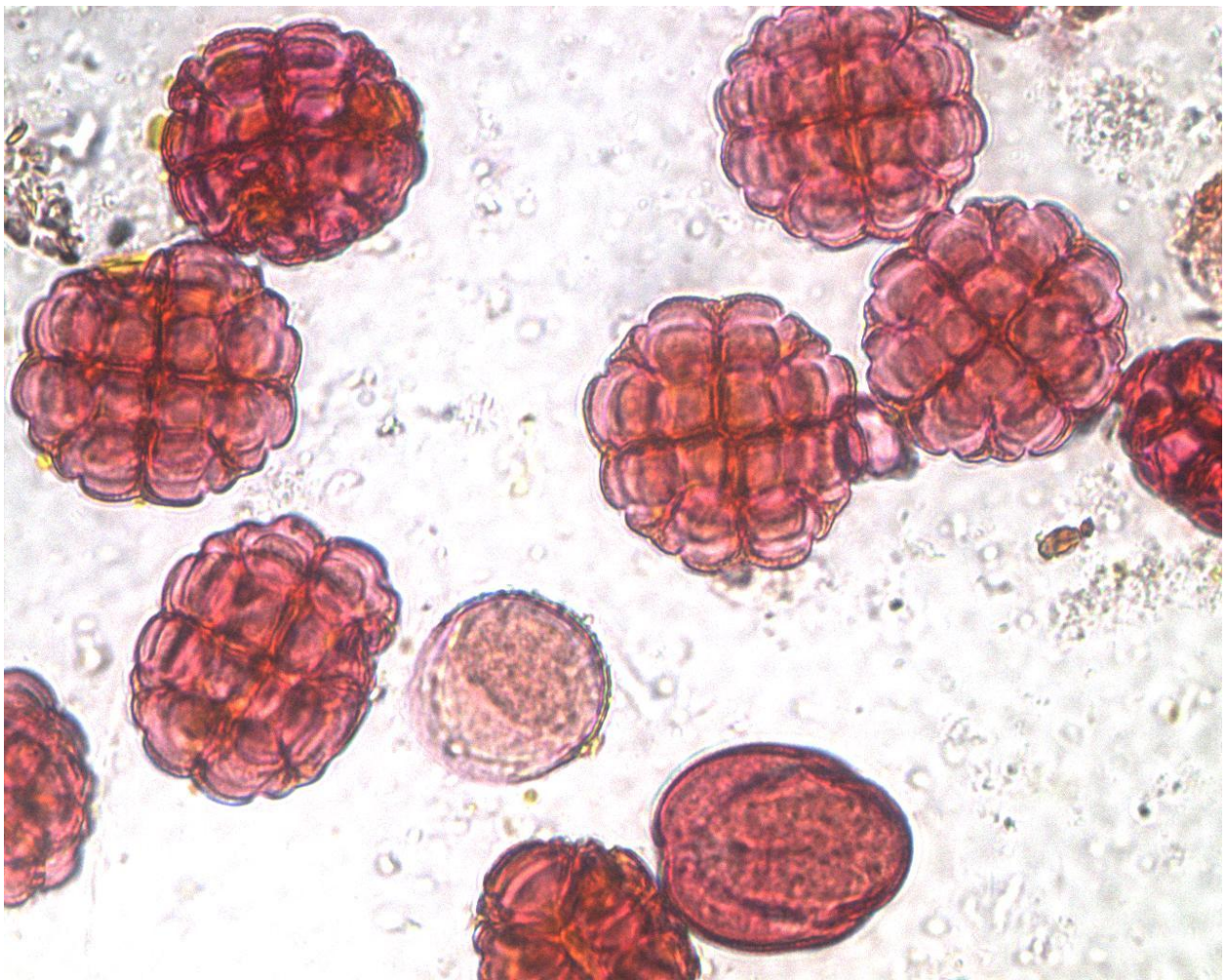


Figure 2.2 Acacia pollen grains stained by Safranin basic stain and observed under the microscope after preparation of the slide following non-acetolytic method of Louveax *et al.*, 1978

2.11 Antibacterial activity

The results reported in Table 2.4 showed that many of the Sudanese honeys exhibited peroxidal activity. This activity expressed as % phenol equivalence is due to the presence of hydrogen peroxide produced by the enzyme glucose oxidase (White *et al.*, 1963; White and Subers, 1964a,b). When catalase was added all the honeys lost their activity, which means there was no non-peroxidal activity. Dold *et al.*, 1937 (Cited in Molan, 1992a) reported that antibacterial activity of honeys due to hydrogen peroxide is affected by exposure to sunlight

(White and Subers, 1964a,b) heat as well as storage condition. While non-peroxidal activity, found in selected Manuka honeys, is not affected by heat and light (Russell and Molan, 1988; Allen *et al.*, 1991). The presence of glucose oxidase was first reported by Gauhe *et al.*, 1941 (cited in Molan, 1992a) later White and his colleagues demonstrated the production of hydrogen peroxide and gluconolactone by glucose oxidase upon the dilution of honey (White *et al.*, 1963). Adcock was the first to discover the destruction of hydrogen peroxide in the presence of catalase (Adcock, 1961).

Figures 2.3, 2.4 and 2.5 present the peroxidal activity of the honeys by the type of hive classification (modern or traditional), Beekeepers practice and collection site. In the figures n refers to the number of honey samples tested. Each sample was tested in triplicate.

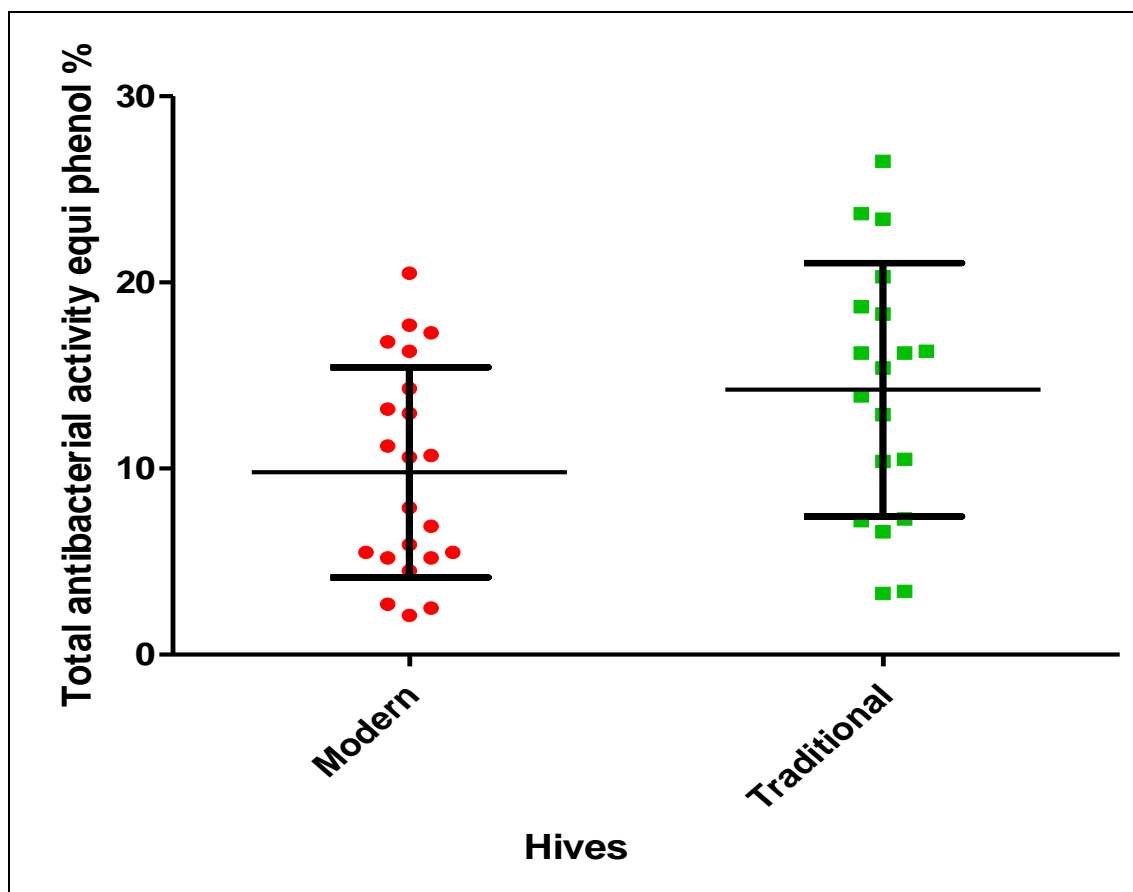


Figure 2.3 Honey total antibacterial activity (% phenol equivalence) vs hive classification. Modern hives, n=29 and traditional hives, n=31. Error bars indicate the mean +/- SD. Difference between means p=0.0278

The type of hive used for production of honey samples had significant impact on the total antibacterial activity as the P value was 0.0278. Honeys from traditional hives had a higher mean peroxidal activity.

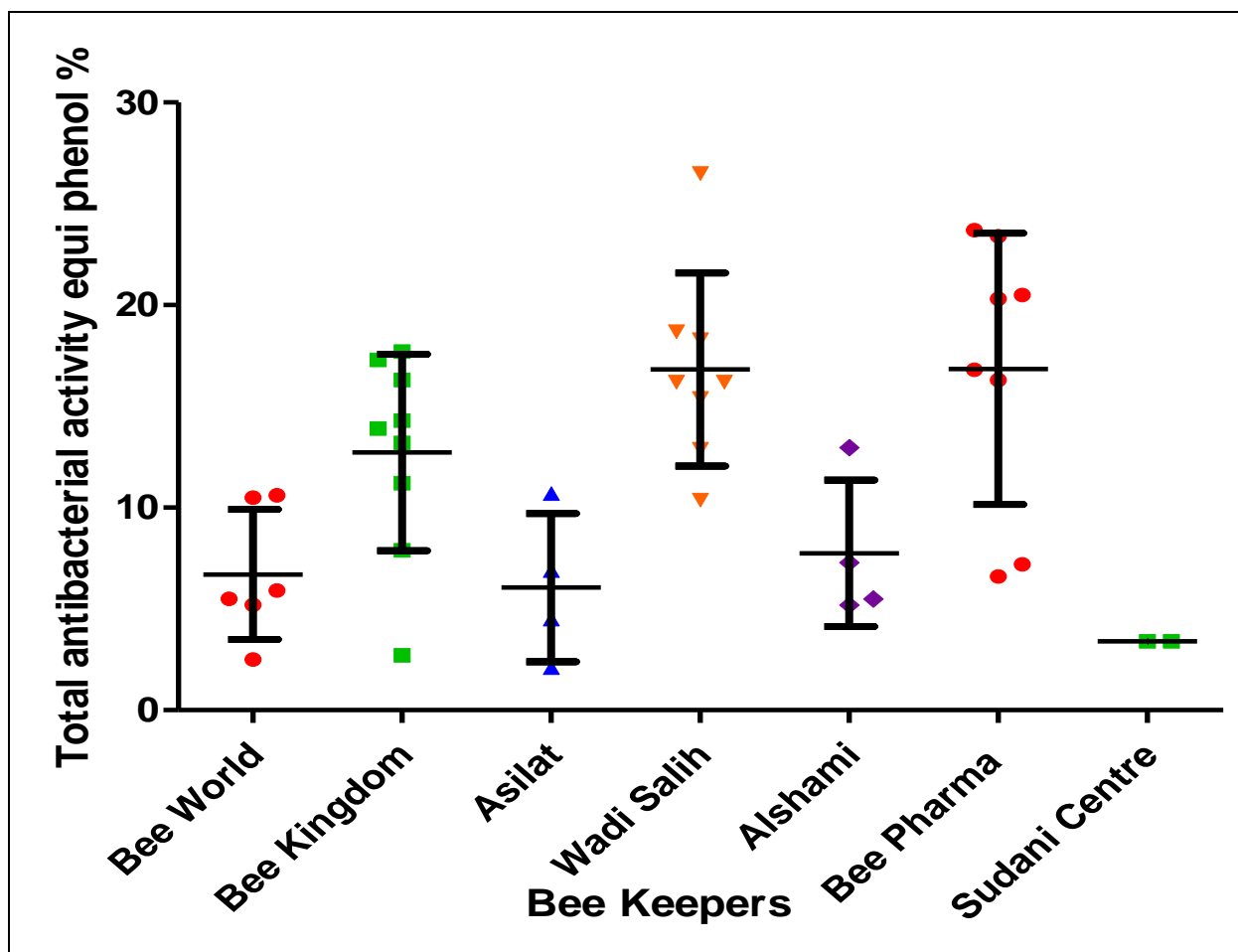


Figure 2.4 Honey total antibacterial activity (% Phenol Equivalence) vs beekeepers practice. Bee world (n=10), Beekingdom (n=12), Asilat (n=8), Alshami (n=6), Wadi Salih (n=8), Sudani Centre (n=8), Bee Pharma (n=8). Error bars indicate the mean +/- SD. Difference between means p=0.0001

The beekeeping practice had significant impact on the antibacterial activity of honey as the P value was 0.0001

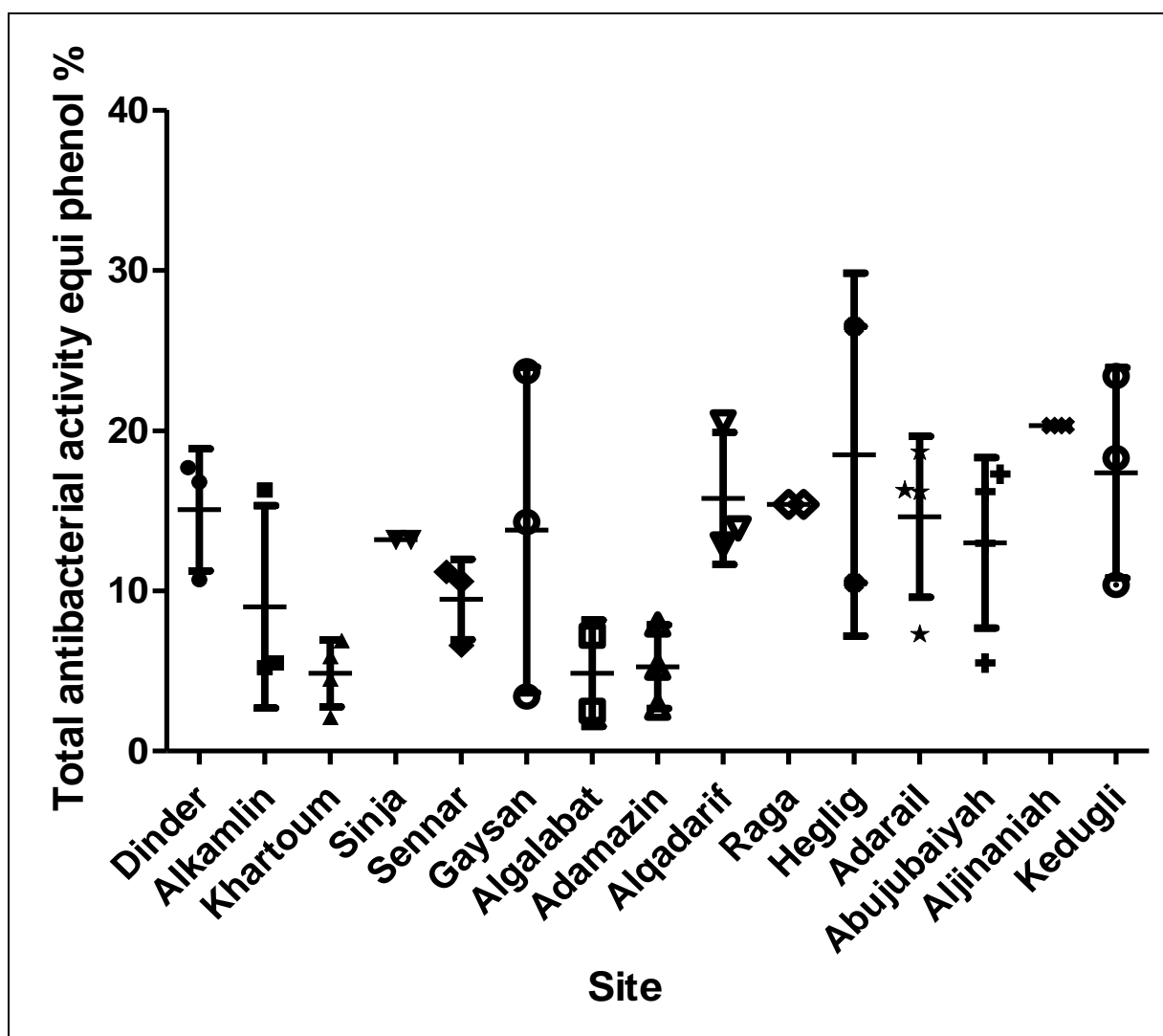


Figure 2.5 Honey total antibacterial activity (% Phenol equivalence) vs collection site. Dinder (n=6), Alkamlin (n=2), Khartoum (n=4), Sinja (n=4), Sennar (n=6), Gaysan (n=4), Algalabat (n=2), Adamazin (n=4), Alqadarif (n=4), Raga (n=2), Adarail (n=4), Abujubaiyah (n=4), Aljinaniah (n=4), Kedugli (n=4). Error bars indicate the mean \pm SD. Difference between means $p=0.0235$

The site of honey production had a significant impact on the antibacterial activity of the honeys with a P value of 0.0235

The bioassay results showed that 40 samples of the Sudanese honeys tested in this study possessed variable detectable levels of antibacterial activity. The antibacterial activity of the Sudanese honeys was between 2.1 and 26.5 % (w/v) phenol equivalent. All the detectable

levels of this antibacterial activity were removed after the addition of catalase which confirms that all the Sudanese honeys tested possessed peroxidal activity and none of the tested samples showed any non peroxidal activity which is a distinct feature of selected honeys such as those produced from New Zealand and Australia (Molan and Russell, 1988; Allen *et al.*, 1991)

In 1991 Allen tested 345 honey samples from different parts of New Zealand in which 25 honeys retained their antibacterial activity in the presence of catalase (Allen *et al.*, 1991). The 25 honeys with non peroxidal activity were identified as manuka (*Leptospermum scoparium*) and viper bugloss (*Echium vulgare*) honeys. In a study of selected Portuguese honeys Henriques found that honeys from *Lavandula stoechas* possessed detectable levels of non peroxidal activity which was justified by the presence of Couramin and cinnamic acid which are known to possess antibacterial activity (Henriques, 2006).

The main advantage of manuka honey which made it the choice for wound care is the high levels of non-peroxide activity which resists the action of catalase produced from wound exudates. The levels of non-peroxide activity reported by Allen were found to reach as high as 59 w/v % in selected manuka honeys while the lowest detectable level was only 2 w/v % (Allen *et al.*, 1991). Although medical grade honeys produced by Comvita and used as standards in some of the bioassays of this study had UMF of 18% w/v, manuka honeys used in other chapters of this thesis were of 12% UMF as the company has abandoned the production of 18% w/v UMF. Wound exudates might contain about (5-8 μ moles/ml) of catalase which is normally present in plasma (Henriques, 2006). The catalase produced from wounds could probably reduce or nullify the peroxide activity which is usually possessed by the majority of honeys such as the honeys in this study, while a non peroxide honey such as

manuka is not affected by the production of catalase (Allen *et al.*, 1991; Molan and Allen, 1996). Another advantage of manuka honey is the heat and light resistant i.e., the antibacterial activity of manuka honey is not affected by moderate heat or light, while peroxidal honeys are greatly affected by heat and light which have a negative impact on the hydrogen peroxide (White and Subers, 1964a,b).

Although honey has been used successfully for the treatment of various ailments since ancient civilisations up to date and plenty of research has been focused on antimicrobial activity of honey, majority of the studies did not determine or quantify the honey used in the study nor did they use standardised methods and reference cultures which makes it very difficult to compare between the outcomes of those studies (Lin *et al.*, 2010).

Since the reintroduction of honey in modern wound care the majority of the conducted research was concentrated on manuka honeys as they have distinct antimicrobial activity and many companies funded the manuka research which is the main source for medical grade honey and dressings for wound care. The introduction of manuka honey in modern medicine encourages the screening of locally produced honeys specially in third world countries like Sudan where honey were used since the ancient Egyptian and anecdotal reports confirmed the success of honey as well as honey combination in the treatment of different ailments. Screening locally produced honeys could solve many problems related to local health and well beings in undeveloped countries. The use of honey could solve many problems by minimising the increased uptake of unnecessary antimicrobial agents which have several impacts such as leading to increased rates of antimicrobial resistance, eradication of intestinal normal flora, increase of morbidity and mortality, drug toxicity, long

hospitalization period, increase of costs, resistant microorganisms and associated infections (Nathwani and Davey, 1992; Aswapokee *et al.*, 1990; Kunin, 1978; Craig *et al.*, 1978).

It seems probable that most countries should have honeys suitable for use as a medicine, whose selection for medical purpose is possible (Henriques, 2006). Local honeys with potential antioxidant and antibacterial activities in countries like Sudan could play a key role in preventing or limiting the wide spread of antimicrobial resistance. Since honey is sweet, natural, inexpensive and has wide acceptance from Sudanese public who can easily get antibiotics over the counter and as a result the majority tends to take antibiotics without prescriptions from the doctor and miss use them. While the high consumption of antibiotics requires restricted and rationalised consumption (Moellering, 1995). Many people in Sudan and probably many other developing countries misuse antibiotics either by not taking the proper dose or taking antibacterial agents for fungal or parasitic infections which harms the normal flora without eradication of the existing pathogen. In addition to the use of antibiotics for treatment of infections which could have easily been treated by others. All these together could lead to the emergence of resistance strains and render many antibiotics use less.

Although there is a scarcity of literature describing the antimicrobial resistance in Sudan, anecdotal reports shows that many antibiotics have lost their sensitivities within a year of their introduction. An example of that is amoxicillin which many doctors use as a placebo today when they do not want to give treatment for the patient. In Sudan and probably in some other undeveloped countries the doctors find themselves in a situation where no prescription is really needed other than pain killer but, they tends to give antibiotics like amoxicillin and sometimes Ampiclox (Ampicillin and Cloxacillin) to convince the patients

that they knew the cause of the illness and they gave the treatments. Doctors sometimes prescribe unnecessary drugs due to the psychology of many Sudanese who think that you do not know their illness if you did not give them treatment. Hence, finding antimicrobials from natural sources including honey and plants could play a significant role especially if the doctors were convinced by honey and they prescribed it instead of giving the unnecessary drugs particularly for certain applications such as wound management.

The Sudanese honeys studied in this research did not show any non-peroxide antibacterial activity; however, this does not mean that no non peroxidal activity could be found because manuka and jelly bush honeys were found after studying 345 honeys from all over New Zealand (Allen *et al.*, 1991).

Moreover, it was found that not all the manuka honeys possess non-peroxidal activity but, only honeys from North Central region (Molan and Russell, 1988) have the non-peroxidal activity which also confirms that the geographical region plays an essential role in the activity of honeys.

In a pilot study (Hashim *et al.*, 2007) four Sudanese honeys showed weak non-peroxidal activity from a floral source presumed as *Azadirachta indica* and attempts have been made to confirm the findings in the current research. Unfortunately, the provider stopped producing that type of honey as consumers were demanding honeys from other floral sources like *Ziziphus Spina Christi* (sidr) and Acacia. This was one of the main reasons that the honeys selected for further studies were from the biggest two producers in the country even though some honeys from those producers yielded lower antibacterial activity since, many of the small companies and individual beekeepers who does not own the lands of their

Apiaries tend to quit the business which makes it impossible to get those honeys for future studies.

Sudan is a very big country and it is difficult to cover all parts of Sudan due to many reasons including the costs of transport, living, and the risks of getting honeys from areas of conflict such as Darfur and Southern Sudan where plenty of honeys are available due to the rich vegetation which form floral sources for *Apis mellifera*. With the separation of South Sudan and the implementation of peace, further research could be launched to investigate more honeys from all over Sudan.

Furthermore, the results obtained in this study confirm that locally produced Sudanese honeys possess some antimicrobial activity which was previously reported by Farouk in a study of treating wounds and ulcers from diabetic patients which was successful in killing pathogens compared to some aminoglycoside antibiotics including gentamicin (Farouk *et al.*, 1988). The honeys used in Farouk's study as well as many other currently used for therapeutical purposes in Sudan are raw honeys which could cause further infections rather than curing the infection as honeys are not sterile and they contain microorganisms (Snowdon and Cliver, 1996). Honeys which are produced and distributed with the intentions of therapeutical applications should be irradiated to sterilise the honey before use as irradiation does not destroy the antibacterial activity of honeys as demonstrated by Molan and coworkers (Molan and Allen, 1996).

The findings in this study have shown that the antibacterial activity of Sudanese honeys is largely associated with peroxide from glucose oxidase. Glucose oxidase is of considerable interest since it causes the production of hydrogen peroxide which not only stabilizes the ripening of nectar against spoilage but also has bactericidal action (Malika *et al.*, 2005;

Cliver, 2000); however, other contents like polyphenols were reported to play a role as well (Bogdanov, 1997). The floral source of honey may also be responsible for some of the antibacterial activities of honey (Molan and Russell, 1988). Sidr honeys were believed to be best honeys in Sudan as well as the whole Middle East however; in this study the highest levels of antibacterial activity were from honeys coming from polyfloral sources which contain Sidr pollen grains as well but they were not dominant; hence, the idea which is understood by many Sudanese and Arabs might not be necessarily correct.

The method by which the honey is harvested and whether a traditional log or modern hive is used has a major impact on quality of the honey; however, it is not as important as the way the honey is processed. The quality of honey is mostly affected by the use of smoke, heat, faulty handling and storage (Crane, 1975) from the time of harvest until it reaches the market or the customer.

Beekeepers should be trained to produce high quality honeys especially from traditional hives which gave an average of antibacterial activity higher than the modern hives in this study. Although traditional hives in this study have yielded a better antibacterial activity their water content was higher compared to those of modern hives which could probably be a reason for a short shelf life of honeys from traditional hives as reports have demonstrated that honeys with water content of more than 18g/100g of honey tends to ferment quickly which probably was the reason for lowering the acceptable moisture content from 21 to 20g/100g of honey as set by the standards of IHC (Bogdanov, 2002). The high water content could have played a role in the antibacterial activity of the honeys, since it was found that honeys with higher water content have a higher antioxidant levels (Gheldof *et al.*, 2003). The antioxidant levels of honeys contribute to the antibacterial activity by the presence of

flavonoids and phenolic components which contributes to wards the antibacterial activity as suggested by Wahdan and Bogdanov (Bogdanov, 1997; Wahdan, 1988).

Probably if a new study is launched covering all parts of Sudan and testing honeys from all the available floral sources with immediate testing of physicochemical characterisation and antimicrobial and antioxidant activity accompanied by preparation of standard pollen slides for every botanical source for reference use it could yield better results which might lead to non peroxidal sources or at least allow for significant correlation between the antimicrobial activity, physicochemical parameters as well as geographical and floral sources.

CHAPTER THREE
SCREENING SELECTED PLANT EXTRACTS FOR THEIR ANTIBACTERIAL AND ANTIOXIDANT
ACTIVITY

3.1 Introduction

Since the dawn of civilization natural products have been essential resources for the production of antimicrobial agents that control bacterial infections; however, with the development of synthetic methods the usefulness of these resources has been limited to folk medicine as conventional medicine continues favouring antibiotics despite the emergence of multi drug resistant pathogens such as MRSA and VRE as the efforts were turned on towards high throughput target-directed screening of synthetic chemical libraries in the search for new antimicrobial leads, eventually natural products programmes were largely abandoned in many cases by pharmaceutical companies (Silver, 2006). However, as this approach fail to give the immediate desired results natural products once again are thought of as an attractive source of novel antibacterial agents (Silver, 2006).

Humans have used plants alone and in combinations since antiquity. Traditional medicine (TM) could be defined as all of the non-allopathic medicine which aims to improve health, whereas ethno-medicine is the utilisation of plants as a source of remedy, based on people's knowledge (Abdalla, 2011). The importance of TM as a source of primary health care has been globally addressed since 1976 by the TM programme of the World Health Organisation (WHO), and was first officially recognised by WHO in the primary health care declaration of Alma Ata in 1978 (Rukangira, 2001). WHO reports in 1994 showed that 90% of the global population use medicinal plants for health related purposes, and 81% have no accessibility to synthetic drugs on the market (Nyiredy, 2004). Moreover, it was estimated that there are 250,000 higher plants worldwide, from which only 6% have been investigated for their biological activities (Fabricant and Farnsworth, 2001).

It was estimated that 75% of the Sudanese population use some of the medicinal plants as home remedies including the fruits of tamarind (*Tamarindus indica*, used as a laxative and for Malaria), tabaldi (*Adansonia digitata*, used as an antidiarrhoeal agent), goddaim (*Grewia tenx*, used for treatment of anaemia) and roselle (*Hibiscus sabdariffa*, for treatment of hypertension and coughs) (Abdalla, 2011). The flora of Sudan consists of 3,137 species of flowering plants belonging to 170 families and 1,280 genera; from which 278 species, 210 genera and 72 families have already been identified as medicinal, culinary and aromatic plants (Elkhalifa, 2003).

The search for new antibacterial agents is essentially important due to the widespread of multidrug resistant pathogens. The search for new alternatives usually starts with screening novel or new candidates which are proposed to have antibacterial activity. The selection of the candidate could be based on empirical knowledge, success in ancient period, or current practice with good results in non conventional medicine in a rural community or third world country which will probably be based on ancient practice. Humans have used plant extracts for centuries for the improvement of food flavour and taste (Holley and Patel, 2005). However, currently there is increased interest in plant extracts with intentions of improving the quality of foods by enhancing the organoleptic properties as well as extending the shelf life of foods.

The increased research on the biological properties of plant extracts motivated by the needs for novel antibacterial and antioxidant compounds from natural sources has proved that plant extracts have the potential to be good antioxidant and antimicrobial sources (Davidson *et al.*, 1983). Although the research into the biological properties has initially been focused on the therapeutic potential of plant extracts (Burt, 2004), consumers'

increased awareness of the possible side effects of synthetic food additives has changed their preferences towards more natural products, which has encouraged food industrialists to search for new alternatives of which plant extracts is promising additive which could enhance the taste and extend the shelf life at the same time.

Large pharmaceutical companies during the 1990s abandoned their legacy of natural product research and development, for many reasons. Many difficulties accompany drug discovery from natural products which might be laborious, expensive and time consuming process. Other difficulties include selection of the plants, suitable handling, phytochemical processing, complexity of plant mixtures, loss or decrease of activity after successive fractionation, batch to batch differences, and variations in final compound yield (Abdalla, 2011).

The research into plant extracts and oils has demonstrated that there is considerable potential for utilization of natural antimicrobials in food, particularly the application to fresh fruits and vegetables, for their oxidative degradation of lipids and improvement of the quality and nutritional value of food. Essential oils derived from spices and plants have antimicrobial activity against *L. monocytogenes*, *Salmonella typhimurium*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, *Bacillus cereus* and *Staphylococcus aureus* at levels between 0.2 and 10 µl/ml (Burt, 2004). For example, combined mild heat treatment with addition of cinnamon and clove essential oil to apple cider significantly reduced the D-value and time to 5-log reduction of *Escherichia coli* O157:H7 (Knight and McKellar, 2007).

Plant extracts and oils should be screened for antibacterial and antioxidant activity before being applied for preservation or therapy. Various methods are used for quantitative and qualitative screening of natural antibacterials such as plant extracts. Screening methods include the agar diffusion method, the disc-agar-diffusion method and drop-agar diffusion

(Burt, 2004). Other techniques in antimicrobial determination are dilution of the antibacterial in agar or broth. Modification of broth-dilution studies with bacterial enumeration by optical density (OD) (turbidity) measurement and application of the redox indicator resazurin as a visual indicator of the MIC demonstrates some different screening methods for evaluating the antimicrobial activity of different plant-origin antimicrobials against different food-borne pathogenic micro-organisms. Identification of antimicrobial activity is significantly affected by the method of assay (Brandi *et al.*, 2006; Holley and Patel 2005).

Despite the rich literature of the antibacterial activity of the medicinal plants only limited reports were focused on the synergistic interactions between plant extracts and antibiotics or plant extracts with natural products. Medicinal and Aromatic Plants Research Institute (of Sudan (MAPRI) screened 446 plant species based on both random collection and traditional medicine (TM) information, for their antimicrobial, pharmacological and phytochemical properties (Ayoub and Kingston, 1981).

Little scientific background has been sought for the traditional use of many plants for their antibacterial and antioxidant potential despite their frequent use in Sudan. The excessive use of those plants often over long periods of time as well as the urgent need for novel antibacterial agents highlights the significance of scientifically validating whether the used plant extracts and their combinations have antibacterial and antioxidant activity and whether these plant extracts or their combinations could fill the gap of synthetic antibiotics to which many pathogenic organisms are getting resistant as well as the possibility of using them as a replacement for BHT and other carcinogenic food preservatives.

Aim

The aim of the work reported in this Chapter was to determine the antibacterial and antioxidant activity of selected plant extracts in order to find an inexpensive sterile and natural product with antibacterial potential.

Objectives

Screening selected plant extracts for antibacterial activity

Evaluating the antibacterial activity of autoclaved plant extracts

Screening selected plant extracts for antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

3.2 Materials and methods

Samples of *Pimpinella anisium*, *Cucurbita maxima*, *Nigella sativa*, *Fennel vulgare*, *Tamarindis indica*, *Aacia nioltica*, *Helyotropium egyptiacum*, *Helyotropium sudanicum*, *Mimosa pigra* F, *Mimosa pigra* L, *Phoenix dactylifera*, Propolis, dimethylsulphoxide (DMSO) Fischer, Nutrient broth and Nutrient agar (Oxoid), *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* NCTC 10418.

3.3 Preparation of extracts:

Five hundred grams of each plant sample listed bellow was shade dried and milled to coarsely powder using manual miller and soaked in 1000 ml of either petroleum ether, methanol or 80 % ethanol (Tables 3.1, 3.2 and 3.3) for about seventy two hours with shaking for the first four hours using shaker apparatus. Solvent was evaporated under reduced pressure using rotary evaporator apparatus and the residue was freeze- dried using freeze dryer machine till complete dryness. Yield percentage of each extract was calculated by dividing the weight of extract over the weight of sample multiplying to one hundred

(Harborne, 1984). Dimethylsulfoxide (DMSO) was the solvent used to prepare the required concentrations for sensitivity screening of the plant extracts and oils.

3.4 Agar well diffusion assay

The antimicrobial activities of all the plant extracts were determined using the agar well diffusion method. The oils (*Pimpinella anisium*, *Cucurbita maxima*, *Nigella sativa*, and *Fennel vulgare*) were dissolved in DMSO while the plant (*Tamarindis indica*, *Aacia nioltica*, *Helyotropium egyptyacum*, *Helyotropium sudanicum*, *Mimosa pigra F*, *Mimosa pigra L*, *Phoenix dactylifera*) extracts were dissolved sterile deionised water to prepare 50 (w/v) % of each. The individual test organisms were standardised by adjusting the absorbance of the inoculum to (0.08–0.13) at OD 625 nm (Wiegand *et al.*, 2008). Hundred µl of the standardised inoculums were spread on the surface of Nutrient agar using disposable sterile glass spreader, and the surface was allowed to dry. Wells (8.0 mm in diameter) were cut from the inoculated medium using a flame-sterilized cork borer, and then filled with plant extracts. The plates were incubated at 37°C for 24 hours. A digital calliper was used to measure the zones of inhibition around each well.

3.5 Effect of autoclaving on the antimicrobial activity of plant extracts

Suspensions (50% w/v) of the various plant extracts as well as propolis (from Bee kingdom Co) were autoclaved at 121°C for 15 minutes and then the antibacterial activity was assessed against *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* NCTC 10418 using the agar well diffusion assay as above 3.4. A non-autoclaved (50% w/v) suspension of each plant extract was used as control.

3.6 Determination of the MIC of Tamarind and Acacia

The MIC of the *Tamarindus indica* and *Acacia nilotica* extracts for *S. aureus* NCTC 6571 and *E. coli* NCTC 10418 was determined using the broth micro dilution method. Cultures of each tested bacterium were diluted in 2 X Nutrient broth and the turbidity of the inoculum was adjusted to 0.5 McFarland standard spectrophotometrically at (0.08–0.13) OD 625 nm (Wiegand *et al.*, 2008).

100 µl aliquots were then aseptically dispensed in wells of a 96- well plate. A series of two fold dilutions of autoclaved Tamarind and Acacia was prepared in sterile distilled water ranging from 50 % (w/v) to 1.5 % (w/v). Diluted tamarind and acacia were sterilised and filtered using a 0.20 µm membrane filter, and 100 µl of diluted extracts were added to wells and incubated for 24 hrs at 37°C. Sterile distilled water with no extracts was used as a control. The lowest concentration of *Tamarindus indica* and *Acacia nilotica* which inhibited the growth of each organism was recorded as the MIC.

3.7 Determination of the MBC of Tamarind and Acacia

In order to determine the MBC, 20 µl of sample were collected from those wells which did not show any growth and inoculated on sterile nutrient agar. The plates were then incubated overnight at 37 °C. The MBC was read as the lowest concentration of *Tamarindus indica* and *Acacia nilotica* which did not permit any visible growth on agar plate.

3.8 Radical scavenging assay

The radical-scavenging activity of the plant extracts was evaluated with the DPPH assay (Zaouali *et al.*, 2010). One millilitre of each plant extracts was added to 3 ml of the methanolic DPPH solution. The mixture was then shaken and allowed to stand at room temperature in the dark for 30 minutes then the decrease in absorbance at 517 nm was

measured against a blank (methanol solution) using a Jenway spectrophotometer. A mixture consisting of 1 ml of methanol and 3 ml of DPPH solution was used as the control. The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH, was calculated according to the formula % inhibition = $[(AB - AA)/AB] \times 100$, where AB and AA are the absorbance values of the control and of the test sample, respectively.

3.9 Results and discussion

Tables 3.1, 3.2 and 3.3 present sample yields from the selected plants using the extraction methods described in section 3.3.

Table 3.4 shows antibacterial activity against *S. aureus* (NCTC 6571) and *E. coli* (NCTC 10418) and antioxidant scavenging activity of the plant extracts while Table 3.5 presents antibacterial activity against *S. aureus* NCTC 6571 and *E. coli* NCTC 10418 after autoclaving the extracts.

Table 3.1 Yield of samples extracted using petroleum ether and methanol as solvents

Sample	Weight of Sample	Petroleum ether		Methanol	
		Weigh of Ex.	Yield %	Weight of Ex.	Yield %
<i>Nigella sativa</i>	500 g	152 g	30.4 %	66.32 g	13.3 %
Argel (harjal)	500 g	15.6 g	3.2 %	96.875 g	19.4 %
<i>Acacia nilotica</i>	500 g	4.398 g	0.9 %	180.258 g	36.1 %
<i>Cucurbita maxima</i>	500 g	147.541 g	29.5 %	43.21 g	8.6%
<i>Tamarindus indica</i>	500 g	3.981 g	0.8 %	199.36 g	39.9%
<i>Alium sativum</i>	500 g	2.358 g	0.5 %	40.258 g	8.1 %

Table 3.2 Yield of essential oils extracted using petroleum ether

Sample	Weight of sample	Volume of oil	Yield %
<i>Fennel vulgare,</i>	500 g	10.17 ml	2.0 %
<i>Pimpemella anisuim</i>	500 g	20.3 ml	4.1 %

Table 3.3 Yield of samples extracted using Ethanol as solvent

Number	Scientific name	Location	Part used	Ethanol 80 (Yield) %
1	<i>Helyotropium egyptyacum</i>	Shendi area	Whole plant	26.9 %
2	<i>Helyotropium sudanicum</i>	Shendi area	Whole plant	18.8 %
3	<i>Mimosa pigra F</i>	Shendi area	Fruits	13.9 %
4	<i>Mimosa pigra L</i>	Shendi area	Leaves	24.8 %
5	<i>Phoenix dactylifera L</i>	Shendi area	Seeds	24.5 %

The plant extracts of *Mimosa pigra* L, *Mimosa pigra* F, *T. Indica* and the oils of *N. Sativa* and *P. anisium* has a relatively strong radical scavenging potential and antibacterial activity as seen in Table 3.4. While Cinnamon, *C. Maxima* and Ginger roots has a moderate radical scavenging potential as well as moderate antibacterial activity. The radical scavenging potential of the selected plant extracts presented in Table 3.4 varied between 31.2 and 78.4%. *Allium sativum* have had the lowest radical scavenging potential and did not show any zone of inhibition. *Phoenix dactylifera* L has the second lowest radical scavenging potential and did not show any inhibition zone. The volatile oil of *P. anisium* might gave better antibacterial inhibition zone if the vapour of the essential oil was tested by means of filter paper as studies has revealed that some essential oils gave better result when the vapours rather the oil were tested (Burt, 2004).

Nigella sativa oil was effective against both bacteria before autoclaving and in effective against both of them after autoclaving. Filtration could be suitable method for sterilising

Nigella sativa as well as other oils, but the cost of filtration is high for developing countries like Sudan. *Tamarindus indica* and *Acacia nilotica* were the most active plant extracts before and after autoclaving of the extracts; however, *Acacia nilotica* was only active against *S. aureus* but not *E. coli* (i.e., has narrow spectrum activity). While, *Tamarindus indica* has broad-spectrum activity being active against both Gram positive and Gram negative organisms (see Tables 3.4, 3.5 and Figure 3.1).

Table 3.4 Antibacterial and antioxidant activity of selected plant extracts. The results are the means of three replicates. Antibacterial activity is shown as the mean zone of inhibition diameter where a diameter of 8mm indicates no zone of inhibition, as the well in the agar was 8mm diameter.

Plant extract	Antibacterial activity (mm)		Antioxidant activity (RSA %)
	<i>S.aureus</i>	<i>E.coli</i>	
<i>Mimosa pigra</i> L	22.3	8	78.4
<i>Phoenix dactylifera</i> L	8	8	42.2
Argel	12.6	8	38.5
Propolis	18	8	75.3
<i>Mimosa pigra</i> F	23.6	8	74.1
<i>N. sativa</i>	22.3	17.8	72.1
Ginger roots	8	15.6	54.6
<i>H. sudanicum</i> whole	18.8	8	42.2
<i>P. dactylifera</i> seeds	26.2	8	42.9
<i>A. nilotica</i>	32.3	10	47.2
Cinnamon	16.2	13.3	56.4
<i>T. indica</i> F	32.1	29.3	73.8
<i>F. vulgare</i>	14.7	8	64.4
<i>P. anisium</i>	8	15.2	79
<i>A. sativum</i>	8	8	31.2
<i>C. maxima</i>	13.6	14	67

Table 3.5 Antibacterial activity of the plant extracts which were still active after autoclaving. The results are the means of three replicates. Antibacterial activity is shown as the mean zone of inhibition diameter where a diameter of 8mm indicates no zone of inhibition, as the well in the agar was 8mm diameter.

Plant extract	Antibacterial activity zone of inhibition diameter (mm)	
	<i>S.aureus</i>	<i>E.coli</i>
<i>Mimosa pigra</i> L	18.7	8
<i>T. indica</i> F	30.5	27.4
<i>Mimosa pigra</i> F	18.5	8
Ginger roots	8	8
<i>H. sudanicum</i> whole	15.7	8
<i>A. nioltica</i> F	28.8	8

Figure 3.1 (overleaf) illustrates the broad-spectrum antimicrobial activity of the methanolic extract of *Tamarindus indica* after autoclaving. Similar sized zones are apparent for both *E. coli* and *S. aureus*.

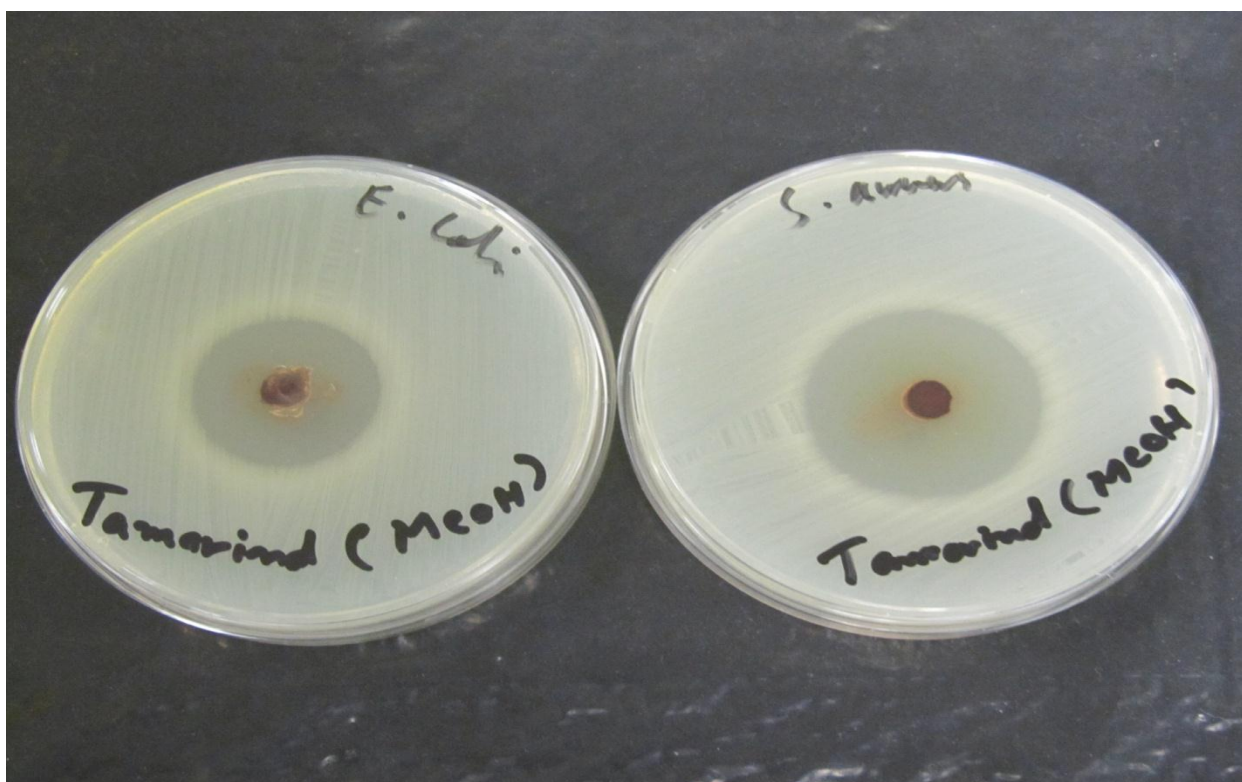


Figure 3.1 Zones of inhibition achieved using autoclaved *Tamarindus indica* methanolic extract against *E. coli* (left hand plate) and *S. aureus* (right hand plate)

As shown in Tables 3.4 and 3.5 autoclaved methanolic extracts of tamarind and acacia were as equally effective in inhibiting the growth of *S. aureus* NCTC 6571 and *E. coli* NCTC 10418 as non-autoclaved material, while the autoclaved acacia extract was only active against the gram positive *S. aureus*. This is particularly important in relation to the possible use of those plant extracts as novel antibacterials as well as food additive since they need to be sterilised before their introduction and those materials were found to resist the destructive effects of autoclaving which makes them suitable sources for expensive antibacterials. In Figure 3.1 the zone of inhibition of *Tamarindus indica* extract against *E. coli* was smaller than that of *S. aureus*, probably due to the complexity of Gram-negative cell wall.

Tables 3.6 and 3.7 show the minimum inhibitory and minimum bactericidal concentrations for Acacia and Tamarind extracts against *S. aureus* NCTC 6571 and *E. coli* NCTC 10418 respectively.

Table 3.6 Minimum inhibitory and minimum bactericidal concentrations for Acacia and Tamarind against *S. aureus* NCTC 6571

Plant extract	MIC % (w/v)	MBC % (w/v)
<i>Tamarindus indica</i>	0.78	1.56
<i>Acacia nilotica</i>	1.56	3.12

Some anomalies in screening of antimicrobial activity against *Staphylococcus aureus*, with a greater reduction of growth at the MIC than at the MBC has been reported (Becerril *et al.*, 2007).

Table 3.7 Minimum inhibitory concentrations for Acacia and Tamarind against *E. coli* NCTC 10418

Plant extract	MIC % (w/v)	MBC % (w/v)
<i>Tamarindus indica</i>	1.56	3.12
<i>Acacia nilotica</i>	>50	>50

The Minimum inhibitory concentration of Tamarind and Acacia against *S. aureus* was 0.78 and 1.56 respectively. While the minimum bactericidal concentration was 1.56 and 3.12 respectively (Table 3.6). The Minimum inhibitory concentration of Tamarind and Acacia against *E. coli* was 1.56 and > 50 respectively. While the minimum bactericidal concentration was 3.12 and > 50 respectively (Table 3.7).

The MIC for *E. coli* NCTC 10418 using *Acacia nilotica* was >50 % (w/v) as presented in table 3.7 and this means that the bacterium was resistant to the inhibitory effects of the plant extract which confirms the results presented in Table 3.4. The resistance of *E. coli* NCTC 10418 to *Acacia nilotica* comes in agreement with reports that plant extracts and essential

oils are mainly effective against gram positive bacteria (Tajkarimi *et al.*, 2010) and this could be justified by the complex cell wall of gram negative bacteria. To understand if the resistant of gram negative bacteria was due to the impermeability of the gram negative cell wall EDTA was added to the plant extract (see Chapter 5) as reports suggested that EDTA could disrupt the cell wall of gram negative bacteria (Gray and Wilkinson, 1965) and make the cell wall permeable to the plant extracts which could lead to the inhibition of the bacterium by the plant extract.

Finding natural products with antibacterial and antioxidant activity is essential particularly for those people in rural areas who do not have access to modern medicines or those who believe folk medicines are better than conventional medicines. Identifying and validating novel bioactive natural products like honey and plants have many benefits in addition to saving lives as it minimizes emergence of resistant strains, reduces the morbidity and mortality rates, and may save governments from spending billions in treating food poisoning and infectious diseases.

Although there are various published reports on the antimicrobial activity of plant extracts, it is difficult to compare the published reports due to seasonal and geographic variations as reports confirmed that even the same plants could yield various amounts of extracts and oils with varied bioactive potentials. The variations between the antimicrobial sensitivity reports could be due to variations in the extraction method, culture medium, size of inoculum, choice of emulsifier and basic test method (Sofia *et al.*, 2007). New approaches and advances in extraction methods, such as crude extraction, high-intensity ultrasound-assisted (HI-US) in combination with proper solvent selection, can improve these approaches (Burt, 2004; Ibrahim *et al.*, 2009; Romeo *et al.*, 2008; Thongson *et al.*, 2004).

Methods such as solvent-free microwave extraction (SFME) and application of crude extracts and enzyme conversions of herbs and spices will improve efficacy and extraction rates, with shorter extraction times and higher levels of active antimicrobial components. Sudanese plants tested in this study showed antimicrobial as well antioxidant potential which agree with reports by (Bendahou *et al.*, 2008; Ibrahim *et al.*, 2009; Kahkonen *et al.*, 1999; Mandalari *et al.*, 2007) on the antibacterial and antioxidant properties of plant extracts. Some plant extracts such as *Tamarindus indica* and *Acacia nilotica* did not lose their antibacterial activity even after sterilization with autoclave which make them good source for natural antimicrobials that can be used for therapy, prophylaxis and food preservation particularly in developing countries like Sudan. Future Studies should include other antioxidant measuring methodologies as well as various extraction methods.

CHAPTER FOUR

THE BIOACTIVITY OF SUDANESE HONEYS

4.1 Introduction

Natural honey is a complex solution which is largely made up of water and sugars in addition to other minor constituents like flavouring materials, pigments, acids, and minerals which are largely responsible for the differences among individual honey types (Doner, 1997). The minor elements such as flavonoids and phenolic acids are known to play a significant role in the antibacterial and antioxidant activity of honeys (Gheldof *et al.*, 2002; Wahdan, 1998).

Although honey has been a well regarded treatment with a good reputation it was relegated to folk medicine with introduction of antibiotics in conventional medicine particularly in developed countries. The emergence of resistant strains such as (MRSA) and (VRE) has led to the re-evaluation of honey which has led to the introduction of licensed honey and honey dressings in developed countries.

The potential of honey in equally eradicating antibiotic resistant as well as sensitive (Henriques *et al.*, 2010) strains has opened a wide area for the research into honeys and the possibility of using honeys for the treatment of other infections; however, research into the evaluation of honey in treating infections of the urinary tract or gastrointestinal disorders were limited to few reports compared to the literature focusing on the management of wound infection. Although there are reports which demonstrates the effectiveness of honey in the treatment of gastroenteritis (Hafejee and Moosa, 1988), research in this area is probably hampered by the complexity of the gastrointestinal environment and the difficulty in simulating that environment while running *in vitro* antibacterial bioassays.

4.2 An investigation into the bactericidal activity of Sudanese honey against

Staphylococcus aureus* and *Escherichia coli

Although the current applications of honey in modern medicine are limited to topical application and the management of wounds, limited research as well as clinical trials have demonstrated that even dilution with in body fluids honey can still inhibit some microorganisms (Lin *et al.*, 2010). The success of any antimicrobial agent in inhibiting or eradicating an existing pathogen in the human body depends on the potency of the antibacterial agent as well as the dosing regimen. If honey will be administered orally for the treatment of any infection in conventional therapy it is essential that one establishes the minimum dose required to inhibit the pathogen causing the infection as well as length of time required by the antibacterial agent to kill the pathogen. It is necessary to investigate antimicrobial potency of honey and determine the mode of action of honey in order to be accepted by healthcare practitioners (Molan and Betts 2004). Research through the last decades has shown that although different antibacterials have the ability to kill the same microorganism it does not necessarily means that they have the same mode of action.

Pharmacodynamics is defined as the relationship between the serum concentration and the pharmacological and toxological effects of the drugs. In the case of antimicrobial agents the primary interest is in the relationship between concentration and the antimicrobial effect. The time course of antimicrobial activity is a reflection of the interrelationship between pharmacokinetics and pharmacodynamics. The pattern of antimicrobial activity over time is an important determinant of effective dosage regimens (Lister, 2001).

The MICs and MBCs have been regarded as the key parameters for the determination of the antimicrobial activity of antimicrobial agents. Although these parameters could give reliable

results and predict the potency of the drug organism interaction, they do not estimate the time required by the antimicrobial agent to inhibit the pathogen. The main advantages of the MICs and MBCs could be that they are easy to perform and you can use minimum amounts of the tested drugs particularly in case of micro broth dilution assays. The disadvantages of MICs and MBCs include: the MICs do not confirm whether the agent is bacteriostatic or bactericidal. The MBCs determine the bactericidal activity, but does not give any idea about the rate of bactericidal activity and the possibility of enhancing the killing rate by increasing the concentration of the antimicrobial agent. The MICs does not evaluate the persistent effects of the antimicrobial agents such as the post antibiotic effect (PAE) and the post antibiotic sub-MIC effect (PAE-SME) (Craig, 1998). The ability to estimate the effect of the increasing concentrations on the bactericidal activity of antimicrobials as well as the magnitude of persistent effects gives a much better description of the time course of antimicrobial activity than is provided by the MIC and MBC (Lister, 2006).

Based on the pattern of bactericidal activity antibacterial agents are divided into two groups. Firstly, antibacterials characterised by concentration- dependent killing over a wide range of concentrations. In this group of antibacterial agents the higher the concentration of the antibacterial agent the greater the rate and extent of bactericidal activity. An example of this group could be the aminoglycosides. Secondly, antibacterials characterised by time- dependent killing. Unlike the first group this group is characterised by minimal concentration- dependent killing. Saturation of the killing rate occurs at low multiples of the MIC—usually around four to five times the MIC (White *et al.*, 1996) Concentrations above these values do not kill the organisms any faster or more extensively. Thus, the extent of killing in this pattern of bactericidal activity is largely dependent on the time of exposure.

The absence of major concentration - dependent killing is a common characteristic of β -lactam antibiotics, vancomycin, clindamycin, and the macrolides (White *et al.*, 1996).

Although the antibacterial activity of Sudanese honey has been documented *in vitro* as well as clinical trials (Wadi *et al.*, 1987; Farouk *et al.*, 1988), this antibacterial activity has never been investigated. Honey is widely used in Sudanese folk medicine, nutrition, food preservation as well in severe cases of diabetic ulcers and wound infection. The application of raw honey in folkloric medicine as well as in the management for severe diabetic wounds in Sudan is based on empirical knowledge and religious belief, motivated by availability and reasonable price compared to conventional medicines which are either expensive or unavailable particularly in rural communities.

Manuka honey was introduced into UK in 2004 (Henriques, 2006) and has been widely accepted since then. The research into the kinetics of inhibition or death through *in vitro* experiments and bioassays including Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) (Stratton, 2003), and time kill curves are important steps prior to acceptance or rejection for medicinal purposes. When resistance is observed *in vitro* it is expected to be present *in vivo*. However, the conditions are far more complicated *in vivo* as susceptibility does not have such a clear cut interpretation, as *in vivo* there are more interfering substances than in the standardised laboratory assays (Varaldo, 2002).

The bactericidal activity of manuka honey as well as other honeys against gram positive and gram negative bacteria has been demonstrated (Henriques *et al.*, 2010; Blair *et al.*, 2009; Sherlock *et al.*, 2010; Kwakman *et al.*, 2010). However, the bactericidal activity of Sudanese honeys has never been reported as prior research was limited to the evaluation of the activity through agar diffusion assays (Farouk *et al.*, 1988). Agar diffusion assay does confirm

the ability of the honeys to inhibit bacterial growth but, this inhibition does not confirm the death of the bacteria. Bacteriostatic agents could inhibit the growth of a pathogen for a while then they might start growing again, whereas bactericidal agents are capable of eradication of pathogens.

In this study the bactericidal activity of selected Sudanese honeys was determined through tube dilution method, microtiter plate method as well as minimum bactericidal concentration.

4.3 An investigation into the radical scavenging potential of Sudanese honeys

Antioxidants are nutritive and non nutritive compounds that can prevent biologically destructive chemical reactions in foods and living organisms by scavenging free radicals and oxidants and absorbing molecular damage that could further lead to diseases (Bistrrian, 2002; Lenaz *et al.*, 2002). The antioxidants play an important role by helping living organisms getting rid of the possible damage that may be caused due to oxidative stress when radicals with unpaired electrons tends to get electrons from other molecules leading to harmful effects on these molecules (Ali *et al.*, 2008).

The imbalance between free radical production and the antioxidant defence system which leads to an excess of oxidation is known as oxidative stress (Gheldof and Engeseth, 2002). During the cellular metabolism oxidant free radical molecules are produced and evidence now suggests that the excessive generation of these molecules could lead to DNA damage (Holmes *et al.*, 1992; Ames *et al.*, 1993) and contribute to the development of certain age related pathologies including arthritis, strokes, atherosclerosis and some cancers (Schramm *et al.*, 2003).

Antioxidants are either found endogenously or provided by dietary sources (Chepulis, 2008). Antioxidants are present in many vegetables, fruits, and food products, such as black tea, coffee and honey. There is sufficient amount of evidence in the literature to suggest that consumption of foods, in particular fruit and vegetables, can boost levels of antioxidants thereby playing a significant role in reducing the incidence of cerebrovascular and other diseases (Machlin, 1995; Steinmetz and Potter, 1996; Van Duyn and Pivonka, 2000).

Honey has a long history of traditional use in various medical systems and in recent time several research groups have increased attention in this product. Although honey has been well known for its healing powers in wounds, it has been used in other therapeutics as well as food preservation (Molan, 1992a,b). Natural honey is rich with antioxidants from different sources including Vitamin C, monophenolics, flavonoids, and polyphenolics. A wide range of antioxidants are present in various types of honeys which includes monophenolics such as 4-hydroxybenzoic and 4-hydroxycinnamic acids predominate in different honeys (Schramm *et al.*, 2003). The success in the reestablishment of honey in recent medicines led to plenty of investigations in the antioxidant properties of honeys and their application in food industry. Antioxidants, which act as preservatives because of their antioxidative activity, include both enzymatic (e.g. catalase and glucose oxidase) and non-enzymatic (e.g., organic acids, Maillard reaction products, amino acids, proteins, flavonoids, phenolics, α -tocopherol, and ascorbic acid) substances. The flavonoid content reaches about 0.5% in pollen, 10% in propolis and about 6 mg/kg in honey (Anklam, 1998).

In the recent years natural foods and additives from natural sources including bees honey as well as a number of spices and plant extracts has gained increased interests from researchers, food and pharmaceutical industries as well as consumers (Siramon and Ohtani,

2007). The increased interest in the benefits of natural foods and additives as well as the increased awareness about the harmful effects arising from synthetic additives and preservatives led to high demands for natural functional foods that will boost the human health rather than products containing substances with deleterious effects. An example of harmful additives include synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) which were banned by some European countries as well as Japan . Both antioxidants (BHA) and (BHT) are reported to be carcinogenic; hence, they have been restricted in foods, pharmaceuticals and cosmetics (Botterweck *et al.*, 2000).

Honey could play an essential role in food industry if the limited research into the antioxidant capacity and the antimicrobial activity against food pathogens is extended. The antioxidant properties of honey has been tested against ground turkey (Mckibben and Engeseth 2002), to inhibit lipid oxidation in ready-to-eat ground beef patties (Johnston *et al.*, 2005), to protect against lipid oxidation, in fruit and vegetable homogenates to inhibit enzymatic browning (Chen *et al.*, 2000) and in living organisms to retard biologically destructive reactions (Gheldof *et al.*, 2003). Those reports encourage more research into the antioxidant activity of honey which could make honey an essential supplement of every day diet as it has been demonstrated to lower the glycemic index (Cortes *et al.*, 2011; Ahmad *et al.*, 2008) and protect against oxidative stress.

Although the previous research into the antioxidant potential of honeys has been promising, the research into the antibacterial activity of honey against food pathogens has been limited to few reports. Taormina has found that both peroxide and non-peroxide components in honey affected the growth of six food pathogens (Taormina *et al.*, 2001). If honey can slow or stop the growth of spoilage organisms or food pathogens, then its incorporation into

foods as a preservative can be explored (Mundo *et al.*, 2004). Due to its antimicrobial properties, honey may serve as a natural food preservative (Lusby *et al.*, 2005; Molan 1992a,b).

There are various methods applied for the determination of antioxidant activity. One of them measures the radical scavenging activity of antioxidants against free radicals like the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical illustrated in figure 4.1, the superoxide anion radical (O_2^-), the hydroxyl radical (OH), and the peroxy radical (ROO). The different methods used for the determination of the antioxidant activity could give different results depending on the specific free radical being used as a reactant.

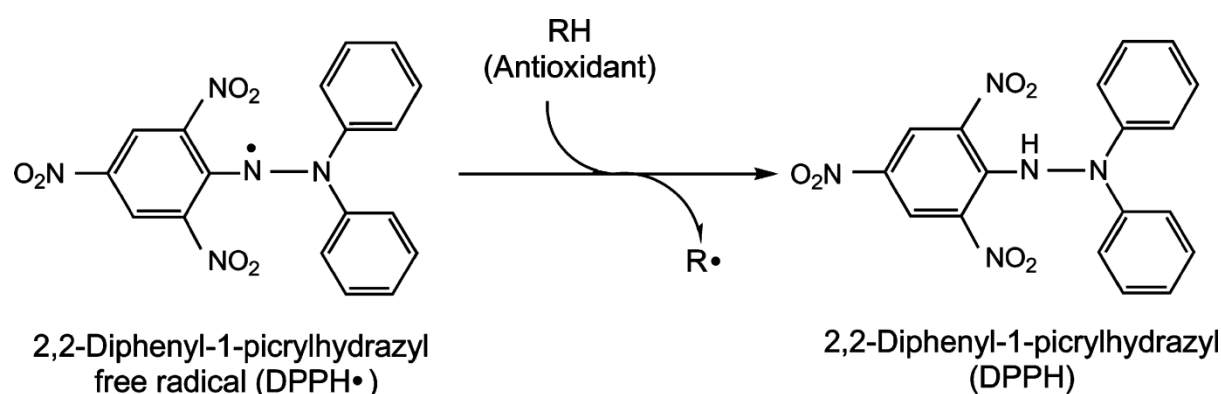


Figure 4.1 Reaction between DPPH• and antioxidant to form DPPH. (From Moon and Shibamoto, 2009)

Aim

To evaluate the *in vitro* bactericidal and antioxidant potential of selected Sudanese honeys

Objectives

To determine the MIC of selected Sudanese honeys

To investigate if the selected Sudanese honeys were bacteriostatic or bactericidal.

To determine the radical scavenging potential of selected Sudanese honeys from different floral and geographical origins using the 2, 2-diphenyl-1-picryl hydrazyl (DPPH) method.

4.4 Materials and Methods

In this study two types of reference microorganisms were used. The Gram positive *Staphylococcus aureus* NCTC 6571 and the Gram negative *Escherichia coli* NCTC 10418. 2, 2-diphenyl-1-picryl hydrazyl (DPPH) (Fischer), ascorbic acid (Sigma), UMF12+ (Comvita®) Manuka honey and seven Sudanese honeys with peroxidal activity were investigated. All the honeys used in this study were from those obtained by Bee Kingdom apiary unless otherwise specified.

4.5 Antibacterial activity of honey

The antibacterial activity of selected Sudanese honeys has been measured three times between January and December 2011 as anecdotal evidence suggested that the antibacterial activities of honey changes with storage time. Antibacterial activity of honey was evaluated using an agar well diffusion assay according to Patton (Patton *et al.*, 2006). Cultures of *Staphylococcus aureus* were cultivated overnight at 37°C in 50ml flasks containing 10ml of nutrient broth and the inoculum was adjusted between (0.8-0.13) at OD of 625nm (Wiegand *et al.*, 2008). Afterwards 0.1ml of the cultures was spread on plates containing solidified Nutrient agar (Oxoid). Wells 8mm in diameter were bored into the surface of the agar medium and 100µl of the honey samples were introduced in the wells. Plates were incubated at 37°C for 24hrs. The zones of inhibition diameters were measured. The experiment was done in triplicate.

4.6 Minimum Inhibitory Concentration (MIC) by tube dilution

Using Khan tubes 1ml of double the desired final concentration of honey made up in sterile deionised water to determine total antibacterial activity. An overnight broth culture of the test organism was added as an inoculum without dilution; each tube was inoculated with 10 µl and incubated at 37°C for 24hrs. Growth or no growth was recorded, by noting turbidity and appropriate positive (single strength broth and inoculum) and negative controls (broth and honey) were included to aid interpretation. Tubes with the lowest concentration without growth were recorded as the MIC (Henriques, 2006).

4.7 Minimum Inhibitory Concentration (MIC) in Microtiter plates

The MIC was determined using the method described above in section 4.6 with minor modifications. The test was done in a sterile 96-well microtiter plate with flat bottom and lid. A total volume of 200µl and an inoculum of 5µl for each well. The plate was incubated at 37°C for 24hrs before measuring turbidity at 620nm in a Tecan plate reader. Appropriate positive (broth and inoculum) and negative controls (broth and honey) were included. Wells with the lowest concentration without growth were regarded as the MIC.

4.8 Minimum Bactericidal Concentration (MBC)

From the microtiter plate wells and the tubes with no growth, 20µl was removed and plated onto nutrient agar (Oxoid) that was then incubated at 37°C for 24hrs to determine the MBC. The lowest concentration that resulted in no growth was the MBC.

4.9 Radical scavenging activity of Sudanese honeys

The same honeys used in 4.4.2 4.4.3 and 4.4.4 were evaluated for their radical scavenging potential by using a spectrophotometric assay based on the reduction in absorbance at 517nm when a stable free radical, 1,1 diphenyl 2 picryl hydrazyl (DPPH), reacts with an

antioxidant. Honeys were dissolved in deionised water ($0.05 < 0.2\text{g/ml}$), and then 0.75ml of this was mixed with 1.5ml of a 0.09mg/ml solution of DPPH in methanol. The mixture was kept in the dark for 30min and the absorbance was read at 517nm (Chen *et al.*, 2000).

4.10 Results and discussion

The antibacterial activity of the honeys was investigated in order to evaluate the effects of storage time on honeys as various studies showed that the antibacterial activity of honey decreases with time depending on the heat, light and storing conditions (White and Subers, 1964a, b).

Figure 4.2 shows the antibacterial activity of 7 Sudanese honeys measured at three points during 2011 using the agar well diffusion assay described in section 4.7.

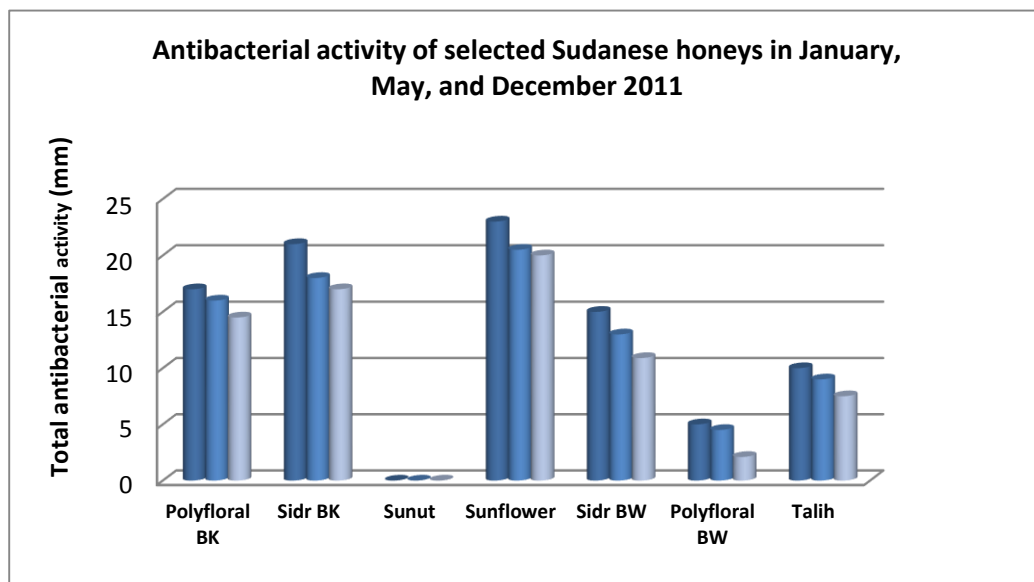


Fig 4.2 Mean antimicrobial activity measured as diameter of zone of inhibition (mm) for 7 selected Sudanese honeys against *S. aureus* in January (■), May (■) and December(■)2011 (n=3 for each honey type at each time of analysis).

The assessment of antibacterial activity of Sudanese honeys over time (Figure 4.2) has shown that the antibacterial potency of Sudanese honeys in this study decreases over the time. A decrease in the antibacterial potency of peroxidal honeys has previously been reported with prolonged storage. This deterioration is greatly dependant on the storage conditions as the peroxidal activity of honey is heat and light sensitive (White and Subers, 1964a,b).

Literature has demonstrated that the activity of manuka honeys increases with time (Henriques, 2006) and this has been explained by the continuous conversion of dihydroxyacetone (DHA) present in nectar of *Leptospermum* trees into MGO particularly in moderate temperatures (Stephen *et al.*, 2010; Adams *et al.*, 2008).

Table 4.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Sudanese and manuka honeys necessary to inhibit 99.9% of the growth of *S. aureus* NCTC 6571 *in vitro* expressed in % w/v (n = 3)

Honey	MIC Tube dilution % (w/v)	MIC in Microtiter plates %(w/v)	MBC %(w/v)
Polyfloral BK	25	25	25
Sidr BK	25	12.5	25
Sunut	>50	>50	>50
Sunflower	25	25	25
Sidr BW	25	25	50
Polyfloral BW	50	50	>50

Talih	50	50	>50
Manuka	6.75	6.75	6.75

The minimum bactericidal concentration (MBC) for Sudanese honey required to yield 99.9% kill of *S. aureus* NCTC 6571 was 25 % (w/v). This was achieved by two of the selected honeys Sidr BW and sunflower. The MBC of the manuka honey control was 6.75 % (w/v).

Table 4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Sudanese , and manuka honeys necessary to inhibit 99.9% of the growth of *E. coli* NCTC 10418 in vitro expressed in % w/v (n = 3)

Honey	MIC Tube dilution %(w/v)	MIC in Microtiter plates %(w/v)	MBC %(w/v)
Polyfloral BK	50	50	50
Sidr BK	25	25	50
Sunut	>50	>50	>50
Sunflower	25	25	25
Sidr BW	50	50	>50
Polyfloral BW	>50	>50	>50
Talih	50	50	>50
Manuka	12.5	12.5	12.5

The lowest MBC of Sudanese honey required to yield 99.9 % of *E. coli* NCTC 10418 killing was 25% (w/v) was achieved by Sunflower honey the same MBC recorded for *S. aureus* NCTC 6571. The manuka honey control MBC was 12.5 % for *E. coli* NCTC 10418 which was higher than the MBC of *S. aureus* NCTC 6571 achieved with the same honey.

Figure 4.3 shows the mean radical scavenging potential of the seven selected Sudanese honeys measured using the spectrophotometric assay detailed in section 4.9. Each honey type was assayed three times and error bars indicate the standard deviation.

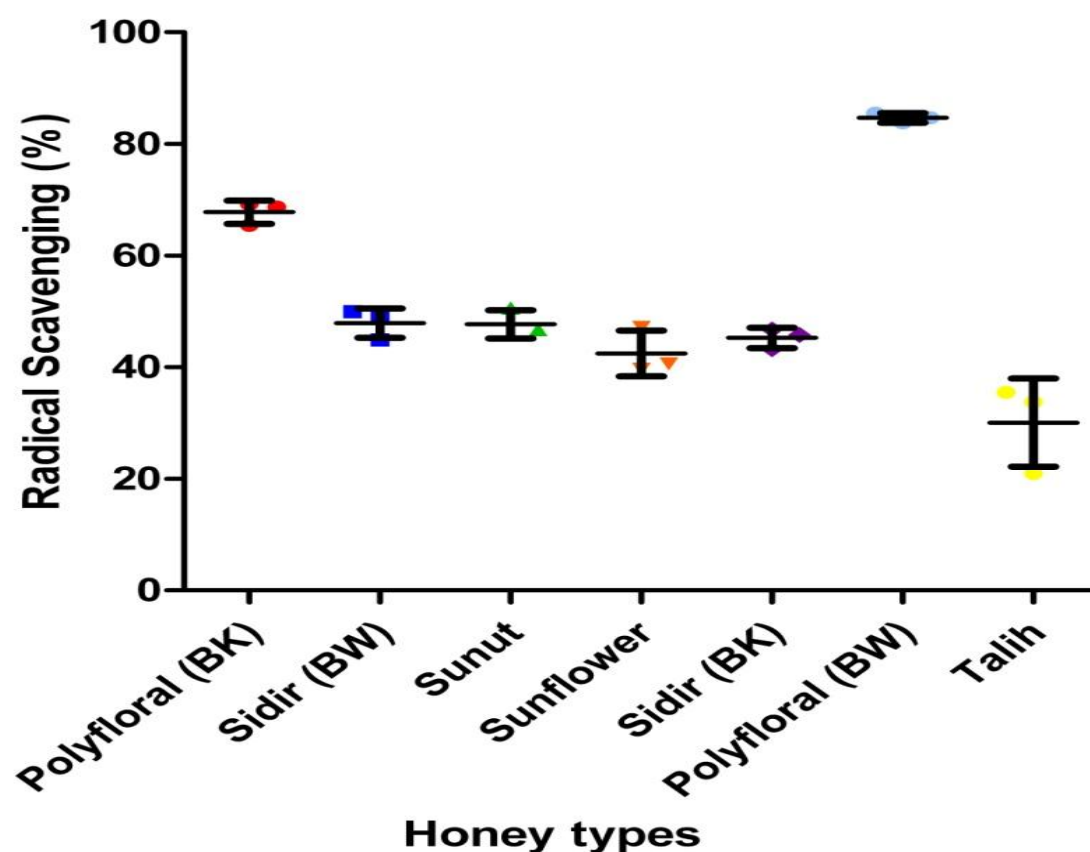


Figure 4.3 Mean radical scavenging potential of Sudanese honeys. . Error bars indicate the mean \pm SD. Difference between means $p < 0.0001$. Controls replaced Sudanese Honey with ascorbic acid. $n=3$

The results presented in Figure 4.3 show that all honeys from different floral sources tested were able to scavenge free radicals (figure 4.3); however their scavenging activity varied in a wide range, on average between 30.1- 84.7%. The polyfloral honey from Bee world apiary had the highest radical scavenging percent giving average of 84.7%, while Talih honey from the Bee kingdom apiary had the lowest radical scavenging percent content.

The results obtained in this study have shown that some Sudanese honeys were bactericidal against *S. aureus* NCTC 6571 as well as *E. coli* NCTC 10418 (Table 4.1, 4.2) and others were not, which confirm the importance of screening honeys for their antibacterial potential before their application in medicine. The lowest minimum inhibitory concentration (MIC) obtained in this study was 12.5% (w/v) for Sudanese honeys against *S. aureus* NCTC 6571 and 25% (w/v) against *E. coli* NCTC 10418, while the manuka honey control MIC was 6.75% (w/v) for *S. aureus* NCTC 6571 and 12.5% (w/v) for *E. coli* NCTC 10418. Although the control manuka honey +12 used in this study gave lower MIC and MBC compared to Sudanese honeys in this study, other honeys were reported to yield lower MIC and MBC indices than manuka honeys (Sherlock *et al.*, 2010; Tan *et al.*, 2009). Honeys from Sudan used in this study showed that some locally produced honeys were able to inhibit gram positive and gram negative bacteria (bacteriostatic), while others were able to achieve 99.9% killing (bactericidal) and one honey sample needed > 50% (w/v) to show any inhibitory effect (Sunut honey). Sunut honey did not show any inhibition zone as demonstrated in Figure 4.2 when tested by agar diffusion method and this might explain the need for >50% (w/v) to show any inhibitory effect.

This study has confirmed that Sudanese honeys from different floral sources have a wide range of antioxidant potential. Talih honey had the lowest radical scavenging potential (RSA) and the MIC of Talih honey against *S. aureus* and *E. coli* was 50% (w/v) phenol equivalent. While Sunut honey, with an RSA similar to Sunflower and Sidr honeys, did not show any inhibition zone in the agar diffusion assay and the MIC was >50% w/v phenol equivalent. These results together suggest that there was no direct correlation between the radical scavenging potential and the antibacterial activity of honey. Idris and his colleagues reported that no significant correlation was established between antioxidant activity and total phenolic contents of Sudanese honeys (Idris *et al.*, 2011).

This study demonstrates marked variations in antioxidant properties and content of Sudanese honeys from different sources. The variations in the radical scavenging potential (RSA) of Sudanese honeys comes in agreement with previous studies (Gheldof *et al.*, 2002; Meda *et al.*, 2005). These variations should be considered when honey is used as a source of dietary antioxidants.

The antioxidant activity of honey could be attributed to phenolic acids, flavonoids, enzymes, ascorbic acid, Maillard reaction products, proteins, amino acids and organic acids (Gheldof *et al.*, 2002). Variations in the antioxidant capacity could be justified by variation of the botanical source and the total phenolic contents (Gheldof *et al.*, 2002). Other reasons for the variation in antioxidant activity might be due to the difference of the enzymatic activity as well as the difference in plant secondary metabolites (Frankel *et al.*, 1998).

Sudan is a large country with a range of climatic conditions, giving rise to many floral sources hence; it is necessary to screen locally produced honeys for their antioxidant

potential and investigate the use of these antioxidants in food preservation (Rasmussen *et al.*, 2008).

Some honeys are used in folk medicine for their perceived action against certain illnesses and this could be due to their antioxidant and antibacterial potential. In a study by Schramm *et al.*, 2003 the effects of the honey on oxidant defence in humans were investigated through determination of the plasma total-phenolic content before and after consumption of buckwheat honey and found that both the plasma total-phenolic content as well as plasma antioxidant and reducing capacities were improved significantly. It was suggested that using honeys with high antioxidant content in foods instead of other sweeteners could help improve the human health; replacing sugars with a source of sweetener rich with phenolic antioxidants (Schramm *et al.*, 2003).

The antioxidant activity of Sudanese honey needs to be evaluated further through various methods, since different methods would allow the detection of different antioxidants depending on the tests employed. A single assay would only give an idea or estimation of the antioxidant properties. Moreover, a single antioxidant assay might lead to scattered results due to the complexity of the honey (Tiwari *et al.*, 2009). Despite being a simple and easy method that could be used for investigating the antioxidant activity of honey, plant extracts or foods, the DPPH should only be used for screening purposes. Future studies involving the antioxidant potential of Sudanese honeys should be conducted using various methods such as the oxygen radical absorbance capacity (ORAC) assay and Thiobarbituric acid-reactive species (TBARS) assay and incorporate controls of well researched honeys such as manuka and tulalang honeys to confirm the effectiveness of Sudanese honeys and encourage the use of those honeys for prophylaxis and therapy.

Although in this study the antibacterial activity of Sudanese honeys was found to be mainly due to hydrogen peroxide, it is possible that the antibacterial activity of Sudanese honeys is due to the interactions of biologically active group of compounds such as the polyphenols and Maillard reaction products. It is predicted that the antibacterial activity of honey is largely due to interaction rather than one main factor since synergistic action of individual groups may be responsible for enhancing or decreasing the final honey bioactivity (Brudzynski and Miotto, 2011). Polyphenols and flavonoids extracted from honey have been shown to inhibit bacterial growth in vitro to varying extents (Aljadi and Kamaruddin, 2004; Estevinho *et al.*, 2008; Russell *et al.*, 1990; Weston, 2000). Furthermore, the intermediate stage products of the Maillard reaction, such as glyoxal and methylglyoxal, showed cytotoxic effects (Riboulet-Chavey *et al.*, 2006). Methylglyoxal has been recently shown to be a dominant component responsible for the antibacterial activity of manuka honey originating from *Leptospermum* spp. (Adams *et al.*, 2008; Mavric *et al.*, 2008).

The antibacterial activity of honey is complicated as research has demonstrated that Maillard reaction products could add to the overall honey antibacterial activity (Brudzynski and Lannigan, 2012) despite being related to the HMF production which is not desired particularly in quality honeys (Zappala *et al.*, 2005) which must not have more than 15mg HMF/kg of honey. Thus the evaluation of the antibacterial activity of Sudanese honeys could be more complicated than those produced in areas with cold or intermediate temperatures as the tropical Sudanese weather could have various implications on the honey quality depending on the extraction, processing and storage which are all related with interactions in foods such as the Maillard reaction which leads to the production of HMF.

In conclusion the Sudanese honeys tested in this studied were found to possess antibacterial antioxidant potential. Unlike Manuka honeys the antibacterial activity of

Sudanese honeys has decreased during a year of storage. The MICs and MBCs of Sudanese honeys have shown that some of them were bactericidal and others were not confirming the importance of screening natural antibacterials before application for treatment or preservation. The current study has demonstrated that Sudanese honeys have antibacterial antioxidant activity. The bioactivity of Sudanese honeys is largely related to hydrogen peroxide as the addition of catalase has nullified this antibacterial activity; however, further research is required in order to determine whether other elements such as Maillard reaction products, flavonoids or other components present in the honey contribute to antimicrobial activity.

CHAPTER FIVE:

SYNERGISTIC INTERACTIONS BETWEEN HONEY, SELECTED PLANT EXTRACTS AND EDTA

5.1 Introduction

The rediscovery of honey in modern medicine as well as the success of manuka honey in eradicating bacterial pathogens from wounds, particularly where conventional antibiotics has failed has led to an increased research in order to find why honey is effective against many antibiotic susceptible as well as resistant strains including potential super bugs such as methicillin resistant *Staph aureus* (MRSA), vancomycin resistant enterococci (VRE) (Cooper *et al.*, 2002a) , *Pseudomonas aeruginosa* (Okhiria *et al.*, 2009; Alandejani *et al.*, 2009; Cooper *et al.*, 2002b), and *Bukholderia cepacia* (Cooper *et al.*, 2000).

The research into the biological activity of bees honey as well as the physicochemical properties of honey has been extensively extended during the last two decades; however, most of the research was focused on honeys from New Zealand and Australia due to their characteristic unusual antibacterial activity which is not available in the majority of the honeys around the globe (Allen *et al.*, 1991; Cooper and Jenkins, 2009; Blair *et al.*, 2009).

The source of MGO in New Zealand manuka honeys was proven to originate from the nectar (Mavric *et al.*, 2008; Adams *et al.*, 2008; 2009) which confirms the early assumptions that the unusual activity of manuka is due to the floral origin (Weston, 2000). The success of UMF trade mark and the wide spread of certified medical grade honeys as well as dressings for the management of wounds throughout Australia, New Zealand, Europe, Hong Kong, USA and Canada has increased the demand for the production of more quality certified Leptospermum honeys. The increased demand for quality and UMF certified manuka honeys has led to an almost 10 fold rise in the original price of manuka honeys (Stephens *et al.*, 2010).

The inexpensive price, effectiveness, and the availability of honeys has been a major motive behind the wide application of honey for healing various ailments particularly in developing countries and rural communities which has led to an increased research into the biological properties of honeys following the approval of medical grade honeys in wound management in modern conventional medicine. However, this fact might not be correct if we consider the increasing rise in the prices of manuka honeys. Hence; there is an urgent need to either increase the production of *Leptospermum* honeys from Australasia or find novel honeys with antibacterial activity similar to that found in *Leptospermum* honeys. Keeping in mind the approval of medical grade manuka honeys and the continuous spread throughout the globe which could only lead to more demand for those honeys as the production of new antibiotics continue to decline, producing more manuka honeys probably wouldn't be enough to cover the increased consumption and this will only lead to increased fraud and adulteration of honeys as well as elevated levels of HMF as many beekeepers will try to incubate their honeys in elevated temperatures to increase the levels of MGO in their honeys (Stephens *et al.*, 2010); hence, the best way to overcome this problem would be finding other honeys or natural products with unusual antibacterial activity.

Although recently some research has been carried out in order to find honeys from other countries with a distinct activity such as the non peroxidal activity of manuka honeys, few reports have been published demonstrating some non-peroxide activity (Henriques *et al.*, 2005; Kwakman *et al.*, 2008); however, they are still weak in comparison to the unusual UMF manuka brands.

5.2. Combination therapy and clinical practice

The use of combination therapy in clinical practice is very common and is employed for the therapeutic advantages it may provide over single agents (Drusano, 2003). Combination therapy is employed to increase the spectrum of antimicrobial activity, to prevent treatment failure when antimicrobial resistance is suspected, prevent the development of resistance, to decrease dose-related toxicity by using less of a toxic antimicrobial agent and more of the non-toxic one and to obtain enhanced antimicrobial killing or inhibition (Berenbaum, 1985, 1989; Eliopoulos G.M. and Eliopoulos C.T., 1988). Combination therapy has been known in conventional medicine for a long time particularly in the treatment of cancers where combination therapy has been applied for over half a century (Mayer and Janoff, 2007). Antibacterial drug combinations apart from Augmentin (Amoxicillin/clavulanic acid) were largely confined to the treatment of tuberculosis. The drugs used in combination may have different mechanisms of action as well as affect different sites of the body; but the overall effect of the combination may either exceed the expected effect (synergism) or nullify each other's biological effects resulting in a reduced effect (antagonism). An additive interaction is defined as the effect where the combined action is equivalent to the sum of the actions of each drug when used alone (Berenbaum, 1985; 1989; Eliopoulos G.M. and Eliopoulos C.T., 1988).

5.3 EDTA a food preservative and potentiator of honey and plants antibacterial activity

A drug may be unable to reach its site of action in the bacteria cell. This may be particularly so with Gram-negative bacteria, the cell wall of which is exceedingly complex and many-layered. However, many Gram-negative bacteria after treatment with ethylenediaminetetraacetic acid (EDTA) become sensitive to antibiotics and other agents to

which they may normally be resistant (Lambert *et al.*, 2004). EDTA releases a large proportion of the lipopolysaccharides (LPS) of the cell wall of these bacteria as well as surface enzymes, and it is clear that such organisms contain an outer permeability barrier preventing access of a drug (Haque and Russell 1974a). EDTA is normally used in conjunction with Tris buffer, but the latter may itself remove components from the cell walls of gram-negative bacteria (Barrett and Asscher). In this study 0.85mM EDTA was used as this is the permissible level in food industry (Alzoreky and Nakahara, 2003).

5.4 Honey in combination therapy

Although the recent approval of honey in modern medicine had been limited to monotherapy or the use of honey as a topical agent without any combinations, in the ancient reports as well as nonconventional medicine honey has been used in combination with plants and other substances such as ghee for the treatment of various ailments. Moreover, honey is used today in combination for the treatment of cough and wounds. Examples for such combinations are the use of honey and lemon for treatment of cough and the treatment of various ailments including wounds with a combination of honey and black seed oil (*Nigella sativa*) which is practiced in Sudan, Arabic Peninsula as well as other Muslim countries including Pakistan and Turkey.

Since Leptospermum honeys with a distinct floral derived antibacterial activity proved to be valuable source for novel antibacterial therapy (Blair *et al.*, 2009) then finding plants with potential antibacterial activity and mixing it with natural honeys could probably lead to a novel mixture having the antibacterial activity of the plant and the healing properties of the honey. In a study of Welsh honeys which has been done for the determination of antibacterial activity and healing stimulation potential it was reported that although, those

honey has demonstrated limited peroxidal antibacterial activity they did demonstrate a strong potential for the stimulation of wound healing (Wheat, 2004). If this approach was found successful then people might be able to get honey with unusual antibacterial activities by feeding the bees (*Apis mellifera*) on selected plants by adopting beekeeping methods such those applied to yield herbhoneys as they were proved to have strong antioxidant activities derived from the plants mixed with the sugars fed to the bees (Socha *et al.*, 2009). Revamil[®] honey which has been approved for wound management is an example honey which has been produced in controlled environment in the Netherlands.

5.5 Black seed and honey Combination

In Sudan as well as other countries in the Arabic Peninsula the application of local honey has been combined with Black cumin (*Nigella sativa*) or Black seed oil. *Nigella sativa* oil has been studied and proved to have antimicrobial as well as antioxidant effects (Chaieb *et al.*, 2011; Zuridah *et al.*, 2008; Hanafy and Hatem, 1991); however, the use of this combination is basically based on religious beliefs as has been explained in the Holy Qur'an as well as Sunnah or Hadith' since the majority of population are Muslims. The black seed oil honey combination is used for various ailments in Sudan including cough, wounds as well as gastrointestinal disorders. The major motive behind the use *Nigella* oil, honey as well as other extracts from medicinal plants such as *Azadirachta indica* and *Tamarindus indica* is the empirical knowledge, availability, ideal price and the difficulties in getting antibiotics as well as the time it takes before the causative agent(s) is known and the treatment is prescribed. Although honey and herbal extracts were rejected in modern medicine until the rediscovery of manuka honey in recent years, they continued to be used in Sudan as well as other third world countries and they continue to save many lives. The continuous spread of multi drug

resistant pathogens in the pan antibiotic era has encouraged researchers to look into the antimicrobial as well as antioxidant potential of natural products particularly those applied in folk medicine. Black seed oil is used alone and in combination with honey for the treatment of various ailments including wound infections in Sudan; however, the research has usually been focused on the antibacterial activity of Black seed oil and honey separately and the antibacterial activity of the pair in combination has not been investigated. In this study the activity of a combination of honey and black seed oil was tested *in vitro* to find out the outcome since, empirical knowledge alone is not enough as certain combinations could lead to antagonistic interaction rather than synergistic one.

5.6 Evaluation of Synergistic interactions *in vitro*

Synergy is a potential benefit of combination antibiotic therapy against infections. While various methods used to detect *in vitro* synergy between antibacterial agents have been described, the checkerboard technique and the time-kill curve are the most widely used techniques. Despite the fact that the checkerboard technique is far easier and less in labour and time consumption, neither the checkerboard nor the time kill curve are routinely used, thus leading to the prescription of antibacterial combinations based on empirical knowledge.

Synergy is usually evaluated through two major *in vitro* techniques *i.e.*, the checkerboard technique, giving the fractional inhibitory concentration (FIC) index, and the time-kill curve (Rybak and McGrath, 1996; White *et al.*, 1996). The checkerboard MIC method is prone to error (Rand *et al.*, 1993) and, by necessity, results from the checkerboard MIC are often confirmed with the more dynamic interaction provided by the time-kill kinetic study format (Jacqueline *et al.*, 2005; Alou *et al.*, 2004; Cappelletty and Rybak, 1996). Although the

checkerboard method is much easier to perform and only minimum amounts of the antibacterial agents are required to run the tests it is not preferred as it gives unreliable results (Wootton *et al.*, 1995). Despite being tedious and labour-intensive in nature allowing only a limited number of drug combinations to be tested, the time kill curve method has been found to be more significant as compared to *in vivo* situations. The time kill curve does not only measure the bactericidal activity but also provides a dynamic picture of the interactions and thus offers an advantageous representation of synergy (Eliopoulos and Moellering, 1991).

For the time kill studies, bactericidal activity is defined as a reduction of $\geq 3 \log_{10}$ cfu/ml. Standard definitions of synergy is a reduction of $\geq 2 \log_{10}$ cfu/ml compared with the cfu/ml of the most potent single drug and antagonism ($\geq 2 \log_{10}$ increase in cfu by the combination compared with that by the most active drug alone. (Lorian, 2005; Antimicrobial Agents and Chemotherapy, 2006).

Despite the reports that many of the antibacterial combinations resulted in either synergy or in difference (Jacqueline *et al.*, 2005; Alou *et al.*, 2004) which is probably one of the reasons which encourages clinicians to prescribe combination of medicines without *in vitro* screening as they think of antagonism as the least expected outcome of the treatment. However, this could only be a motivation to seek *in vitro* assays before prescribing antimicrobial combinations as the implementation of such practice could lead to harmful results if antagonistic or toxic results occur particularly with ever increasing emergence of resistant strains which could only lead to repetition of such practice.

While ancient medicinal recipes and traditional medicine (TM) supports the thesis that honey and plant extracts exhibit antibacterial activity alone and in combination, the continuous emergence of resistant strains of bacteria in the pan-antibiotic era necessitates

the evaluation of natural products such as honey and plant extracts and the possibility of enhancing their effectiveness with the addition of antibiotics (Jenkins *et al.*, 2012) or peptides (Kwakman *et al.*, 2010). Research into the antibacterial and antioxidant activity of honey plant extracts and combinations with other substances such as EDTA or chitosan could pave the way for novel antibacterials that combat the multi resistant strains. Moreover, as the use of honey and plant combinations in traditional medicine in Sudan as well as developing countries continue it is essential to evaluate the antibacterial efficiency of those combinations in vitro as the result of combinations might lead to antagonistic, toxic or harmful effect despite the empirical knowledge which encourages the use of such combinations.

The use of honey and plant extracts could be evaluated further as a possible growth promoter as an alternative to the antibiotics fed to animals to enhance their growth since, these antibiotics play a role in the emergence of resistant strains. In addition, to their use as antibacterials honey and plant extracts might as well be used for prophylaxis and food preservation.

Aim

The aim of this chapter was to investigate the *in vitro* effectiveness of combinations of honey and autoclaved plant extracts against *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571.

Objectives

To determine any synergistic interactions between honey and methanolic extracts of *Tamarindus indica* against *Escherichia coli* and *Staphylococcus aureus*

To introduce a standardised bioassay plate as a mean for testing natural antibacterials combinations

To validate the efficiency of the antibacterial combination of honey and black seed oil with and without EDTA

To evaluate the effect of EDTA in potentiating the antibacterial activity of methanolic extract of *Acacia nilotica* against *E. coli*

To evaluate the effect of EDTA in potentiating the antibacterial activity of a peroxidal honey against *S. aureus*

5.7 Materials and methods

In this study a Sudanese peroxidal honey, a commercial Saudi Arabian black seed oil honey, commercial black seed oil, manuka honey with UMF +12, dimethylsulfoxide (DMSO), and a methanolic extract of black seed oil from MAPRI were used. Ethylenediaminetetraacetic acid (EDTA) Fischer, two reference bacteria *Staphylococcus aureus* (NCTC 6571) and *E. coli* (NCTC 10418), two autoclaved methanolic plant extracts from MAPRI Tamarind and Acacia. Digital camera (*Canon power shot 20*), a computer and Canvas software.

5.8. Investigation of the inhibitory effects of a mixture of honey and *N. Sativa* oil

An agar diffusion assay was used to evaluate the antibacterial activity of a combination of honey and black seed oil. The honeys and black seed oil honey used in this study were used as 50% w/v unless otherwise specified. The black seed oil and methanolic extracts were dissolved in DMSO and used as 50 (%v/v). The peroxidal honeys were tested in combination with *Nigella sativa* oil in DMSO. Nutrient agar (Oxoid) was prepared in 100ml bottles, autoclaved, maintained in the water bath at 45° C for 30 minutes then 100µl of the bacterial inoculums were inoculated and 20 ml were dispensed in 5 petri dishes and kept on a leveled

surface until solidified. After storing in the cold room over the night the plates were taken out from the cold room and labeled. Then using a flamed cooled cork borer 8mm diameter zones were cut in the centre of each plate and the designated plates were loaded with the honey combinations. Peroxidal honeys alone were used as negative control, while manuka honey UMF +12 was used as positive control.

5.9 Determination of synergistic interactions using agar well diffusion assay

The antimicrobial activity of honey in combination with EDTA, and methanolic extracts of *Acacia nilotica* and *Tamarindus indica* were determined using the well diffusion method. The standardised inoculums 0.8-0.13 at O.D 625 (Wiegand *et al.*, 2008) were spread on the surface of Nutrient agar using sterile glass spreader and the surface was allowed to dry. Two wells (8.0 mm diameter) were cut the wells were punched at a predetermined distances so that their inhibitory circles touch each other only tangentially without influencing each other (Dasgupta *et al.*, 2010; Chattopadheya *et al.*, 1988). Then one was filled with honey and the other with either tamarind, Acacia or EDTA. The plates were incubated at 37 °C and observed after 24 hours. Enlargement of inhibition zones indicates a positive interaction or synergism (Ahmad and Aqil, 2007).

5.10 Determination of synergistic interaction in a large bioassay plate

A literature search suggests that interactions of different antimicrobial agents against Gram positive and Gram negative bacteria in a standardised large bioassay plate has not been carried ou before before; hence, this study aimes to evaluate the synergistic interactions between honey, EDTA, *Acacia nilotica* and *Tamarindus indica* methanolic extract under standardised conditions which could be adopted for screening of various bacteriostatic synergistic combinations. The test was done in a large bioassay plate (Nunc bioassay plate

245 × 245mm dish; Corning Inc.) which was used by Allen *et al.*, (1991) for the evaluation of non-peroxidal and total antibacterial activity. The increase or decrease in the zone due to synergy or antagonism was measured by using Canvas 11 (Windows Vista) 2007.

5.10.1 Preparation of bacterial culture for the assay

Three to five colonies of *S. aureus* NCTC 6571 were transferred to 100 ml TSB and incubated at 37°C for 18 hours. The following day, the optical density of the culture was adjusted to 0.08 at 625 nm with TSB. This was used as the inoculum.

5.10.2 Preparation and seeding of the bioassay plates

Two large square plates (245 × 245mm dish; Corning Inc.) were placed on a leveled surface. One plate was for inoculation with *S. aureus* NCTC 6571 and the other was inoculated with *E. coli* NCTC 10418. Two bottles of nutrient agar 200 ml were prepared autoclaved and then maintained at 45°C in a water bath for 30 minutes. Into each 150µl of the bacterial inoculum was transferred, and the agar was swirled well without causing bubbles to be incorporated, then poured into the square plates evenly. The agar plates were covered with the lids and left on the bench for one hour and then stored at 4°C for overnight.

5.10.3 Antibacterial activity test

The plates prepared and inoculated as mentioned above in 5.10.1 and 5.10.2 were used for this assay. The plates were divided into rows, in each row four wells were cut using a cooled flamed 8 mm diameter cork borer. Two wells were cut adjacently while the other two were made distant from those. Honey was filled in one side while tamarind, acacia or EDTA were filled in the other end. The adjacent wells one was filled with honey and the other with either Tamarind Acacia or 0.85mM EDTA. Sterile deionised water was used as a negative control in the bioassay. After loading the wells the plates were incubated at 37°C for 18

hours (Figure 5.2). After overnight incubation image of the plates was taken with a digital camera (Canon *power shot 20*, Japan), then the pictures were transferred to a computer and with the aid of Canvas software the enlargement in zone inhibition were measured applying mathematical formula i.e.,

$$E = \frac{L_f - L_0}{L_0} \times 100$$

Whereas e = the magnitude of extension,

L_f = final length and

L_0 = initial length

5.11 Determination of the time kill curve

The effect of honey and antibacterial combinations on the viability of cells were monitored by inoculating 2mL of an overnight culture of *Staphylococcus aureus* and *Escherichia coli* into 18ml Nutrient broth and incubated at 37°C with shaking (100rpm). Samples were then removed at known intervals, diluted serially and transferred to Nutrient Agar plates (Oxoid) and counted following the Miles and Misra technique then incubated at 37°C for 24 hrs. The number of CFUs was then counted and time kill plots were constructed.

The effects of the antimicrobial combinations were interpreted as follows: synergy was defined as at least a 100-fold increase in killing at 24 hrs with the combination compared to the honey or plant extract alone. Antagonism was defined as at least a 100-fold increase in colony count at 24 hrs with the combination when compared to the honey or plant extract alone. Additivity/indifference was defined as any other scenario not meeting the criteria for either synergy or antagonism (Bonapace *et al.*, 2000).

5.12 Results and Discussion

The combination of honey with black seed oil was suggestive of synergistic interaction due to the enlargement the inhibition zone when the two wells were made close to each other. The enhancement of the inhibition of honey and black seed oil with EDTA was clear as seen in Figure 5.1.

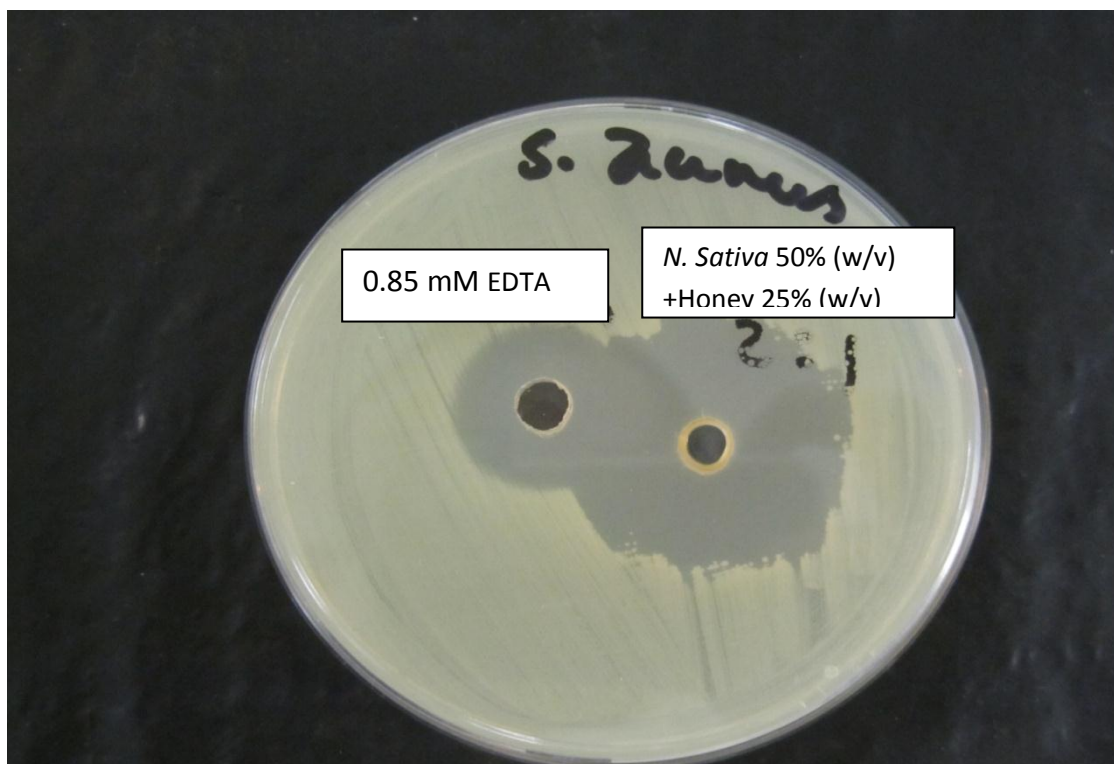


Figure 5.1 Synergy between 50% (w/v) *Nigella sativa* extract and 25% (w/v) honey in combination with 0.85 mM EDTA against *Staphylococcus aureus* NCTC 6571 after incubation at 37°C for 24 hrs.

The synergistic interaction between honey and EDTA as well as the synergy between a plant extract and EDTA yielded enlarged inhibition zones as seen in Figure 5.2 and Figure 5.3. The difference between the enlarged zones due to the effect of EDTA is clear in Figure 5.2 compared to the negative control which is deionised water.

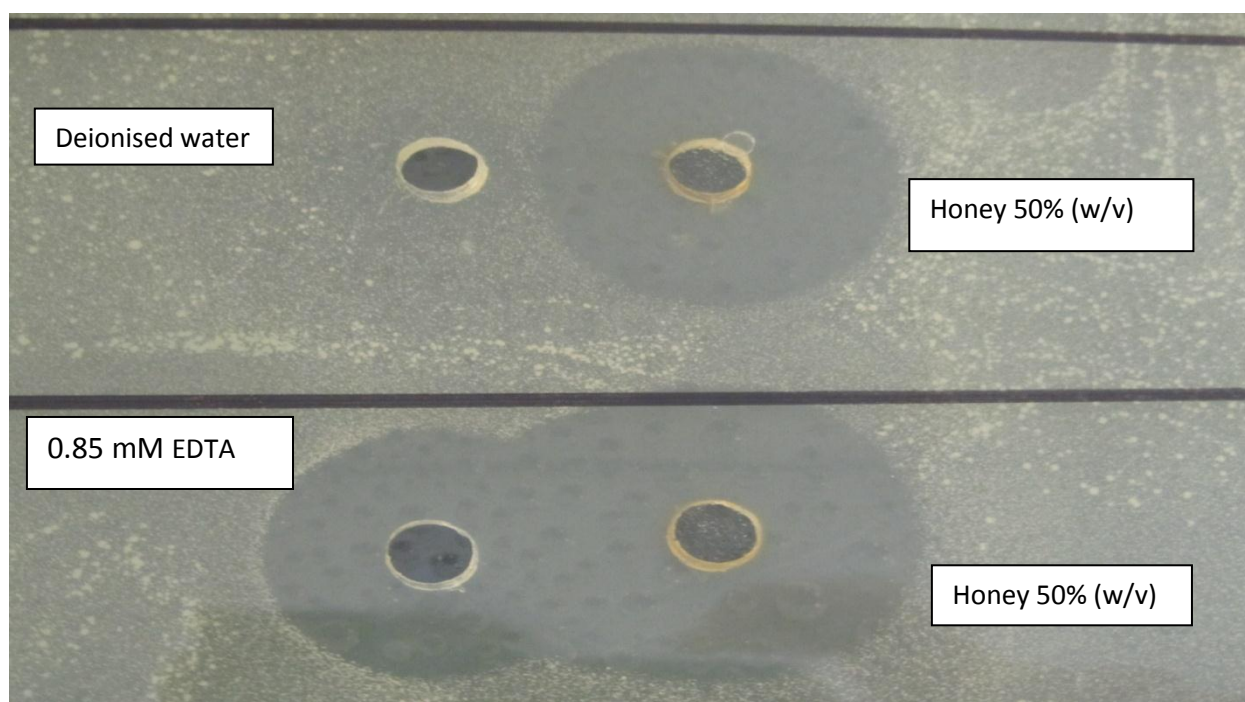


Figure 5.2 Synergy between 50% (w/v) honey in combination with 0.85 mM EDTA against *Staphylococcus aureus* NCTC 6571 after incubation at 37°C for 24 hrs.

The synergistic effect of EDTA with honey is clear above compared to zone of honey alone.

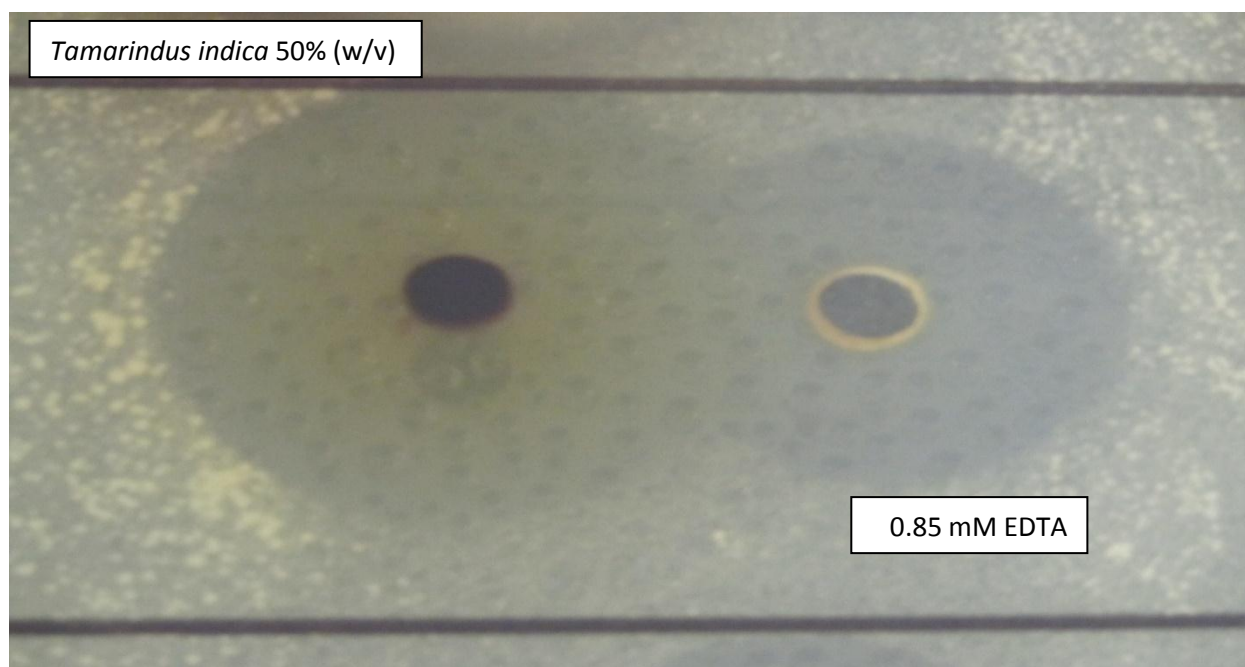


Figure 5.3 Synergy between 50% (w/v) *Tamarindus indica* extract in combination with 0.85 mM EDTA against *Escherichia coli* NCTC 10418 after incubation at 37°C for 24 hrs.

The synergistic effect of EDTA with *Tamarindus indica* is clear above compared to zone of *Tamarindus indica* alone.

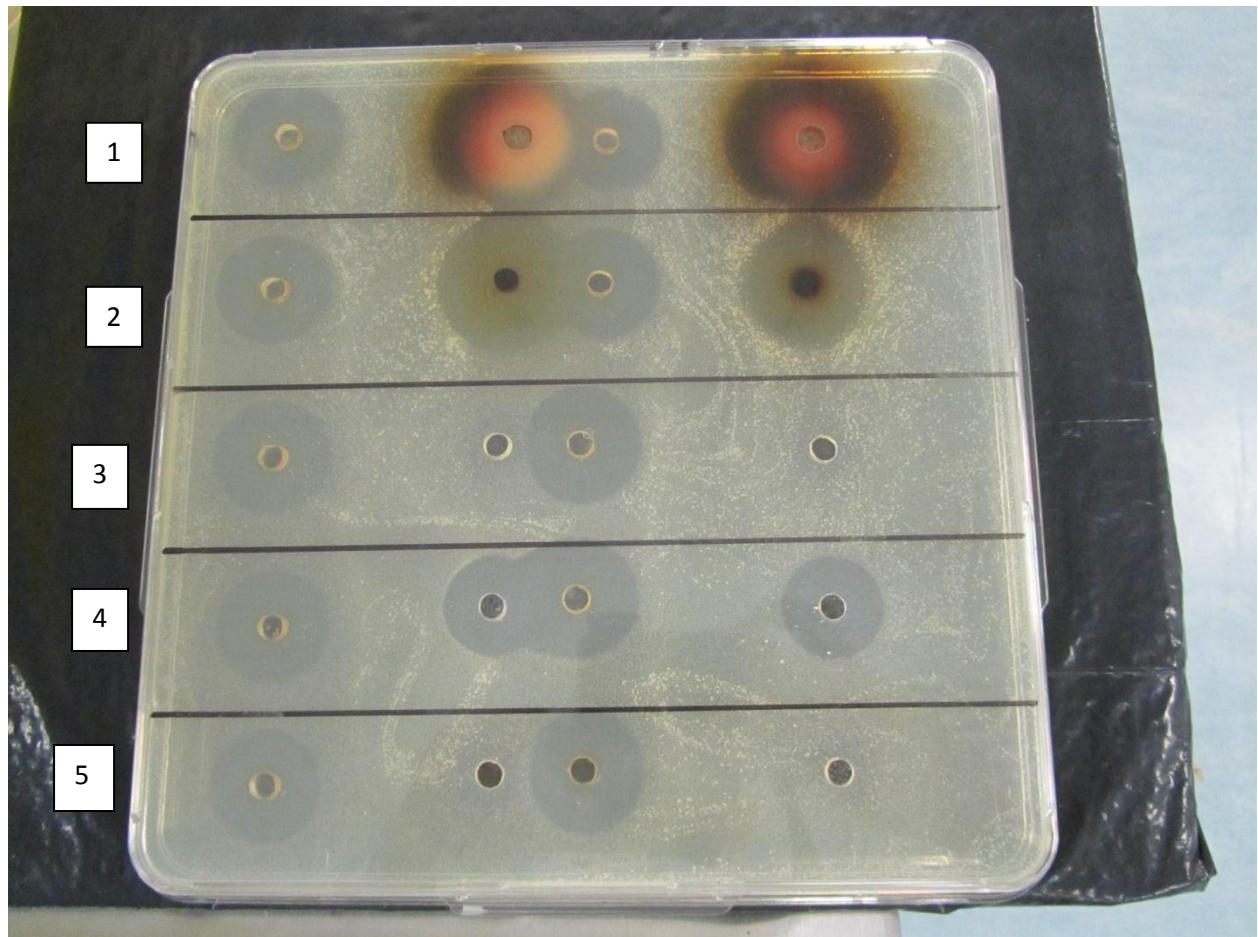


Figure 5.4 A large bioassay plate seeded with *Staphylococcus aureus* NCTC 6571 used to study combination between 50% (w/v) honey, 0.85 mM EDTA, 50% (w/v) Tamarind extract, 50% (w/v) Acacia extract and incubated overnight at 37°C. Distilled water (DW) used as a negative control

The wells from left to right in rows labeled 1-5

1. Honey, Acacia + honey, Acacia
2. Honey, Tamarind + honey, Tamarind
3. Honey, DW + honey, DW
4. Honey, EDTA+ honey, EDTA
5. Honey DW + honey+ DW

Figure 5.5 demonstrates the use of Canvas software for the measurement of synergy between a Sudanese honey and EDTA against *Staphylococcus aureus* NCTC 6571. The Canvas 11 software was used for the measurement of the enlargement of the zone of inhibition upon combination compared to the initial measurement as a novel method for evaluating synergistic interactions using agar well diffusion method.

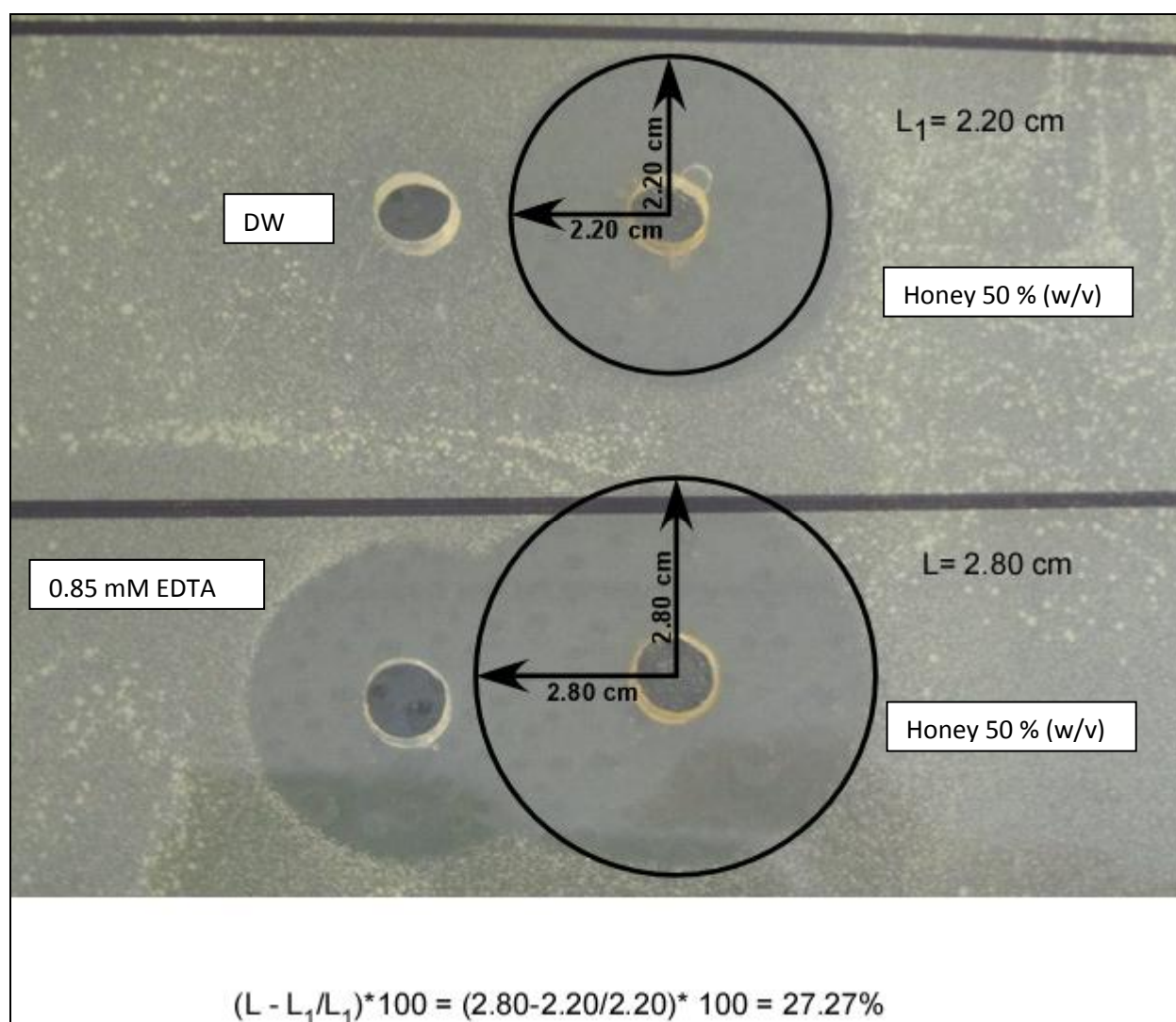


Figure 5.5 Synergy between 50% (w/v) honey and 0.85 mM EDTA against *Staphylococcus aureus* NCTC 6571 after incubation at 37°C for 24 hrs was determined by using Canvas software and applying mathematical formula.

The increase in the diameter of inhibition zones in the agar diffusion assays showed clearly synergistic interactions (Figures 5.1-5.5), which confirmed the need for more precise measurements for synergistic bacteriostatic interactions. In this study the Canvas software

was used to optimize the measurement of the inhibition zones diameter and overcome the problem of overlapping between the edges of the adjacent wells, which is difficult to measure using rulers or digital calipers.

The Canvas software analysis of the combination of honey and Tamarind against *S. aureus* NCTC 6571 yielded a 4 % increase in effectiveness compared with honey alone, Acacia and EDTA yielded 16 % increase and honey and EDTA yielded a 27 % increase compared to Acacia and honey alone respectively.

The agar diffusion method applied in this chapter and the MICs applied in the previous chapters as well as the measurement of synergy using Canvas software are all be useful in the search of combination of natural antimicrobials but in order to confirm the results of these experiments more laborious methods are needed such as the time kill curve method. The time kill curve has the advantage of giving the optimum dose/concentrations of the antibacterials as well time length required for the killing of the target organism.

Figures 5.6-5.17 present the results of single time kill curve experiments for Manuka honey alone and the selected Sudanese honey, alone and in combination with EDTA and selected plant extracts. Experiments were conducted against *Staphylococcus aureus* (NCTC 6571) and *Escherichia coli* (NCTC 10418).

Figures 5.6 and 5.7 present time kill curves for selected Sudanese Honey and Manuka honey respectively against *S aureus* NCTC 6571 over a period of 24 hours.

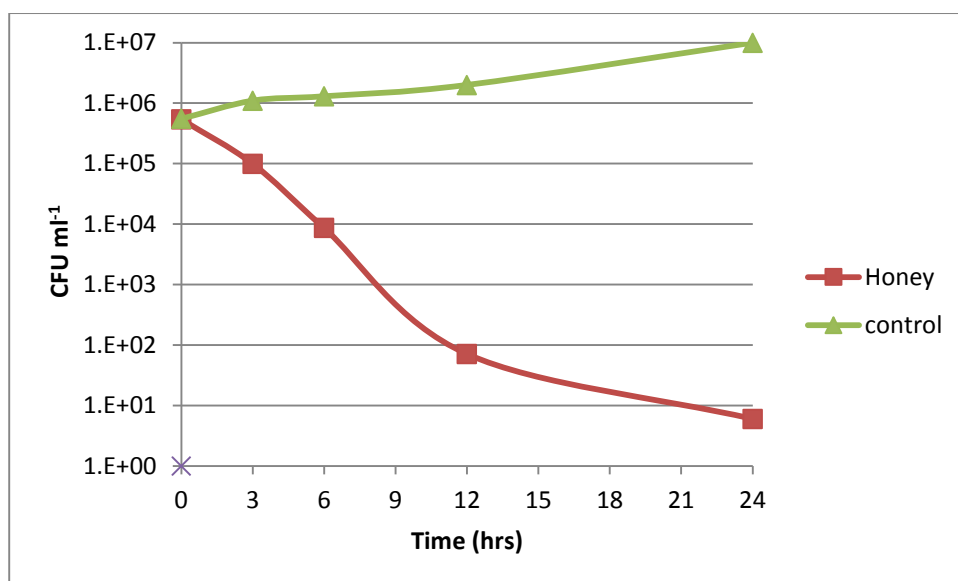


Figure 5.6 Time kill curve (CFU ml⁻¹ over 24 hours) for *S. aureus* NCTC 6571 incubated with Sudanese Honey (50%) in nutrient broth at 37 °C. Controls replaced Sudanese honey with deionised water. n=1

Figure 5.6 shows that a concentration of 50% (w/v) of Sudanese honey was bactericidal against *S. aureus* NCTC 6571 giving a 3 log reduction over 9 hrs, with a 2 log reduction in 6 hrs.

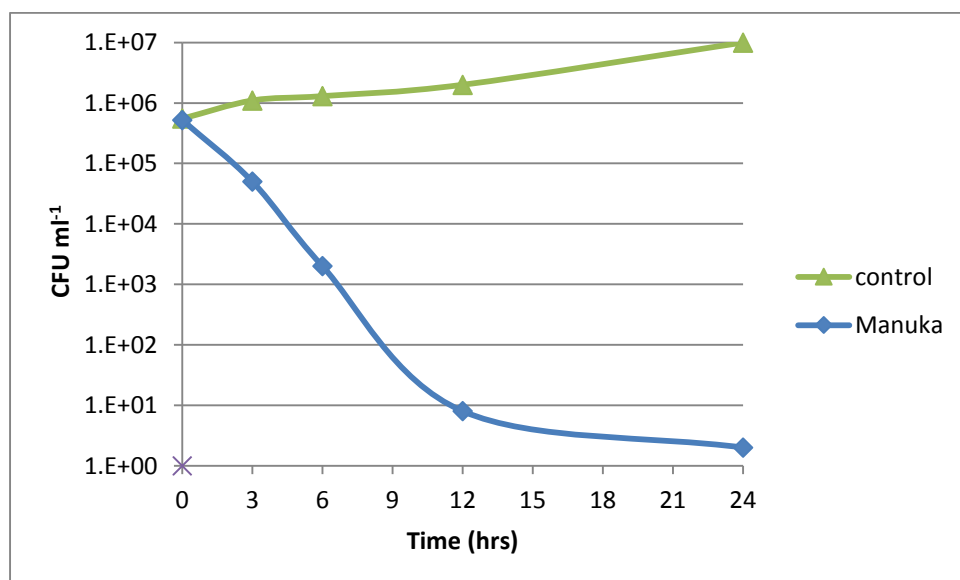


Figure 5.7 Time kill curve (CFU ml⁻¹ over 24 hours) for *S. aureus* NCTC 6571 incubated with 12.5% (w/v) Manuka honey in nutrient broth at 37 °C. Controls replaced Manuka honey with deionised water. n=1

Figure 5.7 shows that a concentration of 12.5 % (w/v) manuka honey UMF 12 + was bactericidal against *S. aureus* NCTC 6571 producing a 3 log reduction in 7 hrs, with a 2 log reduction in 5 hrs.

Figures 5.8 and 5.9 present time kill curves for Tamarind and Acacia extracts respectively against *S aureus* NCTC 6571, while Figures 5.10 and 5.11 present time kill curves for Sudanese honey in combination with EDTA and Tamarind extract respectively against *S. aureus* NCTC 6571

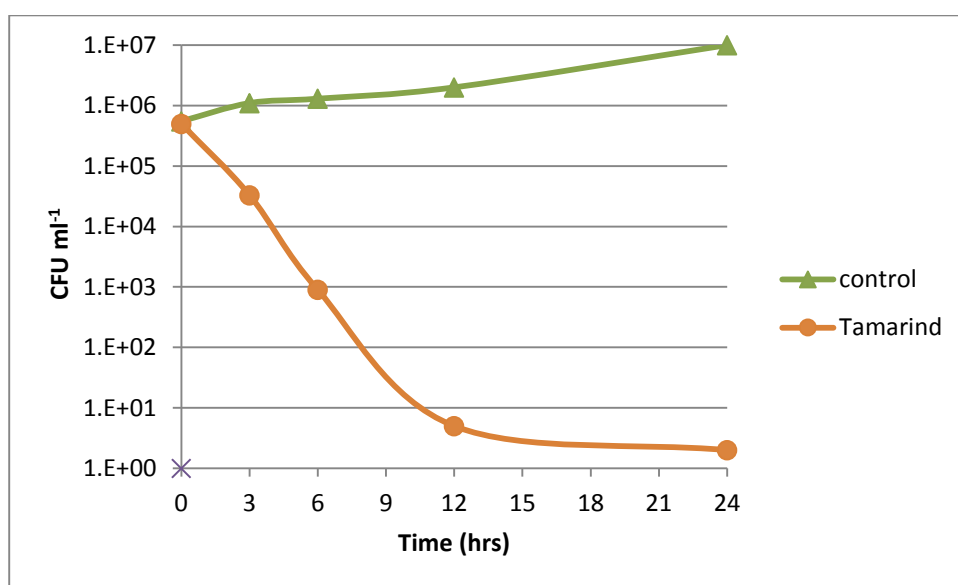


Figure 5.8 Time kill curve (CFU ml⁻¹ over 24 hours) for *S aureus* NCTC 6571 incubated with 0.39% (w/v) Tamarind in nutrient broth at 37°C. Controls replaced Tamarind extract with deionised water. n=1

Figure 5.8 shows that 0.39% (w/v) concentration of *Tamarindus indica* was bactericidal against *S. aureus* NCTC 6571 giving a 3 log reduction in 7 hrs, with a 2 log reduction in 5 hrs.

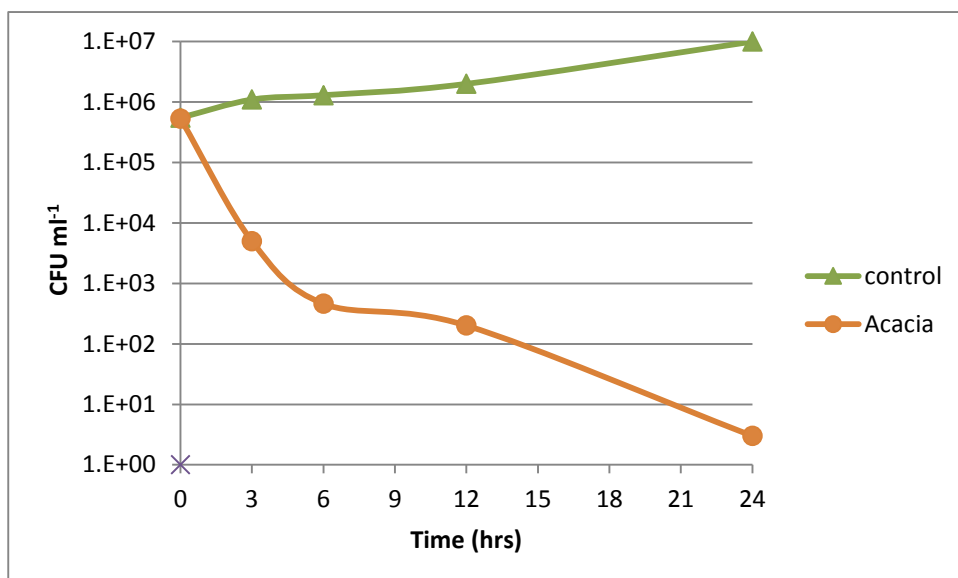


Figure 5.9 Time kill curve (CFU ml⁻¹ over 24 hours) for *S. aureus* NCTC 6571 incubated with 1.56% (w/v) Acacia in nutrient broth at 37°C. Controls replaced Acacia with deionised water. n=1

Figure 5.9 shows that 1.56% (w/v) of *Acacia nilotica* was bactericidal against *S. aureus* NCTC 6571 as it gave 3 log reductions in 6 hrs, while 2 log reductions needed only 4 hrs.

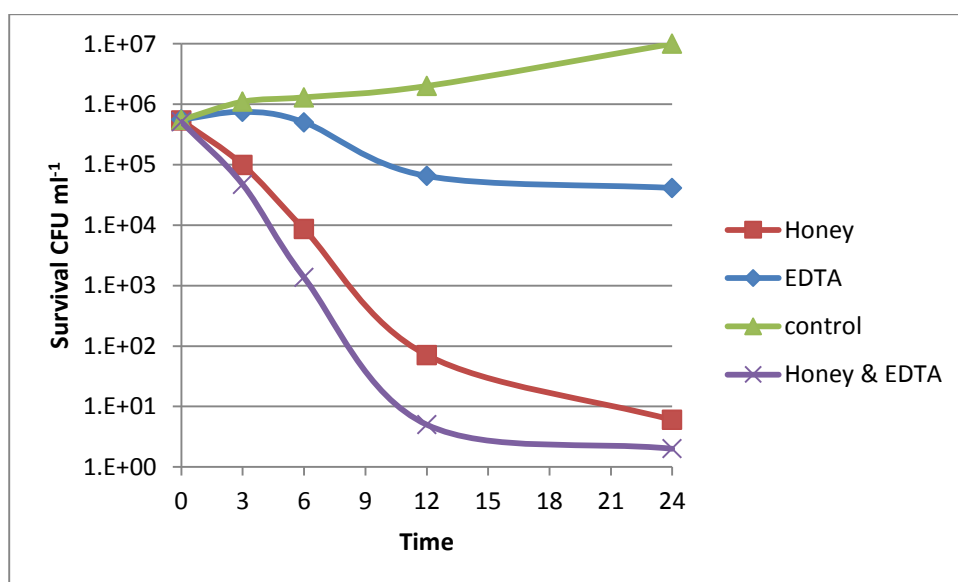


Figure 5.10 Time kill curve (CFU ml⁻¹ over 24 hours) for *S. aureus* NCTC 6571 incubated with 50% (w/v) Sudanese honey and 0.85mM EDTA in nutrient broth at 37°C. Controls replaced Sudanese honey with deionised water. n=1

Figure 5.10 shows the combined effect of the combination of 50% (w/v) Sudanese honey and 0.85mM EDTA against *S. aureus* NCTC 6571. The killing curve shows bactericidal effect by giving a 3 log reduction with in 7hrs, while a 2 log reduction was obtained with in 5hrs. The combination could be classified as synergistic since the combination of honey gave 3 log reduction in 7hrs compared to the activity of honey alone where it took a minimum of 9hrs to give 3 log reduction.

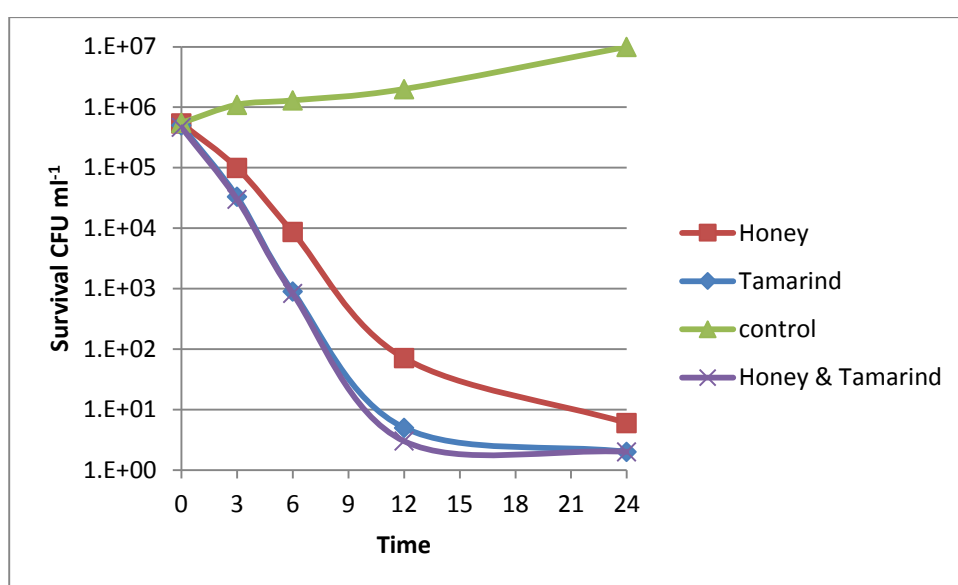


Figure 5.11 Time kill curve (CFU ml⁻¹ over 24 hours) for *S. aureus* NCTC 6571 incubated with 0.39% (w/v) Tamarind and 50% (w/v) Sudanese honey in nutrient broth at 37°C. Controls replaced Sudanese honey with deionised water. n=1

Figure 5.11 shows the combined effect of the combination of 50%(w/v) of Sudanese honey and 0.39%(w/v) of tamarind extract against *S. aureus* NCTC 6571. The killing curve shows bactericidal effect by giving a 3 log reduction within 8hrs, while a 2 log reduction was obtained within 5hrs. The combination could not be classified as synergistic as the combined effect was almost equal to that obtained by using Tamarind extract alone.

Figures 5.12-5.17 present time kill curves for *Escherichia coli* NCTC 10418.

Figures 5.12 and 5.13 present time kill curves for selected Sudanese Honey and Manuka honey respectively against *E. coli* NCTC 10418 over a period of 24 hours.

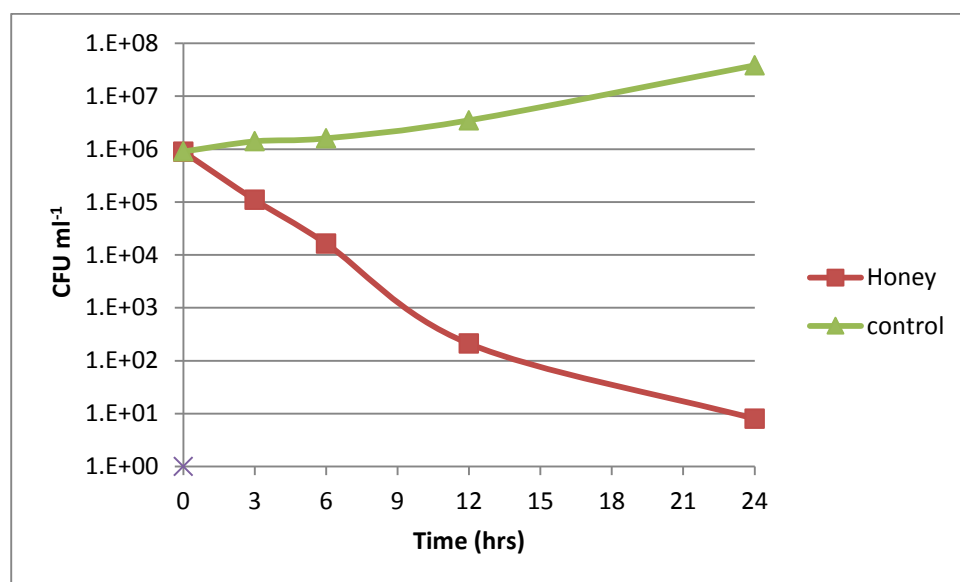


Figure 5.12 Time kill curve (CFU ml⁻¹ over 24 hours) for *E. coli* NCTC 10418 incubated with 50% (w/v) Sudanese honey in nutrient broth at 37°C. Controls replaced Sudanese honey with deionised water. n=1

Figure 5.12 shows that 50% (w/v) of Sudanese honey was bactericidal against *E. coli* NCTC 10418 as it achieved a 3 log reduction in 9 hrs, while a 2 log reduction required 6 hrs.

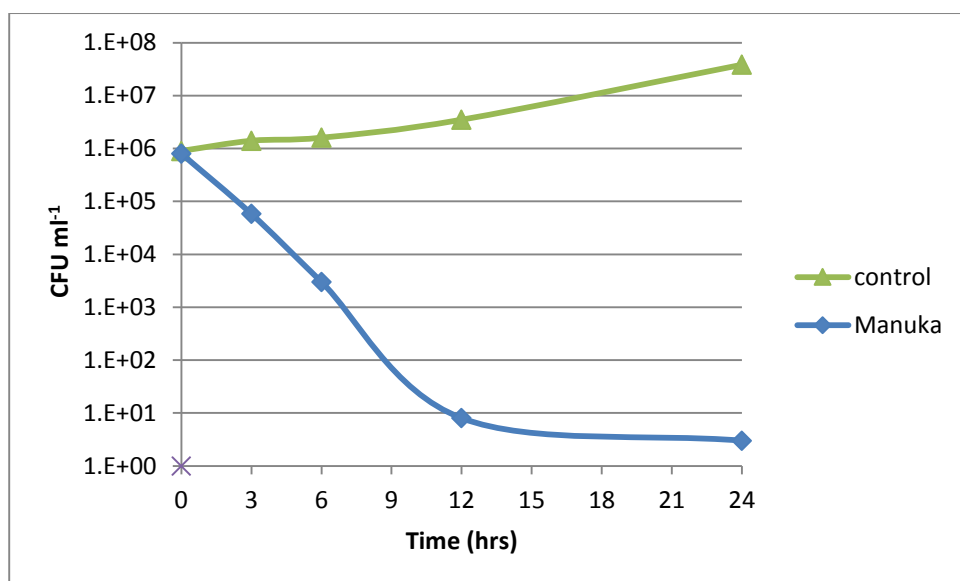


Figure 5.13 Time kill curve (CFU ml⁻¹ over 24 hours) for *E. coli* NCTC 10418 incubated with 25 % (w/v) Manuka honey in nutrient broth at 37°C. Controls replaced Manuka honey with deionised water. n=1

Figure 5.13 shows that 25% (w/v) of manuka honey UMF 12 + was bactericidal against *E. coli* NCTC 10418 as it achieved a 3 log reduction in 7 hrs , while a 2 log reduction needed around 5hrs.

Figures 5.14 and 5.15 present time kill curves for Tamarind extract and Tamarind extract in combination with Sudanese honey respectively against *E. coli* NCTC 10418, while Figures 5.16 and 5.17 present time kill curves for Acacia extract and Acacia extract in combination with EDTA respectively against *E. coli* NCTC 10418.

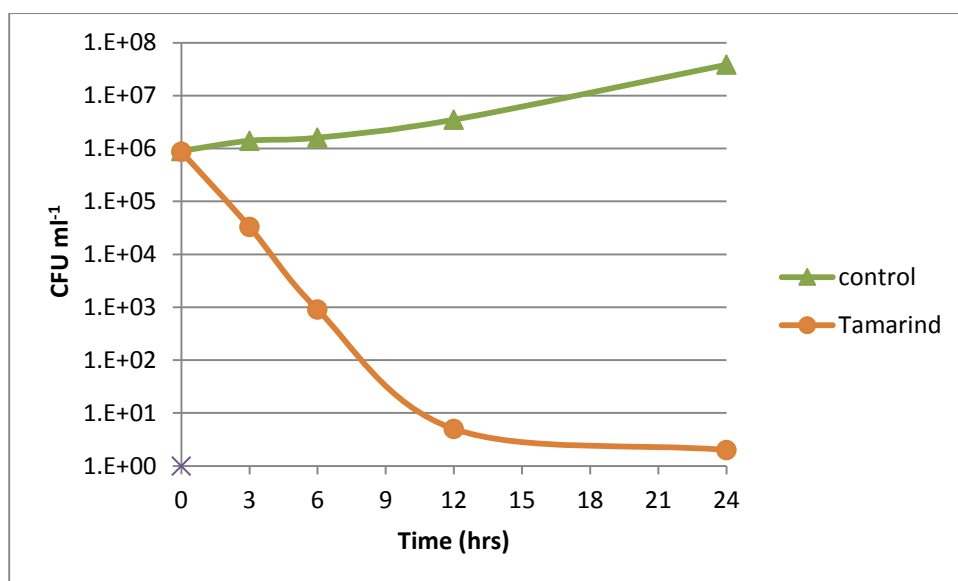


Figure 5.14 Time kill curve (CFU ml⁻¹ over 24 hours) for *E. coli* NCTC 10418 incubated with 0.39% (w/v) Tamarind in nutrient broth at 37°C. Controls replaced Tamarind with deionised water. n=1

Figure 5.14 shows that 0.39 %(w/v) of *Tamarindus indica* was bactericidal against *E. coli* NCTC 10418 as it achieved a 3 log reduction in 6 hrs, while a 2 log reduction needed 4 hrs.

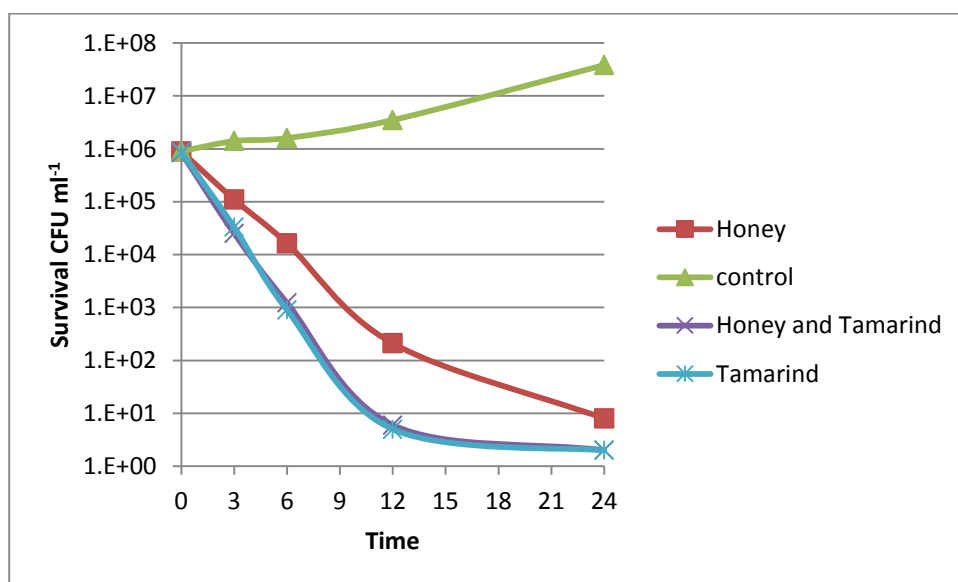


Figure 5.15 Time kill curve (CFU ml⁻¹ over 24 hours) for *E. coli* NCTC 10418 incubated with 0.39 % (w/v) Tamarind and 50% (w/v) Sudanese honey in nutrient broth at 37°C. Controls replaced Sudanese honey with deionised water. n=1

Figure 5.15 shows the combined effect of the combination of 50% (w/v) of Sudanese honey and 0.39% (w/v) of Tamarind extract against *E. coli* NCTC 10418. The curve shows bactericidal effect achieving a 3 log reduction within 6hrs, while a 2 log reduction was obtained within 4hrs. There is no evidence of synergy as the combined effect was very similar to that obtained by using Tamarind extract alone.

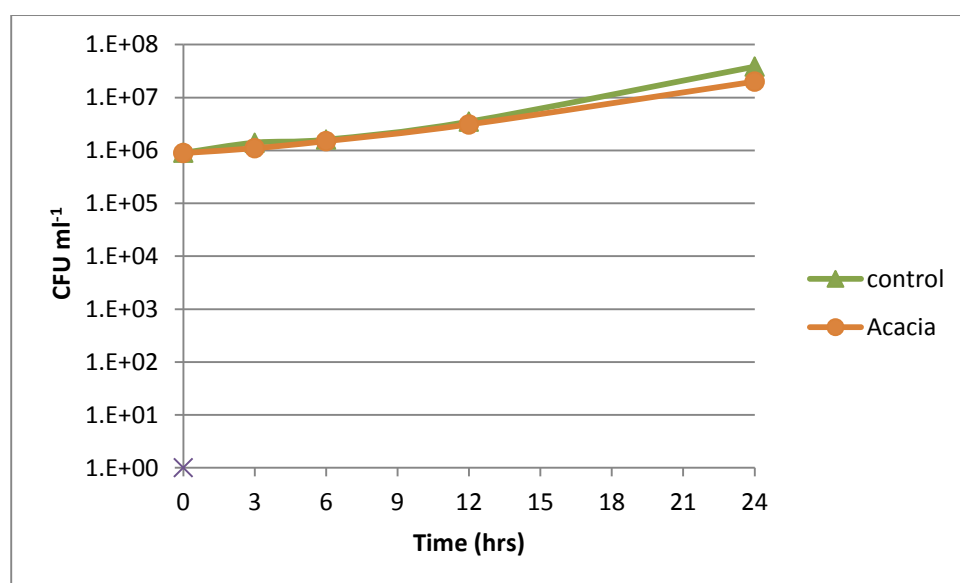


Figure 5.16 Time kill curve (CFU ml⁻¹ over 24 hours) for *E. coli* NCTC 10418 incubated with 1.56% (w/v) Acacia in nutrient broth at 37°C. Controls replaced Acacia with deionised water. n=1

Figure 5.16 shows clearly how *E. coli* NCTC 10418 was not affected by 1.56% (w/v) Acacia as the growth was almost equal to that of the control. This may be due to the impermeability of the bacterial cell wall as Gram negative bacteria have a complex cell wall.

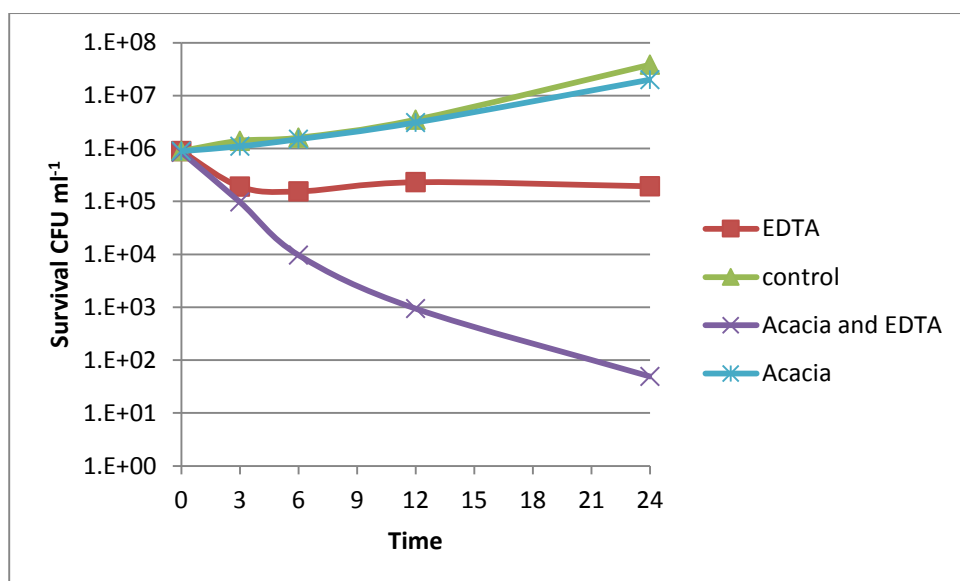


Figure 5.17 Time kill curve (CFU ml⁻¹ over 24 hours) for *E. coli* NCTC 10418 incubated with 1.56 % (w/v) Acacia and 0.85 mM EDTA in nutrient broth at 37°C. Controls replaced Acacia with deionised water. n=1

Figure 5.17 shows the combined effect of the combination of 1.56 % (w/v) Acacia and 0.85 mM EDTA against *E. coli* NCTC 10418. The killing curve shows bactericidal effect by giving 3 log reductions after 12hrs, while 2 log reductions was obtained with in 6 hrs. The combination could be classified as synergistic since the combination of Acacia with EDTA achieved a 3 log reduction in 12 hrs while Acacia extract alone had no effect on the growth of the culture. EDTA is known to increase the permeability of Gram negative cell walls and make it easier for agents to enter the cell.

The initial screening with agar diffusion of honey plant extracts and EDTA combinations has shown encouraging results as antagonistic interactions were not found neither between honey and EDTA nor between honey and selected plant extracts. These results were further confirmed by the use of Canvas software to measure the increase due to synergy based on measuring the difference between the diameters of the zone of the antibacterial agent alone and in combination; with the synergy calculated using a mathematical formula to give

the percentage of enlargement of the inhibition zones. Canvas results were found to be in agreement with the time kill curve results for Honey in combination with EDTA against *Staph aureus* and *Acacia* and EDTA against *E.coli*. The slight synergy indicated by the Canvas software for the combination of Honey and Tamarind extract against *S. aureus* was not supported by the Time Kill curve.

Despite the requirements of this new method which includes the need for digital camera and a Canvas software program this method looks appealing as it is much easier to perform and not as labour intensive as conducting time kill curves. Moreover, future studies could lead to agreement of specific percentages to define synergy or antagonistic interactions for use in rapid screening of bacteriostatic activity. Time kill curves would then be used to evaluate bactericidal effect of the combinations showing most potential. A simple to use screening method will encourage researchers to look for successful combinations that could fight the emerging antibiotic resistant pathogens. The canvas method described and evaluated here is simpler to perform than the checkerboard method which also identifies bacteriostatic combinations that must be confirmed by time kill curves.

The EDTA concentration used in this research was 0.85mM, selected as this is the permissible level laid down by many food legislative organizations (Sikes and Ehioba, 1999; Hansen *et al.*, 2001). This concentration was found to give positive result with honey against *S. aureus* and *Acacia nilotica* against *E. coli*. Despite being resistant to *E. coli* when used alone, there was synergistic interaction between *Acacia nilotica* and EDTA as the addition of EDTA led to the inhibition of *E. coli* in 12 hours. The resistance of *E. coli* to *Acacia nilotica* extract is in accordance with previous reports where Gram negative bacteria were found to be resistant to plant extracts (Brantner *et al.*, 1994; Nostro *et al.*, 2000; Ojala *et al.*, 2000)

and this resistance of Gram negative bacteria is related to lipopolysaccharides in their outer membrane (Sawer *et al.*, 1997; Gao *et al.*, 1999). The ability of EDTA to chelate the divalent cations present in the cell wall such as magnesium (Farca *et al.*, 1997), may explain the synergistic effect exerted by the combination of *Acacia nilotica* and EDTA against resistant bacteria.

The antibacterial and antioxidant potential of plant extracts make them a potential source for inhibitory substances for some food borne pathogens and the combination of EDTA which is a food additive might lead to synergistic interaction adding to their bioactive potential (Alzoreky and Nakahara, 2003). Antibacterial combinations could be useful particularly when the causative pathogen is not identified as it offers a broad-spectrum coverage until the causative pathogens are isolated and identified (Rybak and McGrath, 1996). Clinicians tend to prescribe combination therapy based on empirical knowledge without the use of *in vitro* synergy data, as there is a lack of clinical data to correlate the results of *in vitro* synergy studies with patient outcome (Rybak and McGrath, 1996).

Although *Tamarindus indica* extract in this study has confirmed the broad spectrum activity reported by Al-naimat [Al-naimat, 2011], there was indifference when sub-inhibitory combination of tamarind was combined with 25% (w/v) of Sudanese honey as the time required to inhibit the growth of *E. coli* (Figure 5.14) and *S. aureus* (Figure 5.11) was almost the same time required for 0.39 (w/v) of tamarind alone to inhibit the micro organism.

Steenbergen has demonstrated that synergy in antibiotics such as daptomycin is highly strain and drug specific, and that indifference or an additive response was the most common interaction of daptomycin with a variety of other antibiotics (Steenbergen *et al.*, 2009). Selected instances of synergy were reported and the reasons behind that remain

unknown (Steenbergen *et al.*, 2009). Since synergy is found to be selective between antibiotics without clear understanding behind this phenomenon, this is expected to be found between honey and plant extracts and it might even be difficult to determine the mechanisms of synergy due to the varied and complex composition of honey and plant extracts. Although synergy detected by agar diffusion might not be confirmed by the time kill curve in many cases, the lack of antagonism seen with honey and plant extracts combinations in this study is an encouraging outcome suggesting that honey may prove to be effective in combination therapy as well as in monotherapy.

In conclusion this study has demonstrated that honey could be potentiated by EDTA using Canvas software technique to measure the change in zones of inhibition on bioassay plates. This could be adopted as a novel simple method for screening synergistic interactions as the results came in agreement with results of time kill curve. The study has also demonstrated that EDTA could be used to broaden the antibacterial activity of *Acacia nilotica* against *E. coli* which was resistant to *Acacia nilotica* methanolic extract (Figure 5.16) before the addition of EDTA sensitized the target bacterium (Figure 5.17). The time kill curve method has confirmed the synergistic interactions between honey and EDTA, *Acacia nilotica* and EDTA as well as the bactericidal effect of a Sudanese honey, manuka honey, tamarind and acacia against *S. aureus* NCTC 6571. In addition to the bactericidal effect of Sudanese and manuka honeys, tamarind and a combination of acacia and EDTA against *E. coli* NCTC 10418 as well as the indifference in the combination of honey and tamarind against *E. coli* (Figure 5.15). This study demonstrated that none of the combinations tested in this study showed any antagonistic interaction which encourages the continuity of their use in therapeutics, prophylaxis and food preservation. However, further research with other honeys against clinically relevant isolates with suitable controls is essential before their acceptance by the

medical profession. This study has shown the *in vitro* demonstration of synergy between Sudanese honey, EDTA and plant extracts, however, further research using clinical isolates as well a broader range of honeys is required.

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE WORK

Sudan is a large country with excellent beekeeping potential; however currently relatively small quantities of honey are produced despite having all the resources that are required to make Sudan one of the biggest producers and exporters in the world due to the plentiful supply of water, land and required climatic locations.

The Sudanese government should pay attention to beekeeping as a well managed honey industry could be a major source of revenue and support many farmers who cannot generate sufficient income from farming alone. Moreover, beekeeping practice is a business which can be operated on a small or large scale; it does not need a large workforce and can be undertaken by women supplementing family income. Furthermore, beekeeping maintains the biodiversity of the lands and adds to the overall productivity of agricultural projects as the bees are excellent pollinators.

Despite a widespread belief in the population in the beneficial properties of honey and its use in nutrition and therapy in Sudan, honey needs more attention from the authorities as well as the researchers in Sudan, since the antimicrobial as well as physico-chemical characteristics are not well recognized and the local Sudanese honeys are not authenticated.

The characterisation of any honey sample requires financial commitment as well as time as it is necessary to evaluate the same type of honey throughout different seasons and from various locations to assess the geographic as well as the climatic impacts on the quality of honey in addition to the bee type which plays an essential role as well. This study has shown that characterisation of Sudanese honeys is very difficult due to the difficulties in maintaining a continuous provider for the same honey type over a period of three to four years as the majority of beekeepers are small scale producers with a minimum turnover which does not allow the continuous stability due to the socioeconomic conditions in Sudan.

Many beekeepers involved in the early stages of this study quit honey production due to the poor economic conditions and difficulties finding stable markets for their products. In this study we found that apart from two apiaries none of the the beekeepers own the lands on which they run their beekeeping projects; hence, the selection of the honeys for bactericidal and antioxidant activity was from Bee Kingdom and Bee World apiaries as they alone could provide consistent samples over several seasons.

Research has shown that identification of MGO as the main reason for the unusual antibacterial potential of *Leptospermum* honeys from New Zealand took around two decades and the full characterisation of the antibacterial properties of those honeys is yet to be completed (Allen *et al.*, 1991; Cooper *et al.*, 2000; Henriques, 2006; Blair *et al.*, 2009; Lin *et al.*, 2010). The socio-economic instability in Sudan is a major barrier to conducting similar systematic evaluation of locally produced honeys despite the widespread therapeutic use of honey and plant extracts in the country.

One motivation beside the effectiveness and availability of honey was the inexpensive price of honeys which make it suitable choice for people from all economical levels. However, this is not now the case for manuka honey and some other types which are considered as quality honeys where prices have increased 10 fold as reported for manuka honeys (Stephens *et al.*, 2010). The high price of manuka and medical grade honey will make it unaffordable to many people particularly those from third world countries like Sudan. Thus, it is necessary to evaluate the antimicrobial potential of locally produced honeys and select for honeys from floral sources with broad spectrum antimicrobial activity like honeys from *Leptospermum* trees in New Zealand and Australia.

This Study has demonstrated that Sudanese honeys possess antibacterial activity which was mainly due to hydrogen peroxide as the sixty honeys tested did not show any activity after

the addition of catalase. Failing to identify honeys with unusual antibacterial activity in this study does not mean that all Sudanese honeys will be peroxidal honey alone as Manuka honey was identified after screening more than 300 hundred types from various floral sources in New Zealand. Sudan is very big country as (currently 16th in the world) compared to New Zealand (75th in the world) and despite having areas which are desert there are areas with abundant water and floral sources to study before it is possible to draw any conclusions. Moreover, research has demonstrated that not all New Zealand Manuka honeys are non peroxidal; hence, there is still possibility that even a Sudanese honey from a floral source which has been screened in one area might give outstanding activity if it produced in a different area.

Researchers have conducted intensive research aiming to understand the mysteries behind the unusual antimicrobial powers of Manuka honeys or to find other honeys with distinct activity as was found in Revamil™ honeys from green houses in the Netherlands. However, they are very few reports demonstrating the antimicrobial efficacy of combination of honeys with antibiotics and plant extracts and food preservatives. Although many of the ancient reports demonstrating the medicinal properties of honeys have mentioned it is use with various combinations; this fact has gone unnoticed apart from reports of it is action with ginger, gentamicin (Karlyl *et al.*,1998), and methicillin(Jenkins *et al.*, 2012).

This study reports on the screening of selected plant extracts for their antibacterial activity in order to select for autoclavable plant extracts with broad spectrum activity that could be used as an alternative to manuka honey or be combined with local honeys to harness the healing potential of the honey and the plant extract. Recently a joint project between Cardiff University and the National Botanic Garden of Wales started looking for honeys with antimicrobial potential then testing the DNA profiles of the pollen associated with the most

powerful honeys, looking for the plants which contributed to any distinct antibacterial activity. This approach looks similar to the work conducted in this thesis with the use of DNA analysis helping researchers avoid pollen analysis which as reported here is a tedious job and needs skilled personnel and adequate records of pollens.

As the research is demonstrating the effectiveness of these natural products there is need to find novel methods to test the effectiveness of these antibacterial agents alone or in combination. In this study the Canvas software was introduced for the first time as more precise measure for the zones of inhibition. The Canvas software might be useful tool for screening antibacterial combinations in solid media as it is difficult to find the margins of the inhibition zones when there is additive or synergistic interaction following the method used by Chattopaddeya and coworkers (Chattopaddeya *et al.*, 1991). The Canvas software applied here measures the percentage increase in the size of the inhibition zone using a mathematical algorithm and synergy is identified as the increase in the size percentage compared to the size of each antibacterial alone.

This thesis has demonstrated the importance of screening locally produced honeys as antibacterial agents that could be used for treating wounds, nutrition and prophylaxis. Although the research into those 60 Sudanese honey has yielded moderate to strong peroxidal potential we were able to confirm that a combination of honey and black seed oil (*Nigella sativa*) had the potential for use in wound management as the addition of catalase did not nullify the antibacterial activity. Although the antibacterial activity of this combination was more than the activity of black seed oil or honey alone further research is required to see if this activity was merely due to that of the oil and the sugar content of the honey alone or whether the combination in some way inactivated catalase before it could destroy the hydrogen peroxide.

This research has also confirmed the broad spectrum activity of *Tamarindus indica* fruit and Sudan is one of countries which lie in the tamarind zone in Africa. Sudanese consume tamarind mainly as juice and produce tamarind for local consumption; however, this valuable tree should have more attention as it could be exported as many people in India and other regions around the globe are using it as a food.

This study has shown that although when tamarind is combined with honey it does not give clear synergistic interaction *in vitro*; however, the reaction is not antagonistic and this encourages more research into this combination and the possibility of finding a novel antibacterial having the distinct antibacterial activity of tamarind and the healing potential of honey. Tamarind on its own is used for treatment of wounds and for gastrointestinal problems and honey is also used for management of wounds and there are reports about its use for treating gastrointestinal infections (Mohamad *et al.*, 2012). The use of honey and tamarind in traditional medicine suggest their usefulness alone and combination. Further research to validate effectiveness of honey and tamarind alone and in combination is required. Moreover, this study suggests running beekeeping practices in areas where tamarind tree could be used as floral source by the bees as this might lead to honeys with unusual activity.

Synergistic interactions in this study has shown the potential of using honey and EDTA in combination or plant extracts and honey in combination. However, more research is required to confirm the results of this research particularly with some clinical isolates as the results obtained in this study were based on two reference strains and synergy is not an all-or-none phenomenon and it may need to be assessed on an isolate-by-isolate basis (Bonapace *et al.*, 2000).

Although MICs are used widely in evaluation of the antimicrobial activity of honeys they should not be the basis for judging the antibacterial activity of honeys particularly in case they showed high peroxidal activity or even moderate non peroxidal activity as research has demonstrated that MICs are not reliable on their own for judging the antibacterial activity (Lin, 2010). They could be used for initial screening as they need only small quantities of honey or any antibacterial for running the tests. Time kill curves are laborious and time consuming but they give more reliable results. Moreover, time kill curves show how rapidly the bacteria are killed and give an idea about the time required for an antibacterial agent to kill the microorganism.

The MICs of Sudanese honeys were high compared to the MICs of Manuka honey in this study. However, this does not necessarily mean that those honeys are ineffective against bacterial pathogens as research has demonstrated that bacteria with high MICs for honey respond quickly when tested by the time kill curve. For example, *Pseudomonas aeruginosa* which is currently one of the problematic microorganisms was found to be inhibited by Manuka honey within as little as two hours (Lin *et al.*,)

Herb honeys which are produced by bees fed on sugars mixed with herbs or herbal extracts yielded honeys with physicochemical characteristics resembling floral honeys but, with high antioxidant potential due to the presence of phytochemicals (Socha *et al.*, 2009). Since the majority of research for honeys with the unusual activity of Manuka honeys have only led to many peroxidal honeys and very few honeys with non peroxidal activity it might worth exploring the potential floral sources and feed the bees on them in attempt to get honeys with unusual antimicrobial activity. In case this approach was found to be successful then more plants could be screened and the potential candidates might be selected for

beekeeping. An example of honey which is produced in controlled environment and gives honeys licensed for wound management is Revamil™ honey from the Netherlands which is produced in greenhouses with antibacterial activity claimed to be due to hydrogen peroxide, b- defensin and MGO (Kwakman *et al.*, 2010)

This study has also demonstrated the synergistic interaction between honey and EDTA as well as *Acacia nioltica* and EDTA. Although EDTA is a food preservative and chelating agent which is known to enhance antibacterial activity against Gram negative bacteria, this is probably the first study to demonstrate synergy between EDTA and honey. The results presented here suggest that futher work on peroxidal honeys combined with EDTA or Tamarind paste might lead to a combination that could be be tested in in-vivo on wounds. Any clinical application would need to a supply of irradiated sterile honey which adds significantly to the cost of production.

In conclusion this study did not find any non-peroxidal honeys but has introduced a novel method for evaluation of bacteriostatic synergistic combinations which could be further developed and adopted for future studies. Moreover, it has found synergistic combinations of honey and EDTA and after further studies there is some potential for a combination of black seed oil, peroxidal honey and EDTA as an alternative to Manuka honey in wound treatment.

It is possible to conclude that although Sudanese honeys were found to possess antibacterial activity against Gram positive and Gram negative bacteria, further research needs to be done to confirm the usefulness of those honeys for wound management since, all the honeys were found to possess peroxidal activity which brings questions about the role of catalase produced by wounds in the elimination of this antimicrobial effect.

Moreover, this research has showed that the antibacterial activity of Sudanese honeys varies as some were found to be bactericidal and others were bacteriostatic in nature. This means that more research needs to be established in the mode of action of honeys, particularly peroxidal honeys since the majority of the research about the mode of action of honeys was limited to Manuka honeys.

This study has demonstrated the potential for Sudanese honeys to possess antibacterial and antioxidant activity. This work needs to be further developed with the collection, analysis and characterisation of a larger number of Sudanese honeys. An African network for honey research where ideas could be shared and a larger volume of samples tested and characterised would be valuable. Further studies on the kinetics of death of *Staphylococcus aureus* and *Escherichia coli* are necessary for the characterisation of honey's antimicrobial activity.

The radical scavenging and antioxidant potential of honeys should be further investigated not only for its potential in wound healing, but also because antioxidants have been shown to possess some antimicrobial activity (Sato and Miyata, 2000). Hence the assay of a larger number of honeys for their antioxidant potential and the characterization of the components responsible for this activity, with the use of HPLC, GC-MS or LC-mass spectroscopy (Inoue *et al.*, 2005) for their identification is possible (Henriques, 2006).

The role played by Maillard reaction products such as melanoidins (Brudzynski and Miotto, 2011) as well as effects of elevated levels of HMF as all were found to produce intermediates with antibacterial activity including MGO which is largely responsible for the antibacterial activity of manuka honey.

Further work should include the establishment of a Sudanese honey research group that is supported by honey producers to maintain the production of various honey types and raise funds to support the honey research as the case of New Zealand honeys and the support they got from Comvita and other companies for *Leptospermum* honeys.

In this study only two reference strains were used throughout the research. Future studies should involve the use more reference strains as well as clinical isolates from infected wounds. Antiseptic phenol and manuka honey were used as positive controls without any standard antibiotics or known peroxidal honey (e.g., Pasture honey) as a control in screening the antibacterial activity of Sudanese honeys. Future studies could benefit from the use of standard antibiotics and artificial honey. The antioxidant activity of Sudanese honeys as well as plant extracts was only evaluated through the radical scavenging 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method. The antioxidant activity should be evaluated through other methods such the ferric reducing antioxidant power (FRAP), and the oxygen radical absorption capacity (ORAC).

The study did not look into the details of the antioxidant and antibacterial properties of the screened plant extracts as the focus was mainly on Tamarind, Acacia and black seed oil. Future studies should cover all the literature of the screened plant extracts, various parts of the plants, different methods of extraction, using different solvents as all were found to affect of the bioactivity of the plants tested.

Moreover, future studies could involve interviewing expert traditional healers and search the honey and plant extract combinations they are prescribing for different infections and then start validating each combination in attempt to recommend the successful combinations and rule out those which were found to be ineffective.

Finally more *in vivo* data is required on the action and effectiveness of the action of peroxidal honeys on wound management for it to be accepted by the medical community like medical grade manuka honey.

The findings in this thesis indicate the need for further research to evaluate natural products including honey and plant extracts as novel alternatives for prophylaxis, food preservation and therapy.

CHAPTER SEVEN: REFERENCES

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APPENDIX

Questionnaire

Apiarist

Presumed floral source

Hives Modern () or Traditional ()

Climatic conditions

Site

Vegetation

Bee type

Does the beekeeper (s) own the land?

Comments

Date received at NCR.....