DEVELOPMENT AND PHYTOCHEMICAL CHARACTERIZATION OF A HERBAL PRESERVATIVE FROM *TAMARINDUS INDICA* AND *ZIZIPHUS ABYSSINICA* HERBS

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(Food Science and Technology)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2018

Development and Phytochemical Characterization of a Herbal Preservative from *Tamarindus Indica* and *Ziziphus Abyssinica* Herbs

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A thesis submitted in partial fulfillment for the award of the Degree of Doctor of Philosophy in Food Science and Technology in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To my Children Wendy M. Ongeri, Richard Nyaberi Jnr Ongeri and Winphine Bosibori Ongeri for giving me a reason to work hard in life.

To my extended family members for giving me identity.

And to Men and Women who cherish the knowledge of Science

ACKNOWLEDGEMENT

I most sincerely wish to thank my supervisors Prof. Christine. A. Onyango, Dr. Julius M. Mathara and Prof. Francis M. Mathooko for their extremely valuable guidance and advice throughout this study.

I also wish to express my special gratitude to all members of staff from the Department of Food Science and Technology, particularly, Dr. Arnold Onyango, Prof. C. K. Njoroge and, Prof. M. A. Mwasaru, for their useful suggestions during the inception of the project. I am also grateful to Mr. P. N. Karanja, Mr. D. M Votha, Mrs Jesica Oruka, C. W. Muigai and Mr. M. Okoth for the technical assistance they gave me during the entire period of research.

I am grateful to the National Council for Science and Technology for funding this research project part of my study not forgetting Jomo Kenyatta University of Agriculture and Technology for availing me part time teaching which went a long way in funding the whole study.

Finally I would like to acknowledge with gratitude the support and encouragement my parents, brothers, sisters and friends gave me. I would especially like to thank my friends most particularly Dr. Peter Kahenya for his cooperation, encouragement and assistance whenever need arose. Above all, I give thanks to the Almighty God for giving me the ability, determination, strength and good health, to realize my long cherished goal.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis for variance
AOAC	Association of Official Agricultural Chemists
AZA	*Ziziphus abyssinica* A. Rich
BEA	Benzene/Ethanol/Ammonia hydroxide
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
CE	Catechin Equivalents
CSPI	Center for Science in the Public Interest
CEF	Chloroform/Ethyl acetate/Formic acid
DAD	Diode Array Detector
DMRT	Duncan's Multiple Range Test
DPPH.	2, 2-diphenyl-1-picrylhydrazyl.
DMSO	Dimethyl Sulphoxide
EMW	Ethyl acetate/Methanol/Water
FAO	Food and Agricultural Organization
FRSA	Free Radical Scavenging Activity
FDA	Food and Drug Administration
GAE	Garlic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrophotometry
CFU	Colony Forming Units
TBARS,	Thiobabituric Acid Reacting Substances

TVC	Total Viable Counts
HCl	Hydrochloric Acid
HPLC	High Pressure Liquid Chromatography
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KARI	Kenya Agricultural Research Institute
Lc−QTof−ms	Liquid chromatography quadrupole-time of flight mass spectroscopy
MTI	*Tamarindus indica*
MDA	Melondialdehyde
PG	Propyl Gallate
RE	Rutin Equivalent
PDA	Potato Dextrose Agar
SD agar	Synthetic Define agar
TBHQ	Tert-buly hydroquinone
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
VRBGA	Violet-red bile glucose agar
US	United States
UV	Ultraviolet Rays
WHO	World Health Organization
FRSA	Free Radical Scavenging Activity

ABSTRACT

Food security issues are a major concern in the present day world, where people are coming up with all sought of methods of preserving food using conventional chemicals. Many of these chemicals are harmful to man and have been known to cause diseases. It then becomes necessary to device ways and means of preserving food using natural material such as herbs that are not known to pose a danger to the health of consumers. Many herbs with such preservative qualities have been identified, only that they have a bias to either antioxidant activity or antimicrobial activity. This study therefore, sought to blend two herbs, one of which exhibited dominant antioxidant activity and another antimicrobial activity. These herbs were then used to preserve pork sausages. The first part of the study determined the most efficient method of extraction by comparing soxhlet extraction with methanol as the solvent, cold extraction with methanol as the solvent and water extraction. To identify the active ingredients present in the extracts responsible for antioxidant and antimicrobial activity, profiling of the extract was undertaken using bioautographic, GC-MS and LC-QToF-MS methods. To identify the most effective blending ratio of the two herbs extracts AZA and MTI, herbs blends in the ratios of 1:3, 1:1, and 3:1 (MTI:AZA) were compared by subjecting them to TBARS, TVC and inhibition tests against test microorganisms *E. coli. S. aureus,* and *candida albicans.* The herbs extract blends were then applied in sausage preparations. Three sausage preparations having 1.8 kg each of pork were inoculated with 1.575 g, 3 g, and 6 g respectively of the herb extract blend 1:3 (MTI:AZA). The sausages were stored for five days at 4°C, and tested for development of rancidity (TBARS), protein degradation (PV), and microbial proliferation of TVC, *E. col*i, *S. aureus* and *Candida albicans*. The two herbs were identified as *Ziziphus abyssinica* (AZA) and *Tarmarindus indica* (MTI). It was also noted that water was the best solvent for extracting MTI, while the cold method of extraction using methanol as the solvent was the best for extracting AZA, 21 compounds were identified from the sample that exhibited antioxidant activity and 24 from the extract that exhibited antimicrobial activity against *E. coli. S. aureus,* and *candida albicans*. The

extracts in the ratio 1:3 (MTI: AZA), was identified as the most effective herbs extract blend. In the TBARS test, both batches of sausages in whose preparation 1.575 g, and 3 g of extract were added and stored at 4°C were found to develop decay by the third day while the control and the sausages with 6g extract were still edible with no obnoxious smell on the fifth day. When the sensory analysis was done the sausages with 6 g herbal concentrate were rated acceptable. It was concluded that the blend of the two herbs is best suited to make a preservative that can substitute the use of synthetic preservatives in the market.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Individuals and families everywhere in the world find the peace of mind when they know that the food they put on the table is safe. It is better off from a public health perspective if consumers can choose a healthy, diverse and economical diet without having to worry about food safety. Therefore, food safety clearly tops the list of consumer concerns about food quality. Previously consumers associated the presence of mercury, pesticides, hormones, agricultural chemicals and antibiotics with food of poor quality, but as this is addressed, other items have emerged in the list namely synthetic food additives. These additives are voluntarily added into food for purposes of preserving or adding particular features in the food such as increased shelf life, appealing taste and texture in order to meet the urbanization need for quickly prepared and consumed foods (FAO and WHO 2003).

The synthetic substances added for the purpose of preservation, prevent decomposition by deterring microbial growth or undesirable chemical changes such as lipid peroxidation. The oxidative deterioration of lipids is of great concern in the shelf life of foods as lipid peroxidation leads to development of undesirable off-flavors that decreases the acceptability of foods (Jackobsen *et al*., 2008). In addition, lipid oxidation decreases food safety and nutritional quality by formation of potentially toxic secondary oxidation products during cooking and or processing (Valeria Velascol, & Pamela Williams, 2011). To prevent and/or retard lipid oxidation, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) Parabens have been added to lipid-containing foods (Golden *et al*., 2005). However, synthetic preservatives are suspected to be potential health hazards in foods, by being associated with disorders including possible carcinogenicity, respiratory problems, behavioral changes, bloating, and lethargy, in addition other health problems like

constipation, blood pressure and diabetes (Sharma, 2015). Due to this and more, consumers prefer foods that have no preservatives or are as "natural" as possible. This has led to the search for natural substances that can impart the same protection but without the potential hazard associated with synthetic antioxidants and antimicrobial compounds. It is therefore generally believed that going back to the use of herbs which the pastoralists of West Pokot and other indigenous communities have used for many years would offer a solution to the myriad of problems brought about by the introduction of synthetic products in the market. Therefore, the growing interest by consumers to substitute synthetic chemicals with natural preservatives has elicited research on plant sources that have the same properties as the synthetic compounds. (Taylor, 2012).

Spices and their essential oils have been found to have varying degree of antimicrobial activity (Marija *et al*., 2009; Singh *et al*., 2005). The antimicrobial activity of some essential oil components against foodborne pathogens, including mycotoxin-producing fungi, has been developed and proposed for use in foods as natural antioxidants and antimicrobials. Hence, the antioxidant and antimicrobial property of herbs used to improve the shelf life of food material and at the same time provide safety to consumers has been known to result from many bioactive secondary metabolites that have the potential to kill various microorganisms and also have antioxidant properties (Steinmetz, & Potter, 1996). Examples of these compounds include flavonoids, phenols, phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates (Quiroga, *et al*., 2001). Each of these compounds exhibit complex patterns of bioactivities and ecological function, apparently due to compound specific differences in phenolic structure. Structural differences may determine rates of radical scavenging (Yamasaki *et al*., 1997; Hagerman *et al.*, 1998), protein precipitation (Scalbert, 1991) metal chalation (Mila *et al*., 1996), levels of toxicity (Nishizawa *et al*., 1990; Ayres *et al*., 1997) and rates of prooxidant activity in consumers (Barbehenn *et al*., 2005).

The *Tamarindus indicus* (MTI) and *Ziziphus abbyssinica* (AZA) are such herbs, both commonly used to preserve meat by the pastoralists of West Pokot County in Kenya. In earlier studies these herbs were found to be rich in phytochemicals, mainly reducing compounds, sterols and steroids, alkaloids, saponins, flavonoids, polyphenolics and condensed tannins. The herbs exhibited antimicrobial activity against test microorganisms that included *B. subtillis, P. aeruginosa, S. aureus, E. coli, C. albicans*. The herbs *Tamarindus indicus* (MTI) had very high antimicrobial capacity against all the test organisms while the *Ziziphus abbyssinica* A. Rich (AZA) exhibited very high antioxidant capacity (Nyaberi, 2009).

The present study was designed to assess preservative potential by determining if the antioxidant and antibacterial potential of two herbs MTI and AZA would be enhanced when utilized in combination than when used singly. The study will also assess the phytochemical constituents of the herb MTI and AZA then compare with the already determined constituents of MTI.

1.2 Statement of the problem

In recent times the population of Kenyans has risen exponentially which has resulted in the increased demand for fresh food and processed food products (Kinyua, 2004). To meet this food demand, wastage of food had to be minimized by improving on the storage life of the food products. This was and has been widely achieved by preserving fresh and processed produce using synthetic preservatives which are cheap, readily available and prone to chemical abuse in both urban and rural setting. Unfortunately, the excessive use of these preservatives which include chemicals such as Butylated hydroxytoluene (BHT), Butylated hydrocyanisole (BHA), Tert-buly hydroquinone (TBHQ), Sulphites, sorbic, Sorbates Benzoic, Nitrites, have been associated with health problems such as cancer, hives, asthma, or other allergic reaction in sensitive individuals(Doyle, 2007). Other methods of preservation include freezing, drying, salting. These have been unable to solve the problems of preserving food products including meat and fish for the rural duelers,

due to rampant poverty and unavailability of electricity in the rural areas and poverty in the urban setting (Gracia *et al*., 2015).

Considering the magnitude of the above findings many people are refraining partially or totally from eating foods preserved with synthetic preservatives (Sharma 2015). In mitigation, herbal preservatives come in as a worthy replacement to the conventional preservatives in the market. They are widely available, can't be a hazard even when abused, safe for use by man, they can control spoilage by microorganism and oxidation (Sharma 2015). The only drawback is that there is limited knowledge of their potential, diversity and efficacy, part of which this study will address (Halvorsen *et al.*, 2002).

1.3 Justification

The fact that the pastoralists of West Pokot County have used herbs to preserve their meat for a long time indicates that the herbs have never had negative effects on their health. It is the limited knowledge on the use of the herbs that has since worked to their disadvantage. The health implications of herbs cannot be compared to complications associated with the use of synthetic compounds (Nilsson *et al.*, 2005). The use of herbs is preferred because they are natural, of medicinal importance as an added advantage. They have no known side effects, are abundantly available and are consumed without fear of toxicity or residues. Foods preserved with natural products are more acceptable and perceived as safe and nutritious (Penarrieta *et al*., 2005). While a lot has been documented on the use of herbs as medicine, very little has been documented on their use as food preservative agents. Further still different herbs have been known to have different properties. Therefore, combining several herbs would result in having a product with better preservative potential and utilized in less quantity. It is important that a comprehensive research is undertaken to understand the constituents, efficacy, expiry periods and the possibility of combining some of the herbs to get a more effective herbal preservative utilized in much less quantities. The success of this research will uplift the lives of the local communities who will involve themselves in the conservation and propagation of the herbs for both economic gain and conservation of the environment. The

trade in the preservatives and the benefit of being able to preserve ones food and avoid losses due to spoilage, will enhance food security among the general population. This is in line with the vision 2030 objectives. Research in this area is timely to facilitate the development of a preservative from herbs and use it to preserve meat. Taking into consideration that herbs already enjoy synergy and thus its introduction and use would be easily accepted.

1.4 Objectives

1.4.1 Overall objective

To develop and characterise novel composite herbal preservative from MTI and AZA as a possible substitute for the conventional preservatives used in meat preservation.

1.4.2 Specific objectives

a) To determine the most efficient method for extracting selected compounds from MTI and AZA
b) To quantify the active phytochemical compounds in the herbs AZA and MTI.
c) To determine the preservative effect of the combined herbs AZA and MTI on sausages.
d) To develop herbal substitute for a selected commercial preservative.

1.5 Hypothesis

a) The herbal blend of MTI and AZA produces a better preservative effect on pork sausages than each individual herbal extract.

b) The preservative effect of MTI and AZA blend is greater or equal to that of a comparable commonly used synthetic product

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Africa is one continent that is endowed with the richest biodiversity in the world. It has a variety of plants many of which are not only utilized as food but also for preservative and therapeutic purposes. The major reason is that, this continent consists of a geographical spread, spanning approximately 216,634,000 hectares of closed forest areas. Over 5000 different species of plants have been known to occur in these areas, among which many of them are useful in traditional preservation of food and curative purposes (Iwu, 1993). The use of herbs for preservation has been there for many years dating back as far as 3000BC (Cowan, 1999; Maffi, 1999). Despite enormous advances in conventional practices, preservation and curative practices using traditional herbs has been encouraged by the World Health Organization, partly because some conventional drugs have failed to prove effective, and have had serious side effects, on the users (Juhee *et al*., 2004). This has made the use of herbs for various purposes including preservation enjoy synergy (Halvorsen *et al.*, 2002; Nilsson *et al.*, 2005; Penarrieta *et al*., 2005).

Herbs or short-stemmed plants that are valued for their preservative, flavour, fragrance, and curative properties. They are used in preservation, perfumery, cosmetics and medical industry (Kaur and Saraf, 2010). Most herbs grow to a maximum height of 30-90cm, but the herb AZA grows to a maximum height of 3 – 5m. A few aromatic trees, such as bay, MTI, grow to a height of 6-9 meters, yet they are also considered herbs. The valuable parts of many herbs are the leaf, flower, seed, stem, root, fruit pulp or entire plant. It goes without say therefore, those herbs are, and have been an integral part in the life of many indigenous communities for many centuries. Other than providing building materials, fodder, weapons and other commodities, communities such as Maasai, Borana, Sabaot, Kikuyu, and the West Pokot have extensively used herbs as preservative agents, flavour

enhancers, appetizers (Uebelherr, 2005) and nutritional additives. The herbs MTI and AZA are such herbs used by the pastoralists of West Pokot to preserve meat.

Plate 2.1: A mature tree herb of AZA in West Pokot County (Photo by Nyaberi M. O. 2013)

2.2 Traditional practice

|The herb AZA grows in areas with severe heat and slight frost like in arid or dry tropical or subtropical areas. In Kenya they are predominantly found in West Pokot County. In this region the flowers appear between July and August, fruits start to appear between December and April (Orwa *et al*., 2009).

The herbs MTI and AZA mature and are harvested around the end of December and March. At this time most of the herbs have fruited and the rains have subsided and therefore the concentration of metabolites is at the highest (Freitas and Glories, 1999). The pollination vectors are bees. The fruits of AZA are harvested when ripe and have turned red in colour. While in the case of MTI the pods dry and turn grey in colour. During

processing, the fruit mass of AZA is stripped from the seed together with the seed coat and dissolved to form a paste. In case of MTI the seeds and the outer casing is removed and the remaining fruit mass is dissolved in water to form a paste. Meat that has been cut into strips is immersed in the paste and left to dry. These herbs individually would preserve meat for a reasonable time, free from moulds and any other spoilage indicators for as long as a year, the taste of meat was not affected when the paste was prepared from the fruits of AZA. However, when paste from the fruits of MTI was used, it left a sour taste of the herb in the meat. The herb MTI was found to prevent the accumulation of moulds and other microorganisms that encourage spoilage (Nwodo *et al*., 2011 and Nyaberi *et al*., 2011) while the herb AZA was found to be a very powerful antioxidant (Nyaberi *et al.*, 2011). The application of the herb on the surface of the meat is in line with Lambert *et al.*, (1998) who said that the internal tissue of an animal that has been slaughtered is sterile until it is contaminated while the surface of the meat becomes contaminated with a variety of microorganisms mostly spoilage and pathogenic bacteria, moulds and yeast due to exposure to contaminated environment resulting from poor handling.

2.3 Conventional Preservation techniques

Preservation is an important aspect in storage because it increases shelf life of food. Different preservation methods are available in the market most of which are very expensive and out of reach to many people especially in the rural areas. These methods generally involve temperature manipulation, reduction of water activity, alteration of the pH, oxidation of lipids, Irradiation and application of chemical preservatives. The resultant effect on the meat is the exclusion or elimination of spoilage microorganisms and prevention of rancidity due to oxidation of the lipids in meat, (Dimitrievi*ć et al*,. 2007; Fox and Cameron, 1977).

The high cost of the former, use and abuse of chemical preservatives such as Butylated hydroxytoluene (BHT), Butylated hydrocyanisole (BHA), Tert-buly hydroquinone (TBHQ), Sulphites, sorbic, Sorbates Benzoic, Nitrites has become rampant resulting in these preservatives eliciting health implications such as allergic reactions to chemicals

like sulphur, casinogenic effects (Steinman, 2006 ; Abbas *et al*., 2007). As a result a general shift towards the preference of natural locally available plant materials such as herbs that can serve the same purpose and pose a lower risk to one's health has occurred (Halvorsen *et al*., 2002; Nilsson *et al*., 2005; Penarrieta *et al*., 2005).

2.4 Mechanism of Preservation using herbs

Herbs exhibit various characteristics that include antimicrobial, antioxidant, antifungal, nutritive and anti-nutritive properties (Lupina and Cripps, 1987; Christoffell and William, 1997). This has been brought about by the plants synthesizing chemical substances that bring about these effects such as phytochemicals, and phytoalexins. The reason why the plants synthesize these chemical substances was provided by Majorie (1999) as reported by Aboaba *et al.*, (2005) that plants readily synthesize substances for defense against attack by insects, herbivores and same microorganisms. They also synthesize some of these products to enable them survive adverse conditions in order to propagate themselves.

Prindle and Wright (1977) mentioned that the effect of phenolic compounds is concentration dependent. At low concentrations, phenols affect enzyme activity, especially of those enzymes associated with energy production. At higher concentrations, they denature proteins. The effect of phenolic antioxidants on microbial growth and toxin production could be the result of the ability of phenolic compounds to alter microbial cell permeability, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in their structure and functionality (Fung *et al*., 1977).

Lis-Balchin and Deans (1997) reported that strong antimicrobial activity could be correlated with essential oils containing a high percentage of monoterpenes, eugenol, cinnamic aldehyde and thymol. Conner and Beuchat (1984) suggested that the antimicrobial activity of the essential oils of herbs and spices or their constituents such as thymol, carvacrol and eugenol could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds.

Nychas (1995) indicated that phenolic compounds could denature the enzymes responsible for spore germination or interfere with the amino acids involved in germination. Once the phenolic compounds have crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity. Davidson (2001) also reported that the exact cause–effect relation for the mode of action of phenolic compounds, such as thymol, eugenol and carvacrol, has not been determined, although it seems that they may inactivate essential enzymes, react with the cell membrane or disturb genetic material functionality. Several studies have attempted to determine the efficacy of extracts from selected plants as antimicrobial and antifungal agents (Lopez *et al*., 2000). Some studies have shown that specific essential oils and phenolic compounds can control the growth rate and spore germination time of spoilage fungi (Hope *et al*., 2003). Many antibacterial agents may exhibit their action through inhibition of nucleic acid, protein and membrane phospholipids biosynthesis (Franklin *et al*., 1987 and Dorman *et al*., 2000).

2.5 Origin and uses of MTI and AZA

The herb MTI or Oron (local name), family, Leguminosae, is one such widely used medicinal plant. It is found Native to tropical Africa; this tree virtually grows in all tropical climatic regions from India through Africa to the Caribbean and South America and up to Southern Florida. The tree was so long ago introduced into and adopted in India where it has often been reported as indigenous. It was apparently from this Asiatic country that it reached the Persians and the Arabs who called it *"tamar hindi"* (Indian date, from the date-like appearance of the dried pulp), giving rise to both its common and generic names. Unfortunately, the specific name, *"indica"*, also perpetuates the illusion of Indian origin. The fruit was well known to the ancient Egyptians and to the Greeks in the 4th Century B.C the pulp is made into a variety of products. It is an important ingredient in chutneys, curries and sauces, including some brands of Worcestershire and barbecue sauce, and in a special Indian seafood pickle called "tamarind fish". Tamarind preparations are universally recognized as refrigerants in fevers and as laxatives and carminatives remedy for biliousness and bile disorders (Caluwé *et al*., 2009). The pulp has also been

documented in both the British and American pharmacopoeias as anti-pyretic, antiscorbutic, laxative, carminative and remedy for biliousness and bile disorder. The leaves have antihelmintic and vermifuge properties, destroying intestinal parasites. (Morton, 1987, Iwu, 1993 and Raimondi, *et al*., 2003)

The AZA or *Angau* (local name) of family Rhamnaceae are small trees indigenous to tropical Africa and India, ever green that grows up to 15m high with trunk of 40cm or more in diameter. The flesh is white and crisp, the fruits skin is smooth, glossy, thin but tight. There is no information on nutritional parameters and quantitative phytochemical analysis. Previous research has indicated that the herb has various phytochemicals which include saponins, sterol and steroids, alkaloids, tannins, flavonoids and reducing compounds. It also inhibits the growth of *E. coli, S. aureus* and *P. aeruginosa* to varied degrees and has been previously proved to be a powerful antioxidant (Nyaberi, 2009).

CHAPTER THREE

STUDIES ON THE EXTRACTION EFFICIENCY OF THE HERBS *ZIZIPHUS ABBYSINICA* AND *TAMARINDUS INDICA*, USING DIFFERENT METHODS OF EXTRACTION

3.1 Introduction

Nature has been a valuable source of preservatives and has helped human preserve food since time immemorial (Cowan, 1999). According to the World Health Organization (WHO 2003), almost 80 % of the world's population relies on traditional herbs for various needs such as medicinal and food preservation. This is because herbs are culturally acceptable, known to have fewer side effects and better compatibility with the human body (Kaur and Aora, 2009; Mahesh and Satish, 2000; Arunkumar and Muthuselvam, 2009). These herb Plants are rich sources of many bioactive secondary metabolites that have the potential to elicit preservative effect on different types of foods. Examples of these compounds include alkaloids, tannins, flavonoids, phenols, phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides and glucosinolates (Quiroga *et al*., 2001).

The preservative properties of herbs lie, incidentally, on secondary metabolites with *in situ* functions: proanthocyanidins as deterrents to predation, pheromones attract insects for pollination, phytoalexins protect against bacterial and fungal attacks and allelochemicals which inhibit rival plants (Marjorie, 1999).

Flavonoids are natural substances with variable phenolic structures. More than 4000 flavonoids have been identified and grouped according to their molecular structures. The best described property of almost every group of flavonoids is their capacity to act as antioxidants able to scavenge free radicals and reactive oxygen species. Flavonoids and proanthocyanidins are particularly present widely in the plant kingdom and possess many

functions including anti-inflammatory, antimicrobial, enzyme inhibition, antioxidant and antitumor (Cushnie, *et al*., 2005). Previous studies reported antimicrobial and antifungal activity on many flavonoids rich plants (Rauha, *et al*., 2000; Valsaraj *et al*., 1997; Wachter, *et al*., 1999). Additionally flavonoids are characterized by low toxicity since they are widely distributed in edible plants (Cushnie, *et al*., 2005).

The purpose of this study is to determine the most efficient extraction procedure between soxhlet, cold and aqueous extraction methods. The identified method would preferably be used for extracting secondary metabolites during commercial scale extraction of the herbs MTI and AZA.

3.2 Materials and Methods

3.2.1 Study Area

Samples were acquired thrice from Chepararia and Kongelai Sub Counties of West Pokot County as illustrated in figure 3.1

Figure 3.1: The map of West Pokot County showing the areas from where the samples were collected

3.2.2 Sampling Design

The study was set in a completely randomized design. Fruits in each tree were harvested in three levels, ten healthy mature fruits close to the tip, ten in the middle of the tree herb and ten at the lower end of the tree. This was practiced on each tree that was identified to be healthy and populated. This was done with the help of key informants.

3.2.3 Collection of information and samples

The office of the County Agriculture Officer, Kapenguria assisted in the collection of the Samples. A member of the local community was chosen to accompany the sampling team and give information on prevalence and availability of the herbs. Information on how and why the local communities never used to mix the herbs was also sought, the focus for discussions was guided by a questionnaire (Appendix 1). Three groups of respondents involved elders, women and youth groups. From each group fifteen respondents were interviewed. All the respondents were from Chepararia and Kongelai sub Counties of West Pokot County. Their results were triangulated to validate the findings of the focus group discussion (FGD).

According to the guide and the information collected from the people of West Pokot County, indicated that, the seeds of AZA were dried, the flesh and seed coat and processed by grinding to form a course flour like texture. The MTI fruit was de-husked, resulting with the seeds and fibers. These seeds and fibre of MTI together with ground mass from AZA were all soaked in water. The resulting fleshy mass of both herbs fruits were mixed with water to make a paste and later applied to the product being preserved. This traditional practice was used as the basis of attaining the objective of this study.

Taking the traditional processing into consideration two different sets of fruit samples of AZA and another for MTI herbs were collected in triplicates. Immediately after collection, the herb samples were stored in cool boxes containing ice packs used to maintain a temperature of approximately 4°C while on transit to the Department of Food Science and Technology of Jomo Kenyatta University of Agriculture and Technology (JKUAT) Food Science Laboratory for analysis.

Table 3.1: List of herbs collected for analysis from West Pokot County

Local name (Code)	Scientific Name	Class	Plant Part used	Product preserved
Oron (MTI)	*Tamarindus indica* L	Caesalpinoideae	Fruit paste	Meat
Angau (AZA)	*Ziziphus abyssinica* A. Rich	Rhamnaceae	Fruit paste	Meat

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI)

3.2.4 Test microorganisms

Nonresistant clinical isolates of selected microorganisms including *Bacillus subtillis* (locally isolated in the KARI laboratory Nairobi), *Pseudomonas aeruginosa* (27853 ATCC), *Candida albicans* (90028 ATCC), *Escherichia coli* (25922 ATCC) and *Staphylococcus aureus* (25923 ATCC) were obtained from Kenya Agricultural and Livestock Research Organisation (KALRO). The cultures of bacteria were maintained on nutrient agar slants at 4 ± 2°C until required for use.

3.2.5 Preparation of samples for analysis

A 15 kg fresh portion of each of the fruit of MTI and AZA was gently cleaned using running tap water to remove soil, the portion was then dried at ambient temperatures of 25 ± 2°C in a room for 20 days. In the case of AZA the dried sample was put in a grinder (model M10R Japan) and in the act of grinding it removed the flesh and outer seed coat, the remaining flesh on the seeds was removed manually by a knife and the seeds were discarded. The weight of the resultant mass of AZA after grinding taken and expressed as a percentage of the original weight before grinding (15 kg). The 15 kg dried sample of MTI was taken and soaked in water. The fibrous material and the hard seeds were later removed pressed and to remove excess liquid and then dried directly under the sun for one month. The weight of this dried material was taken and subtracted from the original 15 kg material to get the weight of the dissolved matter. The weight of these dissolved matter was expressed as a percentage.

This percentage in the context of this thesis is referred to as the percentage yield. The purpose of the percentage yield would be to determine the amount of raw material required to provide a given amount of extractable product. The yield realized from AZA was ground into moderately coarse powder using an electric grinder (model M10R Japan) while the yield from MTI due to its gummy nature was simply cut into small pieces, both samples were stored at 4 ±2°C until needed for use as described by (Onoruvwe and Olorunfemi, 1998 and Bautista-Banos *et al*., 2003).

3.2.6 Moisture content determination

Moisture content of the different herbs samples was determined according to the AOAC method 950.46 (AOAC, 1995) using forced air conventional oven set at 100°C for 3 hours. Ten grams (10 g) of each of the two herbs sample was weighed in triplicate and placed in moisture dishes and then transferred into a hot air oven set at 100°C for 3 hours. The moisture dishes were left to cool in the oven for about one hour and then removed and placed in a desiccator to cool for further one hour, weights of the dishes were recorded at equal intervals until a constant weight was acquired and percentage weight loss determined.

3.2.7 Extraction of the active compounds

Each of the ground herb was divided into three 45 g portions. The portions were extracted as follows:

3.2.7.1 Aqueous extraction:

One portion was extracted with distilled water therefore referred to as aqueous extract. A 45 g portion of each of the two herbs was extracted using distilled water to simulate the traditional practice by the pastoralists (Bautista-Banos *et al*., 2003). The water mixture was boiled for one hour and then left to cool for one day. The mixture was later centrifuged at 40,000 rpm for 10 minutes at a temperature of 4°C using a refrigerated centrifuge (Kokusan Corporation, Model 2000C, Tokyo Japan). The supernatant was filtered using No. 1 Whatman filter paper and the filtrate evaporated to dryness at about 80±2°C.

3.2.7.2 Cold Methanol extraction:

In cold extraction, the herbs were immersed in the extracting solvent methanol and placed in an opaque glass container. The container was shaken for 30 minutes to ensure sufficient contact using a labs shaker (Kika Labortechnik, Model KS 250 Basic, and Staufen, Germany). The mixtures were left to stand for four days in a dark enclosure at 25 ± 2°C and then filtered. The filtrates obtained were evaporated to dryness under vacuum at 70 ± 2°C using a rotary evaporator (Model RE 100, Staffordshire, England). All the dried samples were put in labeled and tightly corked light proof glass containers and stored under 4 ± 2°C. This process was repeated three times.

3.2.7.3 Soxhlet extraction:

The soxhilet extraction method was used with Methanol as the solvent (Qing and Dagui 2010). Extraction proceeded overnight until there was no further change in colour of the extracting solvent. The filtrates obtained were evaporated to dryness under vacuum at 70 ± 2°C using a rotary evaporator (Model RE 100, Staffordshire, England). All the dried samples were put in labeled and tightly corked light proof glass containers and stored under 4 ± 2°C. This process was repeated three times.

3.2.8 Thin layer chromatography analysis

Various metabolites extracted were determined using the Thin Layer Chromatography method. The stationary phase consisted of a thin layer of silica gel adsorbent on a flat, thick aluminium plate carrier (Fried and Sherma, 1986). For chemical variation, aluminium backed silica gel F254 plates (Machery-Nagel 20x 20 cm, 0.25 mm) were used. TLC was performed on silica gel aluminum plates using ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27) mobile phase, (Silva *et al*., 2004).

All the dry extracts were re-dissolved in 2 ml methanol and loaded onto well labeled TLC plates at a distance of 2 cm from the bottom of the plate and 2 cm apart. These solvents were left in developing tanks for 10 minutes to allow for saturation of the atmosphere in the tanks with solvent vapours before developing the well labeled TLC plates. Visualization of compounds was done using iodine vapour (Reich and Schibli, 2007).

3.2.9 Determination of total phenol content

Total phenol content was determined according to the Folin and Ciocalteau's method (1927). Gallic acid was used as a standard. Concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Concentrations of 0.1 and 1 mg/ml of plant extracts were also prepared in methanol and 0.5 ml of each sample was mixed with 2.5 ml of a ten-fold diluted Folin-Ciocalteau's reagent and 2 ml of 7.5% sodium carbonate. The mixture was allowed to stand for 30 minutes at room temperature before the absorbance was read at 760 nm spectrophotometrically. All determinations were performed in triplicates. The total phenolic content was obtained for the fruit extract from the regression equation of the calibration curve of gallic acid ($y = 10.454x = 0.0201$, $R^2 = 0.97$), expressed as garlic acid equivalent (GAE).

3.2.10 Determination of total flavonoid content

Total flavonoid content was determined using a method of Miliauskas *et al*., (2004). To 2 ml sample was added 2 ml of 2% $AlCl_3$ in ethanol. The UV absorption was measured at 420 nm after 1 h at room temperature. Concentrations of 0.1 mg/ml and 1 mg/ml of the extract in methanol were used while rutin concentrations of 0.01, 0.02, 0.04, 0.08 and 0.10 mg/ml were used to obtain the calibration curve. Solutions were prepared in methanol. Total flavonoid content was calculated as rutin equivalent (RE) from the concentration of rutin equivalent obtained from the calibration curve. Determinations were performed in triplicates. Total flavonoid contents were obtained from the regression equation of the calibration curve of rutin ($y = 2.9215x + 0.3292$, $R^2 = 0.93$), and expressed as rutin equivalents (RE).

3.2.11 Pro-anthocyanidin content determination

Proanthocyanidin content was determined as previously reported by Ayoola *et al*, (2006). Briefly, concentrations of 0.1 and 1 mg/ml of the sample extract were prepared and 0.025, 0.05, 0.1, 0.2 and 0.4 mg/ml of catechin were prepared as the standard solutions for the calibration curve. Solutions were prepared in methanol. 0.5 ml of HCl was added to each test tube and the solutions were allowed to stand for 15 min. The absorbance was measured at 500 nm. Proanthocyanidin content was measured as catechin equivalent (CE) from the

concentration of catechin obtained from the calibration curve. All chemicals and reagents were obtained from Sigma-Aldrich, UK. All determinations were carried out in triplicates. Proanthocyanidin contents were determined from the regression equation of the calibration curve of catechin ($y = 2.1145x + 0.0145$, $r^2 = 1.0$) and expressed as catechin equivalents (CE).

3.2.12 Determination of the free radical scavenging activity (FRSA) of plant extracts

The antioxidant activity of each extract was measured in terms of hydrogen donating or free radical scavenging activity, using the stable radical 2,2 Diphenyl-1- picrylhydrazyl (DPPH). Briefly, to a methanolic solution (1 ml) of extract of various concentrations (0.02 – 0.1 mg/ml) was added 0.5 ml of 1 mM DPPH solution in methanol. A blank solution was prepared containing 1 ml of methanol and 0.5 ml of 1mM DPPH. The experiments were carried out in triplicates. The test tubes were incubated for 15 min, methanol was used to zero the spectrophotometer and the absorbance was read at 517 nm. The radical scavenging activity was calculated using the following formula:

% inhibition of DPPH = {(AB – AA)/AB} x100

Where:

- AB is the absorption of blank sample and
- AA is the absorption of tested extract solution.

The results are expressed as percentage inhibition of DPPH and mean inhibitory concentrations (IC_{50}) determined from a plot of absorbance of DPPH versus concentration of extract.

3.2.13 Evaluation of antibacterial activity of herb extracts

Pure cultures of the test microorganisms were inoculated into nutrient broth (Oxoid, England), incubated for 24 h at 37°C, diluted with sterile nutrient broth to a density of 9×10^8 cfu/ml by serial dilution. Sterile disposable plates were used and appropriate media (Table 3.2) was prepared and poured into sterile disposable plates according to AOAC method 966.23 (AOAC, 1995). Inoculation of the prepared plates with the organism was

done using a sterilized pipette to transfer 0.1ml of the suspensions into the plates followed by spreading with a canards rod to achieve uniform spread on the plate.

Table 3.2: Microorganisms used to test the inhibition capability of the herbs extracts

Microorganisms	Agar	Incubation time and temperature
Bacillus subtillis (locally isolated in the KALRO laboratory Nairobi)	Nutrient agar	37°C for 24 hours
Pseudomonas aeruginosa (27853 ATCC)	Pseudomonas Agar	37°C for 24 hours
Candida albicans (90028 ATCC)	Potato Dextrose Agar with 10% tartaric acid	25°C for 5days
Escherichia coli (25922 ATCC)	Violet-Red Bile Glucose Agar	37°C for 24 hours
Staphylococcus aureus (25923 ATCC)	Baird Parker with egg yolk Tellurite	37°C for 24 hours

Sensitivity of all the organisms to the various extracts was done using the cork and bore diffusion method of Rojas *et al*., (2003) with some modifications. Using a sterile cork-borer of 6 mm diameter, three holes were made into the set agar in Petri-dishes containing the bacterial culture. Then 0.1 ml of each of the concentrations 0.1, 0.2 and 0.3 g/ml of the extracts was poured in to the wells in triplicates. Standard preservative (sodium metabisulphite 0.1 and 0.2 g/ml) was used as reference or positive control. A control was set up for all the organisms, in which, 50% DMSO (Dimethyl sulphoxide) in water was used instead of plant extract to ascertain that the 50% DMSO did not inhibit growth of microorganisms. A second control was set up to check the viability of the microorganisms. The microorganisms were inoculated in the corresponding agar media and placed in the incubator at 37°C overnight with pure sterile water in the holes. The controls without plant extract were examined for growth and those with the plant extracts were examined for

zones of inhibition of growth. This was estimated by measuring the linear diameter of the inhibition zone. Antibacterial activity was recorded if the zone of inhibition was greater than 9 mm (Hassan *et al*., 2006).

3.2.14 Statistical Analysis

All the data was analyzed for variance (ANOVA) using SAS computer program version 9.1. The comparison of the means, standard error and standard deviations at 5 % level of significance was done using Duncan's multiple range tests (Steel and Torrie, 1980).

3.3 Results and Discussion

3.3.1 Extraction yield and Moisture content

The fruits of MTI realized more yield in terms of raw material than that realized from AZA (Table 3.3). This could also have been attributed to the fact that MTI had smaller seeds with more flesh around it than AZA which had bigger seeds and less flesh. The MTI herbs was able to retain more water than the AZA. This means that more fruits of AZA are needed as sample than MTI for the purpose of extraction.

Table 3.3: Percentage yield of AZA and MTI plant material for extraction and their percentage moisture levels

Item	% Moisture	% Yield
AZA	6.5±0.51	18.9±2.5
MTI	9.88±0.41	35.7±3.45

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI)

The moisture content of MTI was found to be more than that of AZA. This is attributed to the fact the MTI being of gummy texture retains more water than AZA which attains a powdered texture.

When the yields from MTI and AZA were subjected to extraction the amount of extract realized is shown in Table 3.4. The water extract of MTI produced a high amount of extract 35.52±0.7 g from 45 g of ground product compared to cold and water extractions which produced 32.49±1.63 g and 29.6±0.83 g respectively from 45 g of ground product. While the extract from Soxhlet extraction of AZA produced significantly high amount of extract amounting to 21.3±0.96 g compared to cold and water extracts which produced 11.04±1.73 g and 13.89±0.93 g, respectively from 45gms of ground product (Table 3.4). It may indicate that the extract in AZA needs more contact with the solvent a property that is there when using the Soxhlet method of extraction.

Table 3.4: Amount of extract produced in both weight and percentage yield from the two herbs AZA and MTI using various extraction techniques and solvents

Sample	Solvent	Extraction method	Amount of Extract (g)	% Extract
AZA	Methanol	Soxhlet	21.3±0.96^{d}	47.29±2.13
AZA	Methanol	Cold	11.04±1.73^{f}	24.52±3.85
AZA	Water	Water	13.89±0.93^{e}	30.86±2.07
MTI	Methanol	Soxhlet	32.49±1.63^{b}	72.21±3.61
MTI	Methanol	Cold	29.6±0.83^{c}	65.77±1.84
MTI	Water	Water	35.52±0.7^{a}	78.93±1.55

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Values bearing the same small letter within the same column are not significantly different (P>0.05). All the figures are in milligrams, values on mean ± SD of n = 4

3.3.2 Identification of compounds in the extracts

To identify the various compounds in the extracts the Thin Layer Chromatography was undertaken. Since different compounds have different Retention factor (Rf) values, compounds with the same Rf value turn out to be the same compound (Sherma, and Fried, 2003). The compounds extracted from AZA by Soxhlet and cold extraction using methanol, seven of the identified compounds were similar. This meant that despite the

difference in the amount of extract obtained (Table 3.5) the compounds extracted may have been the same. The water extraction similarly, had seven compounds identified but the Rf values of three compounds differed such as 0.18, 0.47 and 0.76 (Table 3.5). This gives the possibilities that some compounds may be soluble in water and not in methanol and may not have been affected by heating. The compounds with Rf values 0.03, 0.14 and 0.28 were present in the extract from water while absent in the extract from soxhlet and cold extraction. This may have been due to the fact that either the compounds were insoluble in water or affected by high temperatures or both insoluble and affected by high temperatures. Compounds extracted from MTI were seven for water and soxhlet while cold extraction realized only six. The absence of the two compounds with Rf values 0.03 and 0.14 in the extracts of soxhlet and cold extraction may be attributed to their inability to dissolved in methanol. This behavior is also seen with the extracts of AZA. The properties of the compound with Rf value of 0.03 may not be attributed with solubility or effect of heat since it is present in AZA water and MTI soxhlet and MTI cold extracts. Therefore, taking TLC into consideration MTI cold extraction method may not be a good method of extraction since it does extract less compounds compared to all the other methods.

Table 3.5: The Rf values of different chemical compounds separated using TLC

	AZA	AZA	AZA	MTI	MTI	MTI
Rf Values	Cold extraction	Soxhlet extraction	Water extraction	Water extraction	Soxhlet extraction	Cold extraction
0.03	nd	Nd	x	nd	x	x
0.09	x	x	x	x	x	x
0.14	nd	Nd	x	x	nd	nd
0.18	x	x	nd	nd	nd	nd
0.28	nd	Nd	x	x	nd	nd
0.47	x	x	nd	nd	x	nd
0.61	x	x	x	x	x	x
0.68	x	x	x	x	x	x
0.76	x	x	nd	x	x	x
0.86	x	x	x	x	x	x
Total	7	7	7	7	7	6

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI), nd - Not detected, x- detected

3.3.3 Total phenol, flavonoid and proanthocyanidin content

When the extracts of soxhlet, cold and water extraction methods of both MTI and AZA herbs were analysed, the total phenol flavonoid and proanthocyanidins content determined, the soxhlet extraction extracts of AZA had significantly higher ($P<0.05$) amount of flavonoids 0.84 g/100 g of sample extract when compared to the cold and water extracts which had 0.57 and 0.48 g/100 g of sample extract respectively. The cold methanolic extract of AZA had significantly higher amounts of total phenols 1.99 g/100 g of sample when compared to both soxhlet and water extracts with 1.51 g and 0.61 g/100 g of sample respectively, and proanthocyanidins 0.09 g/100 g of sample compared with the extracts from soxhlet and water extraction with 41.72 g and 11.00 g/100 g of sample respectively. The results of MTI indicate that the soxhlet and cold methanolic extracts contained a significantly lower amount of all the three compounds compared to the water

extracts (Table 3.6). These results indicate that with respect to MTI the water extraction method is the best in extracting the highest amount of phenolic, flavonoids and proanthocyanidin compounds, while for the AZA the cold methanolic extract may be considered better placed to extract more compounds than the soxhlet and water extraction methods. The antioxidant effect of AZA may be attributed to the high amount of polyphenols and to a lesser extent flavonoids.

Table 3.6: Total phenols, flavonoids proanthocyanidins extracted and antioxidant % inhibition of AZA and MTI using different solvents and methods of extraction

Sample	Solvent	Extraction method	Polyphenols	Flavanoids	Proanthocyanidins	Antioxidant (% inhibition)
AZA	Methanol	Soxhlet	1513.8^{c}	838^{c}	41.72^{b}	89.2^{b}
AZA	Methanol	Cold	1991.2^{d}	574^{ab}	87.25^{d}	94.73^{b}
AZA	Water	Water	617.2^{b}	483^{a}	11.00^{a}	97.3^{b}
MTI	Methanol	Soxhlet	198.0^{a}	789^{bc}	7.03^{a}	96.13^{b}
MTI	Methanol	Cold	189.8^{a}	723^{bc}	4.85^{a}	96.65^{b}
MTI	Water	Water	572.1^{b}	4578^{d}	54.60^{c}	54.4^{a}
LSD			106.1	205.6	9.6	12.5
C.V			7.9%	9.3%	18%	10.7%

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Values are in mg/100 g of sample extract. Differences are separated by Duncan's Multiple Range Test (DMRT). Values with same letter(s) in a column are not significantly different at 95 % significance level (P>0.05), N=3.

3.3.4 Antibacterial activity of the herb extracts

The result of the sensitivity of three microorganisms *E. coli*, *C. albicans* and *S. aureus* indicated that, the extract from AZA cold methanolic extract gave a significantly higher ($P \leq 0.05$) area of inhibition than the extracts from the water and soxhlet methods of extraction of 24 mm, 28.33 mm and 20 mm respectively (Table 3.7).

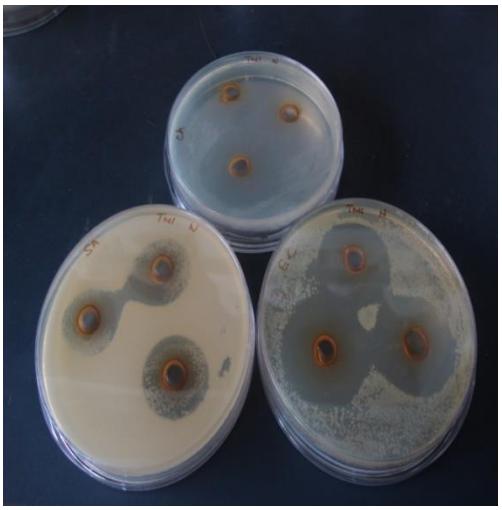
When these results were compared with those of MTI it was noted that all the extracts of MTI exhibited a significantly higher area of inhibition than all the AZA results. Comparing MTI extracts among each other it was noted that its water extract gave the highest area of inhibition. When the results were compared with the controls it was noted that MTI had a significantly higher inhibition capacity than all the controls. This therefore indicated that MTI is a very powerful antimicrobial agent compared to both the AZA extracts and the antibiotic controls. Antimicrobial properties of MTI may be attributed to the high amount of flavonoids and to a lesser extent polyphenols than those in AZA.

Table 3.7: Average inhibition zone diameter IZD (mm) of extracts of MTI, AZA and controls against test organisms

Method	Solvent	Extraction method	E. coli (mm)	Candida albicans (mm)	S.aureus (mm)
AZA	Water	Water	19.00^{b}	18.00^{b}	19.00^{ab}
AZA	Methanol	Cold	24.00^{c}	28.33^{c}	20.00^{b}
AZA	Methanol	Soxhlet	20.00^{b}	15.33^{a}	21.67^{c}
MTI	Water	Water	34.67^{e}	40.00^{e}	24.00^{d}
MTI	Methanol	Cold	30.00^{d}	36.33^{d}	22.33^{c}
MTI	Methanol	Soxhlet	24.33^{c}	35.33^{d}	24.00^{d}
Chlorophenical	Control		16.00^{a}	30.00^{c}	18.00^{a}
LSD			**1.638**	**2.572**	**1.343**
CV			**5%**	**4.6%**	**3.1%**

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Means followed by same letter(s) are not significantly different at 95% significance level. Mean differences separated by Duncan multiple Range Test (DMRT) (N=3)

A



B

C

Plate 3.1: The plates A are the control and B are the plates with extract MTI having *E.coli. S. aureous and C. albicans* as the test organisms, plate C is Cold methanolic extract of AZA against *E. coli, C. albicans* and *S. aureus* showing zones of inhibition

3.4 Conclusion

In the case of AZA the soxhlet and cold methanolic extracts had a very close relationship when considering the amount of flavonoids obtained, but when considering the antioxidant and antimicrobial capacities, then cold methanolic extract was the most efficient method.

The MTI extract that was consistently the most appropriate method of extraction was the water extraction method. The water extraction method compared with the soxhlet and cold extraction methods showed higher antimicrobial activity. It is clear that the high antioxidant capacity of AZA and high antimicrobial activity would compensate when the two are mixed together.

CHAPTER FOUR

PROFILING ACTIVE PHYTOCHEMICAL COMPOUNDS OF THE HERBS *Z. ABYSSINICA* AND *T. INDICA*

4.1 Introduction

Phytochemicals give plants their antioxidant activities (Eminagaoglu *et al*., 2007; Guleria *et al*., 2012 and Anwar *et al*., 2009). Research in plants oils of clove, oregano, rosemary, sage, and lavender has reported the presence of antioxidant and anti-rancidity properties (Adorjan *et al*., 2010). Out of the many phytochemicals present in plants only a few of them have been reported to be responsible for the antimicrobial and antioxidant activities. To identify these compounds many methods have been employed, most notable one is the TLC method whereby bioautography is employed, due to its flexibility, simplicity and accuracy (Olech *et al*., 2012; Cimpoiu *et al*., 2006; Badarinath *et al*., 2010). It also allows localization of activity even in complex matrix and therefore facilitating target-directed isolation of active constituents (Rahalison *et al*., 1991). These TLC methods have been developed and applied successfully for qualitative and quantitative analysis of both antioxidants and antimicrobial compounds (Zhao *et al*., 2010 Jasprica *et al*., 2007). To identify antioxidant compounds the stable free radical 2, 2-diphenyl -1-picrylhydrazyl (DPPH) is often used as a derivatization reagent (Kusznierewicz *et al*., 2012).

To promote the use of herbal plants AZA and MTI as potential preservatives an investigation of their composition and activity is very important to validate their use (Nair *et al*, 2006). This chapter will seek to find the various active compounds that are present

in the herb MTI and AZA and their mixtures that give them the potential to preserve meat foods. Presently MTI has already been widely investigated previously therefore more emphasis will be put on AZA.

Thin-layer chromatography (TLC) is a very commonly used technique in synthetic chemistry for identifying compounds, determining their purity and following the progress of a reaction. It also permits the optimization of the solvent system for a given separation problem. In comparison with column chromatography, it only requires small quantities of the compound (~ng) and is much faster as well.

4.2 Materials and Methods

4.2.1 Preparation of the samples

A 250 g dried portion of the fruits AZA and MTI was extracted using the method described in section 3.2.5. From the extracts obtained, 20 g from each herb was taken and soaked in 50ml absolute methanol overnight. The mixtures were then filtered using Whitman filter paper No. 41 along with 2 gm sodium sulfate which had been wetted with absolute methanol to remove sediments and traces of water in the filtrate. A 50 ml portion of hexane was added to the filtrates to remove organic impurities. The mixtures were shaken and the hexane portion discarded. The filtrates were then concentrated by bubbling with nitrogen to reduce the volume to 1 ml, the extract contained both polar and nonpolar chemical compounds. From the extract obtained phytochemical analysis to identify active compounds was performed using bio-autography TLC analysis.

4.2.2 Extraction and separation of compounds by the TLC system

Analysis of the chemical constituents in the extracts was performed by separation on glass -backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F254). Three eluent systems were used to develop the TLC plates under saturated conditions i.e., ethyl acetate/methanol/water (40:5.4:5): [EMW] for polar and neutral compounds, chloroform/ethyl acetate/formic acid (5:4:1): [CEF] for intermediate polarity and acidic compounds, benzene/ethanol/ammonia hydroxide (18:2:0.2): [BEA] for non-polar and basic compounds. The chemical compounds separated were detected by spraying acidified vanillin (0.1 g vanillin: 28 ml methanol: 1 ml sulphuric acid). Upon spraying, the chromatograms were heated at 110°C in an oven to allow for optimal colour development (Kotze and Eloff, 2002).

4.2.3 Separating Compounds using Bio-autography

4.2.3.1 Initial Preparation

Several TLC plates were loaded with 2 µl methanol extract (1 mg/ml) in a narrow spot directly onto the plate and eluted using the three different mobile solvent systems CEF, BEA, EMW and a control for each solvent system.

4.2.3.2 Separation of Antimicrobial Compounds

The developed plates were dried under hot air of 80°C in an oven with fast moving air for 5 days to remove traces of solvent on the plates. One week old cultures of microorganisms grown on synthetic define (SD) agar were each transferred into 250 ml of freshly prepared broth using a sterile swab. Densities of the cultures used for *C. albicans*, were approximately 8×10^6 cells/ml. In the case of bacteria, overnight cultures grown on Muller

Hilton broth were used and the densities of bacterial organism used for *E. coli*, and *S. aureus* were approximately 3×10^{11}, and 3×10^{12} cfu/ml, respectively.

The prepared chromatograms were sprayed with the fungal or bacterial suspension until wet. This process was carried out in a bio-safety Class II cabinet (Labotec, SA) for fungi, and Laminar flow cabinet (Labotec, SA) for bacteria. Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of p-iodonitrotetrazolium violet (Sigma®) (INT) (Begue and Klein, 1972). White bands, are indication of areas where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested organisms. Portions that were identified to have antimicrobial effect were scrapped from the TLC plates and reconstituted in methanol. The samples reconstituted were passed through a No. 1 Whatman filter paper after standing for thirty minutes. The filtrates were then passed through Millipore (Billerica, MA, USA) membranes (0.22 and 0.45 μm) before being injected into the HPLC for analysis and identification of compounds using mass spectrophotometer (MS) (Suleiman *et al*., 2010).

4.2.3.3 Separation of antioxidant compounds

After separation of the extracts by TLC and the plates developed as explained in section 4.2.2 in situ 2.54-mM DPPH methanol was sprayed on the developed plates and the plates observed with visible light, under UV 254 nm, and UV 366 nm. Samples producing yellowish bands on the red/purple background were considered as antioxidant, usually, the purple background colour was visualized after spraying the plate with DPPH reagent

(Ruiz-Terán *et al,* 2010; Lihua *et al*., 2009 and Rumzhum *et al*., 2012). The background colour of the plate changed from purple to red after 12hrs in darkness. The red background makes the yellowish bands clearly visible. Portions that were identified to have antioxidant effect were scrapped from the TLC plates and reconstituted in methanol. The reconstituted samples were left to stand for thirty minutes before the mixture was filtered using No. 1 Whatman filter paper. The samples were then passed through Millipore (Billerica, MA, USA) membranes (0.22 and 0.45 μm) before being injected into the HPLC for analysis and identification of compounds using MS (Suleiman *et al*., 2010).

4.2.4 Identification of the antimicrobial and antioxidant compounds using HPLC method

The reconstituted samples were filtered and injected into the HPLC machine. The HPLC system consists of solenoid pump with a photo diode array detector (PDA), C18 column was used as the stationary phase, all from Shimadzu Corporation, Japan. The flow rate of the pump was 1 ml/min and a back pressure of < 100 KV with a sample size of 0.2 µl. The mobile phase consisted of 90 % methanol, while the PDA detector was set at a wavelength range of 200 – 400 nm. The output signals were monitored and processed using Shimadzu CFR software. The run time of the method was about 7 min and all analytes were separated within the run time.

4.2.5 LC-QToF – MS analysis of phytochemicals in the herbs extract

The AZA and MTI extracts were concentrated in vacuo to dryness, re-dissolved in 3 ml of LC−MS grade CHROMASOLV methanol (Sigma-Aldrich, St. Louis, MO), and centrifuged at 14,000 rpm for 5 min, after which 0.5 µl of each was automatically injected

into LC−QToF−MS. The chromatographic separation was achieved on a Waters ACQUITY UPLC (ultra-performance liquid chromatography) I-class system (Waters Corporation, Maple Street, MA) fitted with a 2.1 mm × 100 mm, 1.7-μm particle size Waters ACQUITY UPLC BEH C18 column (Waters Corporation, Dublin, Ireland) heated to 40 °C and an auto sampler tray cooled to 15°C. Mobile phases of water (A) and acetonitrile (B), each with 0.01 % formic acid were employed. The following gradient was used: 0−1.5 min, 10 % B; 1.5−2 min, 10−50 % B; 2−6 min, 50−100 % B; 6−9 min, 100 % B; 9−10 min, 90−10 % B; 10−12 min, 10 % B. The flow rate was held constant at 0.4 mL/min. The UPLC system was interfaced by electrospray ionization (ESI) to a Waters Xevo QToF−MS operated in full scan MSE in positive mode. Data were acquired in resolution mode over the m/z range of 100−1200 with a scan time of 1s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature of 100°C, and desolvation temperature of 350°C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan function, a collision energy ramp of 25−45 V was applied in the T-wave collision cell using ultrahigh purity argon (≥99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; [M + H] + = 556.2766) was sampled at 10s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50−1200 Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 2-propanol/water (v/v). MassLynx version 4.1 SCN 712 (Waters Corporation, Maple Street, MA) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using monoisotopic masses with specifications of a tolerance of 10 ppm

deviation and both odd- and even-electron states possible. The number and types of expected atoms were set as follows: carbon, ≤100; hydrogen, ≤100; oxygen, ≤50; nitrogen, ≤6; sulfur, ≤6. The LC−QToF−MS data acquisition and analysis were based on the following defined parameters:

Mass accuracy (ppm) = 1,000,000 × (calculated mass − accurate mass)/calculated mass; Fit confidence % is the confidence with which accurate mass (measured data) matches the theoretical isotope models of the elemental composition in the list; elemental composition is a suggested formula for the specified mass. This is a summation of the quantities of elements, isotopes, or super atoms that can compose the measured data, calculated using the following atomic masses of the most abundant isotope of the elements: C = 12.0000000, H = 1.0078250, N = 14.0030740, O = 15.9949146, and S = 31.9720718. The empirical formula generated was used to predict structures that were proposed based on the online database, fragmentation pattern, and literature.

4.2.6 Identification of components using the Electrospray ionization mass spectrometry

For identification of key compounds present in the fruit paste extract of MTI and AZA, their aqueous and methanol extracts respectively were analysed by liquid chromatography/electrospray ionisation mass spectrometry (LC/ESI-MS) using Bruker UHPLC 3000 chromatography coupled to quadrupole ToF mass selective detector

(microToF-QII). The experimental conditions used for LC-MS analysis of MTI and AZA were followed as per procedure described by Krishnamachary *et al*., 2013.

4.2.7 Gas Chromatographic Analysis of the herbs extracts

The two AZA extract samples that exhibited strong antioxidant activity and two that exhibited strong antimicrobial activity against *S. aureus* with the bio autography tests were randomly chosen from the EMW (polar) samples. These samples were analysed by the GC-MS to identify the active compounds responsible for the antimicrobial and antioxidant effects of AZA. The samples of MTI were not considered in this case due to wide research already undertaken on MTI. The AZA sample extracts were subjected to GC, analysis, carried out on Shimadzu GC-14A chromatograph equipped with flame ionization detector fitted with 25 m× 0.22 mm (id.) SE-30 capillary column at carrier gas flow rate of 2 ml/min with split ratio was 1:100 and sample size 0.2 l. The column temperature was programmed at 70˚C for 4 min. with 4˚C/min rise to 220˚C while detector and injector temperature were maintained at 300˚C and 220˚C respectively Percentage composition of individual components was calculated on the basis of peak area using Shimadzu C-R4A chromate electronic integrator.

4.2.8 Mass Spectrometry

A Jeol model JMS-AX505H mass spectrometer combined with Hewlett Packard 5890 gas chromatograph were used for GC-MS analysis of the extracts from AZA. The herb extract was injected on a 25 m × 0.22 mm BPS (5% phenyl-methyl silicone) capillary Column using helium as a carrier gas with split ratio1:100 and interface temperature 230°C. Data acquisition and processing were performed by Jeol JMADA 5000 system. Various

components were identified by their retention time and peak enhancement with standard samples and MS library search.

4.3 Results and Discussion

4.3.1 Thin layer chromatography

The TLC plates with CEF and BEA all gave results that could not be visualized after elution. On the other hand the results of EMW could clearly be visualized as shown in Plate 4.4. This result of TLC analysis eluted with EMW revealed several spots as shown, this indicated that the extract contained various compounds some of which may be responsible for the antimicrobial and antioxidant activity of the extract.

The first three samples 1a, 1b and 1c were MTI in triplicate, three compounds separated as seen from the three spots. Sample No. 2 was an MTI extract filtered using active charcoal to obtain a clear liquid. No compound was seen to separate indicating that charcoal may have filtered out all active compounds. On the other hand it may be that the active compounds were the compounds responsible of the colour of the sample. It also suggested that most active compounds in the sample had colour pigments. Sample 3a, 3b, and 3c were AZA in triplicate, from this sample five compounds separated. When the same sample was filtered with active charcoal as seen in sample No. 4 nothing separated. A mixture of MTI and AZA yielded a combination of all the compounds in both MTI and AZA. In order to identify the activity of the compounds visualized on the TLC plate, autographic and Bio-autographic TLC was undertaken (Plate 4.4).

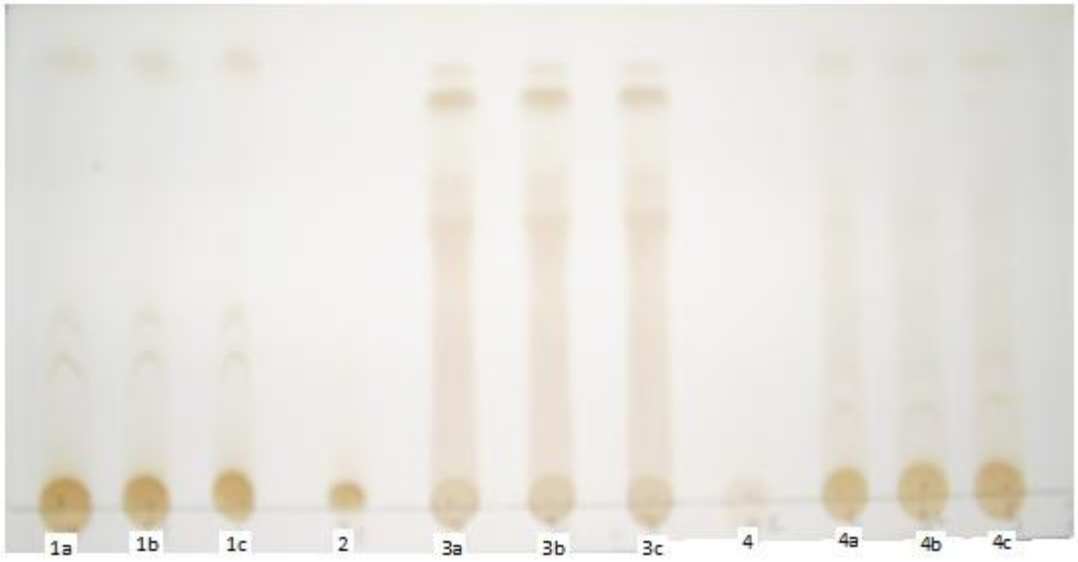


Plate 4.1: Indicates how each of the extracts separated upon elution with EMW. 1 was MTI, 2 and 4 was MTI and AZA extracts filtered with charcoal respectively. 3 was AZA and 4 was the blended extract at the ratio 1:1.

4.3.2 Identification of antimicrobial compounds using Bio-autography

The bio-autographic EMW solvent system targeting polar and neutral polar compounds in the extracts MTI, AZA and their mixture at a ratio of MTI: AZA 1:1 produced a distinct area of inhibition in triplicate when *S. aureus* and *Candida* were inoculated, indicating the presence of a compound or compounds that inhibited the growth of the two microorganism at an Rf of 8.2 and 9.2 respectively (Plate 4.5). The growth of *E. coli* was not inhibited by the compounds separated in the TLC solvent system. The solvent MTI did not separate any compound that produced any kind of inhibition in any of the microorganisms.

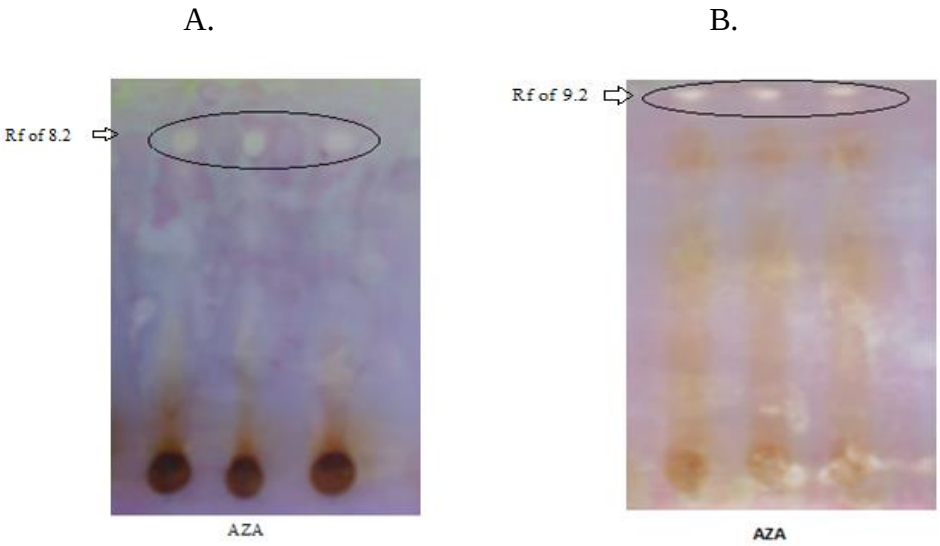


Plate 4.2: The bio-autographic EMW (A and B), solvent system targeting polar and neutral polar compounds in the extracts of AZA inoculated with *S. aureus* (A), Circled are the areas of microbial inhibition.

The bio-autographic BEA solvent system targeting non-polar compounds in the extracts of MTI, AZA and their mixture at a ratio of MTI: AZA 1:1 produced a distinct area of inhibition with *E. coli, S. aureus* and *C. albicans* this indicated that there were compounds that inhibited the growth of the three microorganisms *E. coli, S. aureus* and *C. albicans* at Rf 7.9, 7.8 and 7.4 respectively. The compounds separated from MTI did not produce any kind of inhibition to any of the microorganisms.

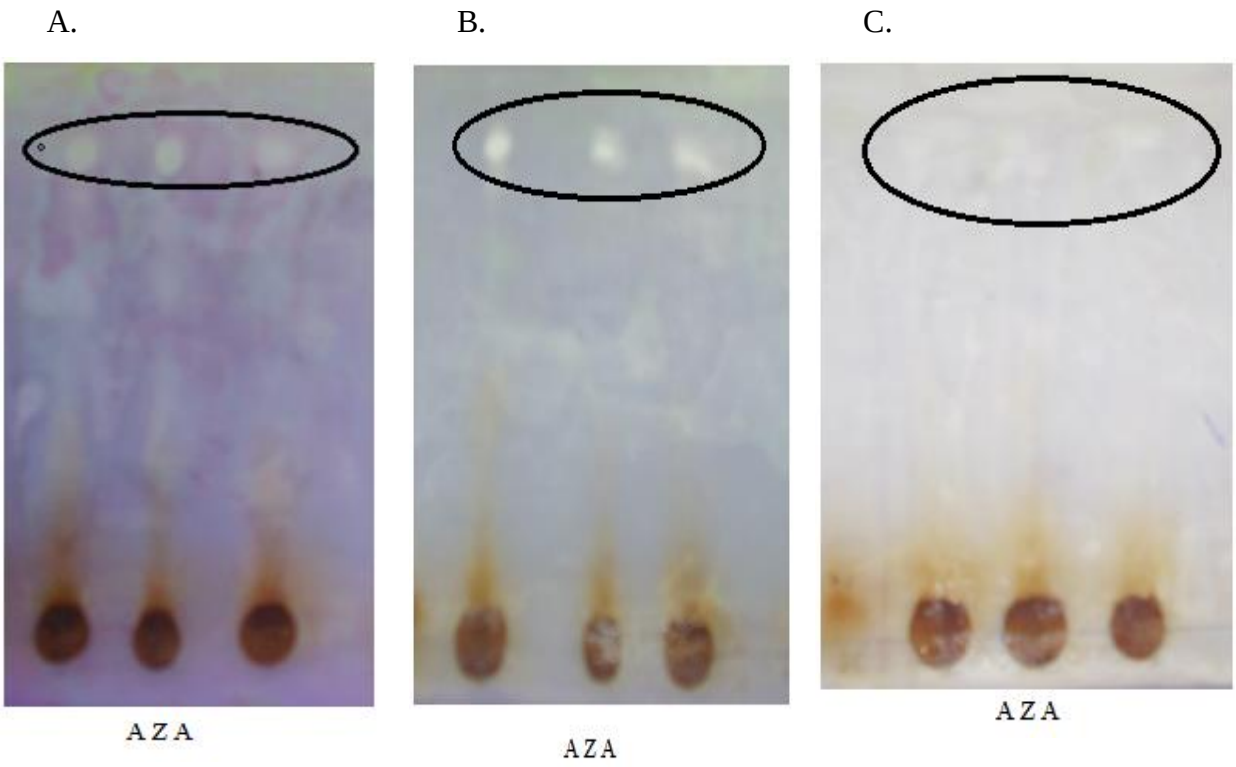


Plate 4:3: bio-autographic BEA solvent system targeting non-polar compounds in the extracts of AZA inoculated with *E. coli* (A), *S. aureus* (B) and *C. albicans* (C) Circled are the areas of microbial inhibition.

The bio-autographic CEF solvent system targeting Intermediate polarity and acidic compounds in MTI and AZA extracts, indicated no activity on the three microorganisms *E. coli, S. aureus* and *Candida*.

Most of the antimicrobial agents detected in this study were present in extracts of relatively non-polar solvents. These findings agreed with previously published results (Masoko and Eloff, 2005, 2006) that the substances responsible for the antimicrobial activity were mainly non-polar in nature (Suleiman *et al*., 2010). The study also revealed that MTI did not have a compound(s) that had antimicrobial effect on the test organisms or the tests undertaken could not separate the compound(s) that had antimicrobial activity, therefore, it may be insinuated that the antimicrobial effect of MTI may have been attributed to the acidity which may have been neutralized during the preparation stage.

4.3.3 Identification of antioxidant compounds using autography

In qualitative analysis of antioxidant activity, the 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates is used as a screening test for the radical scavenging ability of the compounds present in different extracts. The TLC autographic system used with solvent EMW for polar and neutral polar compounds reported most activity with AZA at Rf value of between 4.8 and 7.4. Indicating that the compounds that separated were of varying volatility. The compounds in MTI separated at an Rf Value of between 1.2 and 7.8. The solvent system BEA had compounds from MTI and AZA separating at very low Rf value of between 1.2 and 2.9 respectively. The solvent system of CEF of intermediate polarity and acidic compounds had no antioxidant activity reported. These clearly brings out the fact that AZA has properties that have antioxidant activity as visualized by Suleiman *et al*., (2010) in their analysis. These antioxidant properties can be further authenticated by using GCMS to identify particular compounds.

4.3.4 The antioxidant activity reported for AZA and MTI extracts with different solvent regimes

It was noted that when the extracts of AZA with MTI were blended their individual activity was seen implying that the act of blending did not diminish the individual capacity (Plate 4.7).

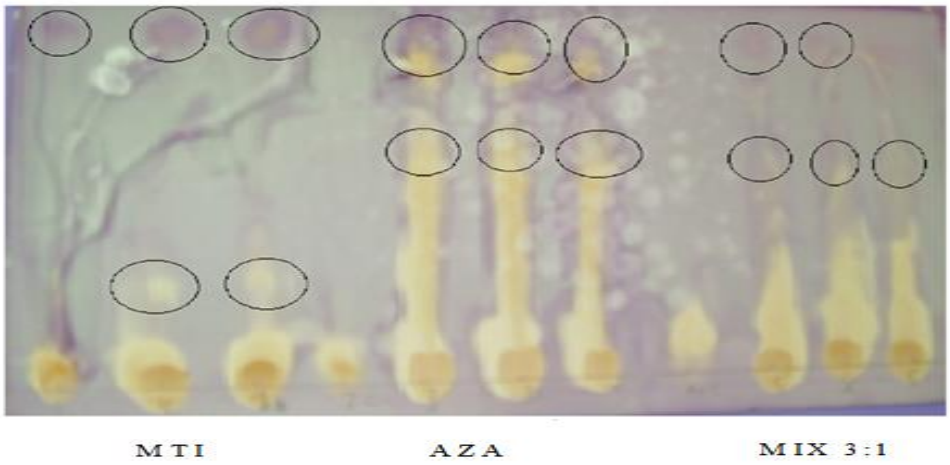


Plate 4.4: Antioxidation activity with the solvent system EMW (Polar and Neutral compounds) having MTI, AZA and a blend of the same

4.3.5 Identification of active compounds by HPLC analysis for the samples

To identify the compounds responsible for the activity of the extracts, each of the active spots on the TLC plates were scrapped and reconstituted with methanol, each of the samples were analyzed by HPLC to obtain peaks that would indicate the presence of any compound(s). The results obtained indicated that each spot on the TLC plate constituted of several compounds though one compound was predominant over the others judging from the peak eluting time and area under the graph as shown in the HPLC graphs in Annex I. These results clearly indicate that, as the compounds move up the TLC plate a

number of compounds remain fixed to the main compound that had attained maximum separation (Table 4.1). The prominent peaks are presumed to be from the most abundant compound responsible for the activity at that particular Rf point. When the retention time and peak areas of the compounds with the highest peak areas were analysed it was noted that the retention times of the separated compounds were related and not significantly different ($p \geq 0.05$) from the mean.

Table 4.1: Summarized HPLC results for antioxidant and antimicrobial samples dissolved in BEA and EMW solvent regime

Extract	Activity	MO's	TLC Solvent	RF	RT (sec)	Area (µV"sec)	Height (µV)
1:3MTI:AZA	Antimicrobial	*S. aureus*	BEA	1	5.212	4168478	270992
1:3 MTI:AZA	Antimicrobial	*E. coli*	BEA	7.5	5.256	2246261	151895
1:3 MTI:AZA	Antimicrobial	*S. aureus*	BEA	8	5.207	488242	32796
AZA	Antimicrobial	*S. aureus*	BEA	7.8	5.217	1173893	77838
AZA	Antimicrobial	*S. aureus*	BEA	7.6	2.233	19673	1505
AZA	Antimicrobial	*E. coli*	BEA	7.7	4.917	340648	22922
AZA	Antimicrobial	*C. albicans*	BEA	7.4	5.215	914564	61654
AZA	Antimicrobial	*S. aureus*	BEA	8	5.221	1174365	77654
AZA	Antimicrobial	*S. aureus*	EMW	8	5.233	1100427	74692
AZA	Antimicrobial	*S. aureus*	EMW	8.2	5.236	1447557	99403
AZA	Antimicrobial	*S. aueus*	EMW	9.3	5.184	403306	26986
AZA	Antimicrobial	*C. albicans*	EMW	9.4	5.216	912604	61884

AZA	Antioxidant	BEA	3	4.868	260030	20039
1:3MTI:AZA	Antioxidant	BEA	1.4	4.871	329602	20777
1:3MTI:AZA	Antioxidant	BEA	7.4	3.531	502432	29825
MTI	Antioxidant	BEA	1.5	4.866	662046	42257
MTI	Antioxidant	BEA	1.5	3.508	993076	37883
MTI	Antioxidant	BEA	7.2	4.441	74381	4614
MTI	Antioxidant	BEA	7.5	4.844	704184	44014
MTI	Antioxidant	BEA	7.4	3.517	694465	40301
1:3MTI:AZA	Antioxidant	EMW	4	6.553	354158	20877
1:3MTI:AZA	Antioxidant	EMW	7.9	3.515	397063	24788
MTI	Antioxidant	EMW	2.5	5.188	706820	38583
MTI	Antioxidant	EMW	2.5	4.365	520811	33801
MTI	Antioxidant	EMW	7.5	4.422	51118	4051
MTI	Antioxidant	EMW	8	4.824	445887	30041
AZA	Antioxidant	EMW	4.9	4.882	647123	41518
AZA	Antioxidant	EMW	4.9	3.515	316788	18866
AZA	Antioxidant	EMW	7.5	4.871	400552	25609

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI), Ethyl Acetate; Methanol; water (EMW) Benzene; ethanol; Acetone (BEA)

Four EMW samples were randomly taken from the re-dissolved extracts for further analysis, two that exhibited distinct antimicrobial activity and two that exhibited

antioxidant activity, they were run through the electrospray ionisation mass spectrometry: Lc−QTof−MS and GC-MS as described below.

4.3.6 Electrospray ionisation mass spectrometry: Lc−QTof−MS

The probable compounds identified included 1-(5-Benzoyl-2-quinolinyl)-1, 2, 3, 4, 5-cyclopentanepentayl, (2E)-2-(1H-Benzimidazol-2-yl)-3-(7-quinolinyl) acrylonitrile and 2-Methyl-5-[(2-methylbenzyl) oxy]-1-benzofuran-3-carboxylic acid (Table 4.9). The RT (Retention time) for all these compounds was similar ranging from 1053-1074 an indication that the compounds had similar properties. These are probably the compounds responsible for antioxidant effect separated by the polar and neutral polar solvent.

Another compound that was prevalent in three out of the four samples had a mass of 299. These compound had a possibility of only two compounds, 9-benzotriazole-1-ylmethyl-9H-carbazole, and 2-[2-(1-Naphthyl)-2-oxoethyl] isoquinolinium, with a retention time of 1.148.

Another compound that was prevalent had a mass of 274 identified as N-Lauryldiethanolamine. This compound may have been responsible for antimicrobial properties based on the fact that the compound was only available in three of the four samples analysed. Considering that the AZA sample was a strong antioxidant then the compound with mass of 299 must have been overshadowed (Table 4.2). These results were clearly not conclusive, further tests were undertaken using the GC-MS to confirm the results.

Table 4:2: Identification of components using the electrospray ionisation mass spectrometry: Lc−QTof−MS analyses on four samples two with antioxidant properties (1 and 2) and two with antimicrobial properties (3 and 4)

SAMPLE +A1:F67	RT	ACC MASS	CONFIDENCE %	EMPRICAL FORMULA (M+H)	IDENTITY
1	1.064	297.1141	99.61	C_{21} H_{15} N O	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
			95.03	C_{19} H_{13} N_4	(2E)-2-(1H-Benzimidazol-2-yl)-3-(7-quinolinyl)acrylonitrile
			99.94	C_{19} H_{13} N_4	(2E)-2-(1H-Benzimidazol-2-yl)-3-(7-quinolinyl)acrylonitrile
	1.148	299.1303	98.35	C_{19} H_{15} N_4	9-benzotriazole-1-ylmethyl-9H-carbazole
			53.34	C_{21} H_{17} N O	2-[2-(1-Naphthyl)-2-oxoethyl]isoquinolinium
			99.2	C_{21} H_{17} N O	2-[2-(1-Naphthyl)-2-oxoethyl]isoquinolinium
		High Energy			
	1.069	297.113	99.53	C_{19} H_{13} N_4	(2E)-2-(1H-Benzimidazol-2-yl)-3-(7-quinolinyl)acrylonitrile
			99.94	C_{21} H_{15} N O	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
			57.06	C_{21} H_{15} N O	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
2	1.053	297.1138	99.45	C_{19} H_{13} N_4	(2E)-2-(1H-Benzimidazol-2-yl)-3-(7-quinolinyl)acrylonitrile
			98.8	C_{21} H_{15} N O	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
			76.9	C_{19} H_{13} N_4	(2E)-2-(1H-Benzimidazol-2-yl)-3-(7-quinolinyl)acrylonitrile
	1.148	299.1331	30.96	C_3 H_{15} N_{12} O_5	NON
			30.32	C_{21} H_{17} N O	2-[2-(1-Naphthyl)-2-oxoethyl]isoquinolinium
			17.66	C_3 H_{15} N_{12} O_5	NON
	2.955	274.2735	76.06	C_{16} H_{36} N O_2	N-Lauryldiethanolamine
			100	C_{14} H_{34} N_4 O	
	2.976	318.2991	98.51	C_{18} H_{40} N O_3	Phytosphingosine

			100	$C_{16}H_{38}N_4O_2$	
			96.14	$C_{16}H_{38}N_4O_2$	
	3.605	302.3044	93.41	$C_{18}H_{40}NO_2$	DErySphinganine
			99.99	$C_{16}H_{38}N_4O$	
			72.47	$C_{18}H_{40}NO_2$	DErySphinganine
		High Energy			
	1.069	297.1134	94.81	$C_{19}H_{13}N_4$	
			98.29	$C_{21}H_{15}NO$	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
			69.61	$C_{19}H_{13}N_4$	
3	1.074	297.1137	68.22	$C_{18}H_{17}O_4$	
			89.91	$C_{21}H_{15}NO$	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
			41.67	$C_{21}H_{15}NO$	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
	1.148	299.129	99.85	$C_{19}H_{15}N_4$	
			93.37	$C_{21}H_{17}NO$	2-[2-(1-Naphthyl)-2-oxoethyl]isoquinolinium
			99.62	$C_{19}H_{15}N_4$	
	2.955	274.274	99.95	$C_{16}H_{36}NO_2$	N-Lauryldiethanolamine
			100	$C_{14}H_{34}N_4O$	
			56.3	$C_{14}H_{34}N_4O$	
	4.71	322.1436	99.7	$C_{20}H_{20}NO_3$	
			99.36	$C_{18}H_{18}N_4O_2$	4-[(3-Aminophenyl)amino]-6-(dimethylamino)-3-quinoline carboxylate
			82.06	$C_{20}H_{20}NO_3$	
	0.697	226.9529	100	H_3O_{14}	
4	0.857	305.1597	58.51	$C_{14}H_{25}O_7$	2-[(Propionyloxy)methoxy]-1,4-butanediyl dipropanoate
			99.84	$C_{12}H_{23}N_3O_6$	

		96.19	$C_{12}H_{23}N_3O_6$	
1.074	297.116	74.13	$C_{18}H_{17}O_4$	2-Methyl-5-[(2-methylbenzyl)oxy]-1-benzofuran-3-carboxylic acid
		88.45	$C_{21}H_{15}NO$	1-(5-Benzoyl-2-quinolinyl)-1,2,3,4,5-cyclopentanepentayl
		45.04	$C_{18}H_{17}O_4$	
4.71	322.1436	99.7	$C_{20}H_{20}NO_3$	
		99.36	$C_{18}H_{18}N_4O_2$	4-[(3-Aminophenyl)amino]-6-(dimethylamino)-3-quinoline carboxylate
		82.06	$C_{20}H_{20}NO_3$	
6.538	341.2625	99.06	$C_{15}H_{37}N_2O_6$	
		78.09	$C_{13}H_{35}N_5O_5$	
		97.68	$C_{15}H_{37}N_2O_6$	
0.697	High Energy 226.9529	100	H_3O_{14}	
	362.9298	99.76	$C_6H_3O_{18}$	
		99.58	$C_4HN_3O_{17}$	
		56.34	$C_4HN_3O_{17}$	

4.3.7 Gas Chromatographic Analysis

The sample that exhibited antioxidant effect after analysis produced twenty one compounds. From the graph (Fig 4.1) it was noted that most of the compounds lay between the RT of 20 and 32. Some compounds appeared in very low peak area while others had high peak areas. All the compounds were individually investigated for their activity during previous encounters. It was noted that the compounds with the highest antioxidant properties included *Benzenamine*, 3,4-*dimethyl*- MW 121.17, *Butylated hydroxytoluene* MW 220.35, *Phenol*, 2,5-*bis*(1,1-*dimethylethyl)*- MW 206.36, *Phenol*, 2,2'-*methylenebis*[6-(1,1-*dimethylethyl*)-4-*ethyl*- MW 368.55, *Phenol*, 2,5-*bis(1,1-dimethylethyl*)- MW 206.32, *Butylated hydroxytoluene* MW 220.35, *Methyl decanoate* MW 186.291 and *Hexadecanoic acid / Palmitic acid* MW 256.43. From these findings it was noted that the compounds that gave the highest peaks in the HPLC tests were not necessarily the compounds having antioxidant activity since most of them appear to be probably the colour compounds. *Butylated hydroxytoluene* may be the compound largely responsible for the antimicrobial activity exhibited by the extract. The compounds were found to be rich in long chain hydrocarbons as listed on table 4.3.

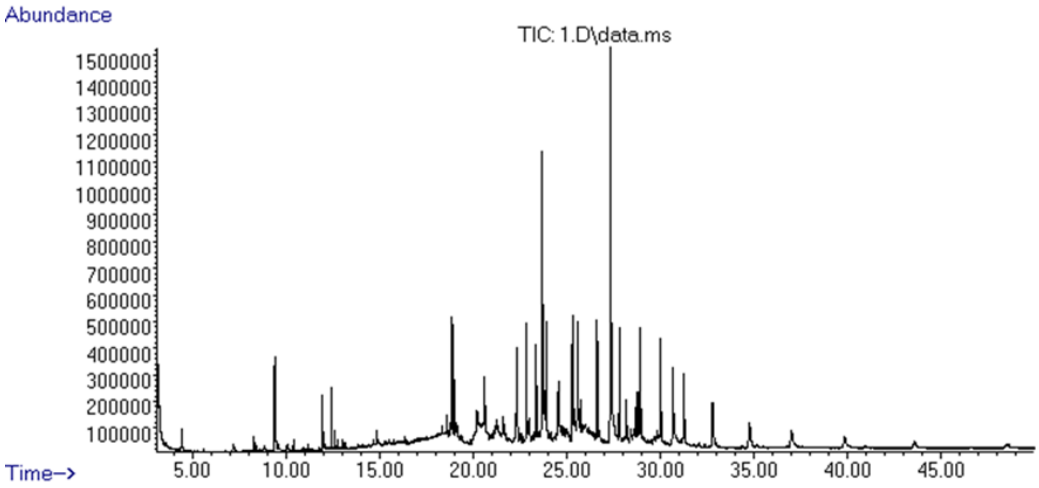


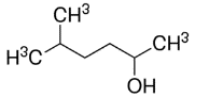
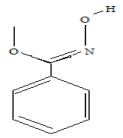
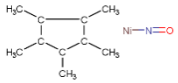
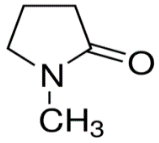
Figure 4.1: GC-MS peak distribution of the AZA sample that showed antioxidant activity in the TLC plate analysis

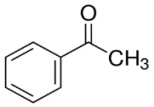
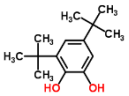
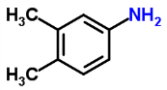
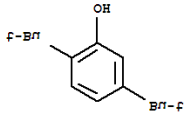
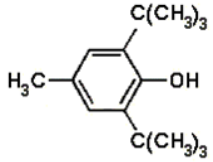
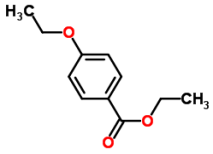
Table: 4.3: Compounds identified by the GC-MS from the AZA sample that had antioxidant activity

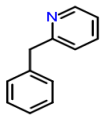
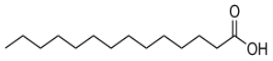
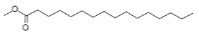
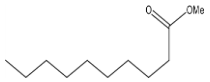
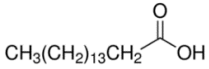
Library/ID	RT	Peak height	Corr area	Corr % max	% of total
2-Hexanol, 5-methyl- (Aroma-Herbaceous)	8.299	30,520	819,615	3.4	0.54
Oxime-, methoxy-phenyl-_	9.32	299,536	8,837,459	36.9	5.819
Nickel, nitrosyl [(1, 2, 3, 4, 5-.eta.)-1,2,3,4,5-pentamethyl-2,4-cyclopentadien-1-yl]-	9.586	23,432	1,121,702	4.7	0.739
2-Pyrrolidinone, 1-methyl-	11.932	209,106	4,921,262	10.3	0.973
Acetophenone	12.418	70,644	1,357,525	5.7	0.894
1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	12.576	69,705	1,804,883	3.8	0.357
Benzenamine, 3,4-dimethyl-	12.757	38,237	990,231	2.1	0.196
Phenol, 2,5-bis(1,1-dimethylethyl)-	18.806	61,312	1,350,856	5.6	0.890
Butylated hydroxytoluene	18.870	466,536	10,493,086	21.9	2.074
Benzoic acid, 4-ethoxy-, ethyl ester	19.005	112,622	3,686,869	15.4	2.428
Pyridine, 4-(phenylmethyl)-	20.198	48,315	1,274,126	5.3	0.839
Docosane	21.105	25,812	537,732	2.3	0.354
Methyl tetradecanoate (floral)	21.245	59,386	2,509,443	10.5	1.652
Methyl hexadecanoate	23.345	859,033	23,716,207	99.1	15.617
Methyl decanoate	23.345	362,059	7,331,195	15.3	1.449
Hexadecanoic acid	23.685	1,076,883	27,587,042	57.6	5.452
Methyl octadecanoate	25.258	874,734	23,941,074	100.0	15.765
Octadecanoic acid	25.568	443,842	17,685,771	36.9	3.495
Hexadecanamide	25.756	159,159	5,866,074	12.3	1.159
9-Octadecenamide, (Z)-	27.34	1,484,338	47,871,541	100.0	9.461
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-	28.616	33,441	935,093	3.9	0.616

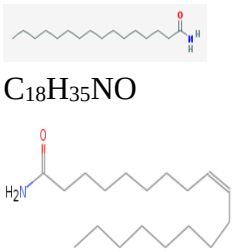
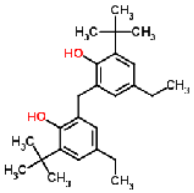
Retention Time (RT)

Table 4.4: Compounds identified by the GC-MS from the AZA sample that had antioxidant activity, their molecular formula, Molecular weight, nature of the compound and bioactivity

Compound	MW	Molecular formular/Structure	Nature of compound and bioactivity based on literature
2-Hexanol, 5-methyl- (Aroma-Herbaceous)	116.2	$C_7H_{16}O$ 	Alkane,
Oxime-, methoxy-phenyl-	151.2	$C_8H_9NO_2$ 	Phenolic, Aroma compound. an amine
Nickel, nitrosyl[(1,2,3,4,5-.eta.)-1,2,3,4,5-pentamethyl-2,4-cyclopentadien-1-yl]-	223.9	$C_{10}H_{15}NNiO$ 	
2-Pyrrolidinone, 1-methyl-	99.13	C_5H_9NO 	A nonvolatile solvent, able to dissolve diverse materials (Harreus *et al*., 2011). low toxicity to aquatic life
Acetophenone	120.1	C_8H_8O or $C_6H_5COCH_3$	Is the simplest aromatic ketone. It's a flavoring agent in foods.

			
1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	222.3	$C_{14}H_{22}O_2$ 	Aromatic compound
Benzenamine, 3,4-dimethyl-	121.17	$C_8H_{11}N$ 	Has antioxidant activity. (Aziz et. al. 2014)
Phenol, 2,5-bis(1,1-dimethylethyl)-	206.32	$C_{14}H_{22}O$ 	Phenolic compound, Has antioxidant antimicrobial, and antifungal properties
Butylated hydroxytoluene	220.35	$C_{15}H_{24}O$ 	It has antioxidant properties (Babu and Wu, 2008) (Williams and Iatropoulos, 1996)
Benzoic acid, 4-ethoxy-, ethyl ester 17.28 Benzoic acid, 4-ethoxy-,ethyl ester C11H14O3 Unknown Antifungal Aromatic Esters	194.2271	$C_{11}H_{14}O_3$ 	Aromatic Ester,

Pyridine, 4-(phenylmethyl)- 2-Benzylpyridine	169.24	$C_{12}H_{11}N$ 	Antifungal property on Candida albican and yeast (http://www.chemicalbook.com/ChemicalProductProperty_EN_CB0320119.htm) Online 2016 28/09/2016
Methyl tetradecanoate (floral)	242.397	$C_{15}H_{30}O_2$ 	Also called myristic acid odour, taste and flavor compound (Fenaroli 1975, and Rezetende *et al.*, 2003).
Methyl hexadecanoate	270.459	$C_{17}H_{34}O_2$ 	Fatty acid ester. It has anti-inflammatory effect on human.
Methyl decanoate	186.291	$C_{11}H_{22}O_2$ 	Alkane, An aroma compound, with antiviral, antioxidant and antimicrobial properties. (Malapaka 2011; Kumar 2011; Radwan and Aboul-Enein 2002; and Sengupta and Ghosh 2011).
Hexadecanoic acid / Palmitic acid	256.43	$C_{16}H_{29}O_2$ $CH_3\ (CH_2)_{14}COOH$ 	Fatty acid aroma compound with antibacterial, antioxidant and antifungal activities. Hussain and Kumaresan 2014.
Methyl octadecanoate/ Stearic acid/Octadecanoic acid	298.50	$C_{19}H_{38}O_2$ 	Fatty acid methyl, a synthetic flavoring substance and adjuvant, and a saponifier Soluble in ether and chloroform and Insoluble in water.
Hexadecanamide	255.44	$C_{16}H_{33}NO$	Fatty amide.

9-Octadecenamide, (Z)-	281.4766	$C_{18}H_{35}NO$ 	
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl-	368.55	$C_{25}H_{36}O_2$ 	Antioxidant compound, antibacterial and antifungal

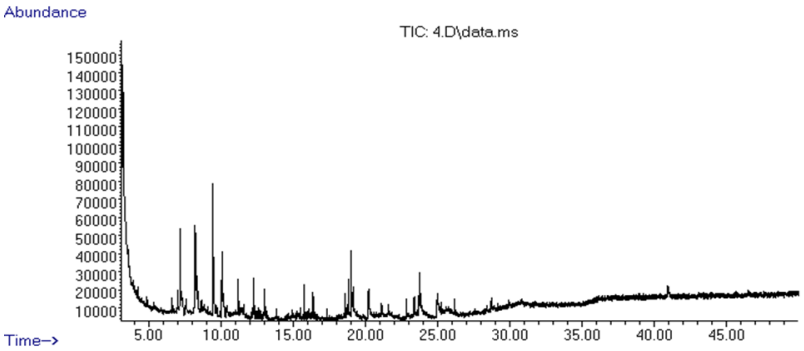
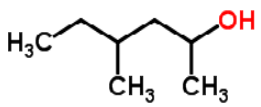
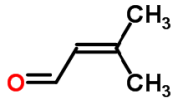
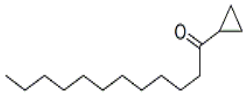
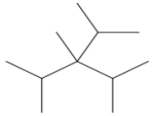


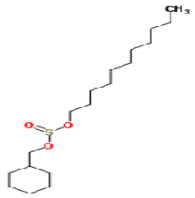
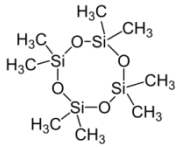
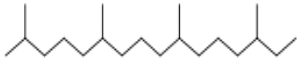
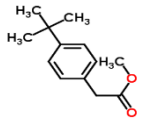
Figure 4.2: GC-MS product distribution of the *AZA* sample that showed antimicrobial activity in the TLC analysis

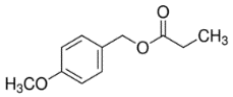
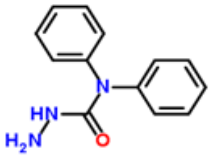
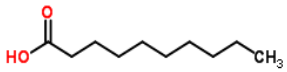
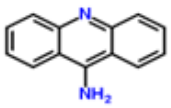
Table 4.5: List of compounds generated by the GC-MS from the AZA sample that had antimicrobial activity

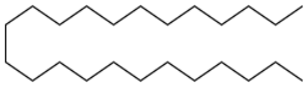
Library/ID	RT	Peak height	Corr area	Corr % max	% of total
4-Methyl-2-hexanol	8.1999	43521	1108747	59.86	9.201
Heptanol<2->	8.2759	20946	506327	27.33	4.202
2-Butenal, 3-methyl-	9.4167	70549	1473593	79.55	12.23
1-Hexene, 3,5,5-trimethyl-	9.4693	31947	1001989	53.83	4.185
1-Dodecanone, 1-cyclopropyl-	9.4634	30479	994324	53.68	8.252
Pentane, 2,3,4-trimethyl-	10.0368	19150	365085	19.71	3.030
Sulfurous acid, cyclohexylmethyl undecyl ester	10.0953	31381	735388	39.70	6.103
Cyclotetrasiloxane, octamethyl-	11.1775	18993	466242	25.17	3.869
Hexacosane	12.2246	21140	439653	23.74	3.649
Heptadecane (C17)	12.991	15206	304334	16.43	2.526
Hexadecane, 2,6,10,14-tetramethyl-	16.3664	14529	336404	18.16	2.792
Tetradecane (C14)	17.3492	110287	1861272	100.0	7.775
Methyl p-tert-butylphenyl acetate	18.8	19500	366576	19.79	3.042
Anisyl propanoate	19.0106	35041	1004803	54.25	8.339
Docosane	19.1335	13903	352438	19.03	2.925
Hydrazinecarboxamide, N,N-diphenyl-	20.2098	15252	605010	32.66	5.021
Decanoic acid	23.6613	28704	1082150	58.14	4.520
9-Acridinamine	24.9659	11485	658024	35.52	5.461
Eicosane (C20) Alkane	28.3881	23119	821454	44.13	3.431
Hexacosane	29.1603	23580	619178	33.27	2.586
Untriacontane	29.9208	33283	932324	50.09	3.894
Octacosane	30.7749	19307	927876	49.85	3.876
Tetracosane	31.7752	30327	1604838	86.22	6.704

Table 4.6: List of compound resulting from the GC-MS analysis of the antimicrobial sample

Compound	MW	Molecular formular and structure	Nature of compound and bioactivity based on literature
4-Methyl-2-hexanol	**116**	$C_7H_{16}O$ 	Aroma compound and an antimicrobial agent against Staphylococcus aureus.
Heptanol	**116.2**	$CH_3(CH_2)_6OH$ 	It has a spicy taste hence used in food flavors.
2-Butenal, 3-methyl-	84	C_5H_8O 	Flavors and Fragrances. And also an antibacterial effect against Pseudococcus affinis.
1-Hexene, 3,5,5-trimethyl	126	C_9H_{18} 	Acyclic, Alkenes, Building Blocks, Chemical Synthesis, Organic Building Blocks
1-Dodecanone, 1-cyclopropyl-	224	$C_{15}H_{28}O$ 	It is one of the components used as anti-ticks repellants.
Pentane, 2,3,4-trimethyl-	114	C_8H_{18} 	A branched alkane. It is one of the isomers of octane. Have carcinogenic effect, very toxic to aquatic life with long lasting effects

Sulfurous acid, cyclohexylmethyl undecyl ester	332	$C_{18}H_{36}O_3S$ 	Boosters for antimicrobial agents.
Cyclotetrasiloxane, octamethyl-	297	$C_8H_{24}O_4Si_4$ 	Silicone polymers
Heptadecane (C17)		$C_{17}H_{36}$ 	Smallest "impossible" alkane. May be incompatible with strong oxidizing agents like nitric acid.
Hexadecane, 2,6,10,14-tetramethyl- 15 27.3 9-hexadecenol C16H32O 238 9.0	282.6	$C_{20}H_{42}$ 	It is used as a bio-marker in petroleum studies
Tetradecane (C14)	198	$C_{14}H_{30}$ 	Volatile compound that is insoluble in water, Antifungal and Antibacterial
Methyl p-tert-butylphenyl acetate Acetic acid or Benzeneacetic acid	206	$C_{13}H_{18}O_2$ 	flavor and fragrance agents

Anisyl propionate	180	$C_{10}H_{12}O_3$ 	Fragrance, Flavoring agents and adjuvants (McGinty et al, 2012). It is a food additive It is a benzyl alcohol
Docosane	310.6	$CH_3(CH_2)_{20}CH_3$ 	Fatty acid (hydrocarbon). Linear Aliphatic hydrocarbon. Insoluble in water, Antibacterial.
Hydrazinecarboxamide, N,N-diphenyl-	227.27	$C_{13}H_{13}N_3O$ 	Antifungal of antiviral, anti-infective and antineoplastic. Semi carbazide products are known to have an activity of antiviral, anti-infective and antineoplastic through binding to copper or iron in cells.
Decanoic acid (capric acid) saturated medium chain fatty acids	172	$C_{20}H_{40}O_4$ 	Flavors and as an intermediate for food-grade additives. Cosmetic, flavor and fragrance agents. It has antimicrobial Antiviral and antifungal activity. http://www.chemicalbook.com/ChemicalProductProperty_EN_CB1669961.htm Decanoic acid is a saturated fatty acid. Salts and esters of decanoic acid are called decanoates or "caprates".
9-Acridinamine	194	$C_{13}H_{10}$ 	Anti-infective, local antiseptic, antibacterial and incompatible with strong oxidizing agents such as hydrides. Insoluble in water.

Eicosane (C20)	282.55	$C_{20}H_{42}$ 	Aliphatic saturated hydrocarbon and mostly unreactive. Incompatible with strong oxidizing agents. Insoluble in water. Antibacterial, antitumor, antifungal, cytotoxic.
Hexacosane	366.7	$C_{26}H_{54}$ 	Aliphatic saturated hydrocarbon and mostly unreactive. N-hexacosane is a colorless crystals. Occurs in many natural products. Hydrocarbons, Aliphatic Saturated
Untriacontane	436.8	$C_{31}H_{64}$ 	Hydrocarbon linear alkane. Acyclic Alkanes. It is a food additive: carrier solvent, chewing gum base compound, clouding agent, glazing agent, texturizer and thickener.
Octacosane	394.8	$C_{28}H_{58;}$ 	High alkane but no information on this compound is available.
Tetracosane	338	$C_{24}H_{50}$ 	Alkane hydrocarbon responsible for colour.

The compounds with the highest peaks were not necessarily responsible for antioxidant or antimicrobial effects, this was seen with Tetracosane, despite having a high peak it is an alkane hydrocarbon responsible for colour.

The compounds identified to have antimicrobial effects were 2-Furancarboxaldehyde $C_5H_4O_2$ which is an aldehyde compound with antimicrobial and antifungal activity. The differences in the GC-MS profiles of AZA collected from different locations could be explained by the fact that plants often produce different amounts of phytochemicals when growing in different geographical regions. (Shankar *et al*., 2005).

4.4 Conclusion

The extracts from various compounds may be contributing to the overall antioxidant and antimictobial effects on the food. Some of the compounds identified to be responsible for antioxidant effects were Benzenamine, 3,4-dimethyl-, Phenol, 2,5-bis(1,1-dimethylethyl)-, Butylated hydroxytoluene, Methyl decanoate, Hexadecanoic acid / Palmitic acid and Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl- while the compounds identified to be responsible for antimicrobial effects were 4-Methyl-2-hexanol, Sulfurous acid, cyclohexylmethyl undecyl ester, Decanoic acid (capric acid) and Hydrazinecarboxamide, N,N-diphenyl. Therefore, the antimicrobial and antioxidant activity of the herbs was caused by a combination of several compounds as stipulated above.

CHAPTER FIVE

DETERMINATION OF THE MINIMUM AMOUNT OF THE HERBAL COMBINED PRODUCT THAT ELICIT PRESERVATIVE EFFECT ON PORK SAUSAGES

5.1 Introduction

The moment an animal is slaughtered, spoilage begins immediately. This results from the introduction of spoilage microorganisms such as bacteria and fungi from the equipment, personnel, contaminated surfaces and holding the products in high temperatures. Spoilage can also result from oxidation of meat fats due to poor handling resulting in oxidative rancidity manifesting as rancid odour and flavor (Zhou *et al*., 2010).

Meat can remain edible for long periods of time if proper hygiene is maintained during slaughter, processing and if measures are taken including food safety, storage, and preservation. All the measures that exclude the use of preservation have been applied and found to pose challenges that have been difficult to address in society (Nilsson *et al.*, 2005). This then resulted in the development of synthetic preservatives, which have been recently associated with diseases such as cancer (Kinyua, 2004). Consequently, development of a preservative from natural sources that would not have dire consequences has led to the search for a suitable herbal preservative.

Herbs used by pastoral communities to preserve their food, though in small quantities are considered to be viable alternatives to synthetic preservatives. The main challenge that most herbalists meet is being able to determine the dosage of particular herbs. This has resulted in over dose or under dose. That would not be desirable in the preservation of food because the flavor and aroma of food need to be precisely maintained.

Previous research has indicated that when AZA was applied to food it did not alter the taste or aroma of the meat sample yet it maintained its antioxidant capacity. However.

MTI was found to slightly alter the taste of meat sample due to its astringent taste and also maintained its antimicrobial properties. Therefore, finding the right concentration of the herbs to be used in the meat samples would be most appropriate.

This study aimed at determining the preservative effect of the combined herbs AZA and MTI. In order to achieve that, the right concentration and ratio of AZA and MTI to be used in the effective preservation of the meat sample without changing the original taste was sought.

The main spoilage bacteria and fungi that are likely to contaminate the meat are *S. aureus, S. typhi, C. botulinum, E. coli* and *C. albicans.* It is against these microorganisms that the herbal blend was formulated. Previous chapters reveal that the most ideal ratio of the herbs AZA to MTI extracts with the most activity was 1:3. This was thus used as the reference point of developing a ratio that will be effective against the spoilage microorganisms and would not have taste implications on the product (Nyaberi *et al*., 2010).

5.2 Materials and Methods

5.2.1 Determination of the most active herbal blend ratio

In order to determine the minimum amount of herbal mixtures that would elicit maximum antimicrobial and antioxidative effect, 45 g portions of the herbs MTI and AZA were taken and extracted as described in Section 3.2.7. The two extracts MTI and AZA were mixed in the ratios provided below on weight to weight basis.

Table 5.1: The ratios of AZA and MTI herb extracts used to identify the most potent blend

Z. abyssinica (AZA) and *T. indicus* (MTI)	AZA:MTI	AZA:MTI	AZA:MTI	AZA:MTI
1 g sample in 10 ml distilled water	1:1	1: 0	1 : 3	0:1
2 g sample in 10 ml water				
5 g sample in 10 ml water				

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI

The extract mixture obtained was used to perform a sensitivity test with various microorganisms which are listed in Table 3.2. Sensitivity was done using the cork and bore diffusion method of (Rojas *et al*. 2003). Using a sterile cork-borer of 6 mm diameter, three holes were made into the set agar in petri-dishes containing the bacterial culture. Then 0.1 ml of each concentration of the extracts in different ratios 1:1, 1:0, 1:3, 0:1 of AZA: MTI respectively were poured into the wells in triplicates. A positive control was incorporated using a preservative sodium metabisulfite.

5.2.2 Minimum inhibitory concentration of sample extracts

The MIC of the water extracts from the selected concentration of herb mixtures used to preserve meat was determined using the standard method of Doughari (2009) with modifications. Plates that showed significant inhibitory activity (more than 9 mm inhibitory diameter) on the test microorganisms were considered for this test. Nutrient broth was prepared and sterilized in an autoclave at 121ºC for 15 min. A sample of 1 ml of the prepared broth was dispensed into the test tubes numbered 2-12 using a sterile pipette and 1 ml plant extract containing 0.1-0.2 g/ml extract in water was dispensed into each of the tubes numbered 1 and 2. Subsequently from tube 2, serial dilution was carried out and 1ml from tube 2 was transferred up to tube number 10 and 1 ml from tube number 10 was discarded. Tube 11 was control for sterility of the medium and tube 12 for viability of the organisms.

Pure cultures of the organisms were inoculated into nutrient broth (Oxoid, England), incubated for 24 h at 37°C, diluted with sterile nutrient broth to a density of 9×10^8 cfu/ml by serial dilution. From this dilution a 0.1 ml of inoculam were transferred into each of the tubes 2- 12 with the exception of tube 11, to which another sterile broth was added. The final concentration of the plant extract in each of the test tubes numbered 1-10 after dilution were 100,000; 50,000; 25,000; 12500; 6250; 3125; 1562.5; 781.25; 390.625; 195.3125 µg/ml, respectively. They were incubated at 37ºC for 24-48 hrs and examined for growth. The last tube in which growth failed to occur was the MIC tube.

5.2.3 The effect of the combined herbal preservative on the meat product samples

The search for the concentration of the herbal mixture that exhibited antimicrobial and antioxidant activity was taken into the next phase where the herbal blend were introduced into pork sausage samples. Pork sausages were prepared by incorporating all ingredients as shown in Table 5.2, apart from the preservatives. The mix was divided into four batches. The first batch (control) 4.5 g sodium metabisufite was added as a preservative which is normally used in conventional sausages. The second, third and fourth batches were preserved with 1.575 g, 3 g, and 6 g of the AZA: MTI (1:3) ratio herbal extract respectively. The sausages prepared were stored raw at 4±2°C for two weeks. Every alternate day a sample was taken from each batch and analyzed for TBARS, TVC, *E. coli, S. aurious*, moulds and yeast and later a sensory analysis was undertaken that involved triangle test to find out if the preservatives alter the taste of the sausages. An appropriate vehicle to assist in application was determined followed by packaging of the preservative.

Table 5.2: Ingredients used in preparation of sausages

No	Ingredients	Batch 1 (g)	Batch 2 (g)	Batch 3 (g)	Batch 4 (g)
1	Lean meat	1,800	1,800	1,800	1,800
2	Fat	600	600	600	600
3	Water	540	540	540	540
4	Salt	33	33	33	33
5	Sugar	30	30	30	30
6	Protein binder	120	120	120	120
7	Non protein binder	450	450	450	450
8	Colour	0.015	0.015	0.015	0.015
9	Phosphate	6	6	6	6
10	MSG	4.5	4.5	4.5	4.5
11	Mixed spices	8.1	8.1	8.1	8.1
	Preservatives				
1	Sodium metabisulphite	4.5	0	0	0
2	1:3 (MTI:AZA)	0	1.575	3	6

(Njoroge *et al*., 2006). MSG Monosodium glutamate, AZA- *Ziziphus abssynica* and OTI- *Tamarindus indica*.

5.2.4 Evaluation of rancidity

Rancidity development was measured using the thiobarbituric acid reactive substances (TBARS) test. The test measures the deterioration in extractable and non- extractable lipids (Papastergiadis *et al*., 2012).

The method of Tarlandgis *et al*., (1960) as modified by Izunimoto *et al*. (1990) was applied. A 10 g sample of the pork sausage was homogenized with 50 ml distilled water and allowed to stand for 30 minutes at room temperature before adding 20 ml of 20 % trichloroacetic acid (TCA) solution (BDH Ltd. England). The sample was mixed

thoroughly before filtering, active charcoal was used to eliminate interferences caused by the colour pigmentation in the extract. A 5 ml sample of the filtrate was drawn and mixed with 5 ml of 0.02 M thiobarbituric acid (TBA) reagent (BDH Ltd England) in test tubes then heated in a water bath for 35 minutes. Absorbance of the reddish/pink colour formed was measured at 532 nm using a Shimadzu UV- VIS spectrophotometer mini 1240, and recorded as D value. A blank was prepared in a similar way excluding the sample. The TBARS was calculated as Malondialdehyde (mg/kg) according to the following equation: E532 x 12.9 where E is the extinction value at 532 nm, 12.9 is a conversion factor. The evaluation was done in triplicates.

5.2.5 Determination of total viable count (TVC), E. coli, S. aureus, yeast and moulds

Total viable counts were determined according to the method of Ogunbanwo *et al*., (2003) with modifications. A 10 g meat sample was homogenized in a stomacher blender (400 Circulator, Seward stomacher, England) using 90 ml of sterile diluents (0.1 % Tryptone water) and then serial decimal dilutions were made from $10^1 – 10^9$. Triplicate plates of each dilution were done using the spread plate technique onto dry sterile plates of plate count agar (Hi Media Ltd, Bombay), Violet-red bile glucose agar (VRBGA) (Hi Media Ltd, Bombay), Baird Parker medium (Hi Media Ltd, Bombay) with egg york tellurite (Hi Media Ltd, Bombay) and Potato Dextrose Agar with 10 % tartaric acid for TVC, *E. coli* and *S. aureus* and yeast and moulds respectively. The plates were inverted and incubated at 37ºC for 24 – 48hrs. *E. coli* colonies were identified as those surrounded by a purple zone of growth. *S. aureus* colonies were circular, smooth, convex, moist 2-3 mm in diameter, gray-black to jet black. The plates with colonies were counted and results expressed in log cfu/ml.

Yeast and molds were enumerated by the surface plate method using potato dextrose agar (PDA) (Hi media Ltd, Mumbai) supplemented with 75 ppm chloramphenicol antibiotics using a sterile glass rod (Harrigan 1998). Aliquots of 0.1 ml of sample dilutions were

spread onto the pre-dried agar. All the plates were incubated at 25°C for 5 days. Enumeration was done and represented in a graph of log cfu/g

5.2.6 Sensory analysis

The sensory analysis of the pork sausages in which the combined herb extract was incorporated, was undertaken by a team of fifteen untrained panelists from the Department of Food Science and Technology. Each of the panelists was presented with three deep fried pork sausage samples. One was the control with no preservative, another with sodium metabisulphite as preservative and the last one with the combined herbal preservative. The samples were coded using random numbers and placed on plastic plates and presented to the panelists. Each panelist was asked to evaluate the samples for colour, appearance, flavour, texture, hardness and general acceptability. Mouth rinsing with water was done between testing samples. The assessment was carried out under natural light at a room temperature of 26 ± 2°C. Evaluation was done using a 9-point hedonic scale (Ihekoronye and Ngoddy, 1985). The parameters evaluated were all scored between 9 (like extremely) and 1 (Dislike extremely) using the questionnaire shown in Annex II.

To determine if the samples with the herbs could be differentiated from those without the herb, a triangular test as described by Jellinek (1985) with some modifications was done. Panelists were asked to identify the sample that tasted different, from three samples of which two were the same and one was different. The results were analyzed using SAS© statistical program.

5.3 Results and Discussion

5.3.1 Determination of the most active antioxidant herbal blend ratio

The extracts blend MTI: AZA at a ratio of 1:3 had the highest antioxidant activity. In this case percentage antioxidation was 28.56±56 % which increased to 55.60±1.14 % and then 76.82±1.2 % at a concentration of 1 g in 10 ml, 2 g in 10 ml and 5 g in 10 ml respectively. This is an indication that the most appropriate extract mixture was the one at a ratio of MTI: AZA 1:3 (Table 5.3).

Table 5.3: Percentage antioxidant of AZA and MTI under three different concentrations and blend ratios using DPPH

Concentration	Percentage antioxidation %				
	MTI:AZA (3:1)	MTI:AZA (0:1)	MTI:AZA (1:0)	MTI:AZA (1:3)	MTI:AZA (1:1)
1 g in 100 ml	22.65±1.76[a]	45.95±2.96[a]	23.61±2.38[a]	28.56±2.82[a]	23.69±1.08[a]
2 g in 100 ml	29.77±2.13[b]	79.60±2.72[b]	37.81±2.27[b]	55.60±1.14[b]	26.92±1.84[b]
5 g in 100ml	67.91±1.50[c]	80.00±0.60[c]	67.24±3.26[c]	76.82±1.20[c]	73.66±1.47[c]

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Means followed by same letter(s) are not significantly different at 95% significance level. Mean differences separated by Duncan multiple Range Test (DMRT) (N=3)

When the extract was clarified through filtration with active charcoal the result indicated that the effectiveness of the extract as an antioxidant was greatly reduced, as can be seen in Table 5.4. The MTI: AZA 1:3 ratio extract gave 23.84±0.58, 34.40±1.94 and 46.32±2.61 for 1 g in 100 ml, 2 g in 100 ml and 5 g in 100 ml respectively which is far much lower than in the case where the extract had not been clarified. This is an indication that active charcoal removes colour. It may also be that some of the active phytochemicals are removed (Table 5.4)

Table 5.4: Percentage radical scavenging activity of the fruit extract of AZA and MTI, upon filtration with charcoal

Concentration	Percentage antioxidation				
	MTI:AZA (3:1)	MTI:AZA (0:1)	MTI:AZA (1:0)	MTI:AZA (1:3)	MTI:AZA (1:1)
1 g in 100 ml	22.87±1.54[a]	22.97±1.16[a]	20.16±0.58[a]	23.84±1.31[a]	17.76±2.49[a]
2 g in 100 ml	38.36±1.94[b]	27.10±1.72[a]	24.13±2.60[b]	34.40±1.94[b]	21.31±2.41[a]
5g in 100 ml	38.41±1.31[b]	64.74±2.61[b]	30.23±2.38[b]	46.32±2.61[c]	22.19±1.83[a]

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Means followed by same letter(s) are not significantly different at 95% significance level. Mean differences separated by Duncan multiple Range Test (DMRT) (N=3)

5.3.2 Determination of the most active antimicrobial herbal blend ratio

The herbal extract of ratio MTI: AZA 1:3 when diluted in different ratios gave varying zones of inhibition when *E. coli* was inoculated. The best results were realized, in dilutions of 1 g in 100 ml, 2 g in 100 ml and 5 g in 100 ml had a zone of inhibition of 3.33 mm 6.33 mm and 8.33 mm respectively. This indicated that the most effective mixture ratio was, MTI: AZA, 1:3 blend. It was also noted that the effectiveness of the extract increases with increase in concentration as shown in Table 5.5. In this case it was also noted that the control preservative was significantly more effective when it was compared with the extract blends.

Table 5.5: Zone of Inhibition in (mm) of *E.coli* by extract diluted in different ratios

E.coli	zone of inhibition (mm)		
Conc/ ratios	1g in 100 ml	2 g in 100 ml	5 g in 100 ml
MTI:AZA (3:1)	3.33 ± 0.57^{ab}	6.33 ± 0.57^{a}	8.33 ± 0.57^{b}
MTI:AZA (0:1)	2.33 ± 0.57^{a}	6.33 ± 1.15^{a}	6.33 ± 0.57^{a}
MTI:AZA (1:0)	2.33 ± 0.57^{a}	6.33 ± 0.57^{a}	6 ± 0^{a}
MTI:AZA (1:3)	5 ± 1^{b}	5.66 ± 0.57^{a}	9.6 ± 0.57^{b}
MTI:AZA (1:1)	5.30 ± 0.57^{b}	6.33 ± 0.57^{a}	12 ± 1^{c}
Control	10.66 ± 1^{c}	12.66 ± 0.57^{b}	18.33 ± 1.52^{d}

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Means followed by same letter(s) are not significantly different at 95% significance level. Mean differences separated by Duncan multiple Range Test (DMRT) (N=3)

When *C. albicans* was subjected to the extract all the ratios gave zones of inhibition but the MTI:AZA of 1:3 ratio gave the highest area of inhibition, amounting to 2.66 mm, 7.0 mm and 13 mm for 1g in 10 ml, 2 g in 10 ml and 5 g in 10ml respectively. The control was significantly more effective than the herbs extracts (Table 5.6).

Table 5.6: Zone of Inhibition in (mm) of *Candida albicans* by extract diluted in different ratios

Conc/ ratios	1g in 100 ml	2g in 100ml	5g in 100ml
MTI:AZA (3:1)	2.6 ± 0.57^{a}	4 ± 1^{a}	12 ± 1^{b}
MTI:AZA (0:1)	4 ± 0^{c}	5 ± 1^{a}	10 ± 1.52^{a}
MTI:AZA (1:0)	4.9 ± 1^{c}	7.66 ± 1.15^{a}	10 ± 1^{a}
MTI:AZA (1:3)	2.66 ± 0.57^{ab}	7 ± 0^{b}	13 ± 2.6^{b}
MTI:AZA (1:1)	3.66 ± 0.57^{abc}	4.33 ± 1.52^{a}	16 ± 2.51^{c}
Control	9 ± 1^{d}	11.33 ± 0.57^{c}	20 ± 0^{d}

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Means followed by same letter(s) are not significantly different at 95% significance level. Mean differences separated by Duncan multiple Range Test (DMRT) (N=3)

When *S. aureus* was subjected to the herb extract, at a ratio of MTI: AZA, 1:3 the zone of inhibition gave the following area of inhibition, 3.33±0.57 mm, 6.33±4.5 mm and 12±1.15 for 1 g in 100 ml, 2 g in 100 ml and 5g in 100 ml respectively (Table 5.7).

Table 5.7: Zone of Inhibition in (mm) of *Staphylococcus aureus* by extract diluted in different ratios

Conc/ ratios	1g in 100 ml	2g in 100 ml	5g in 100 ml
MTI:AZA (3:1)	2.33 ± 0.57^{b}	8.33 ± 1.52^{d}	11 ± 1^{b}
MTI:AZA (0:1)	1 ± 0^{a}	4.33 ± 0.57^{b}	10.33 ± 1.52^{b}
MTI:AZA (1:0)	3.3 ± 0.57^{bc}	2 ± 2.51^{a}	6 ± 1^{a}
MTI:AZA (1:3)	3.33 ± 0.57^{bc}	6.33 ± 4.5^{c}	12 ± 1.15^{b}
MTI:AZA (1:1)	4 ± 1^{c}	9.33 ± 1.52^{d}	15 ± 1^{c}
Control	9 ± 1^{d}	15.33 ± 1.52^{e}	20 ± 0^{d}

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Means followed by same letter(s) are not significantly different at 95% significance level. Mean differences separated by Duncan multiple Range Test (DMRT) (N=3)

5.3.3 Minimum inhibitory concentration of sample extracts

The MIC undertaken gave results that suggested that the herb extracts mixed in the ratio of MTI: AZA 1:3 respectively was the most effective for all the three microorganisms. This is because the MIC of *S. aureus* was 25 mg/ml, *E. coli* 3.125 mg/ml and *C. albicans* 12.5mg/ml which is lower than when compared with the extracts mixed in the ratio of 1:1 MTI: AZA, Table 5.8. This means that when the extracts are mixed in the ratio of MTI: AZA 1:3 then the most effective concentration would be 25mg/ml. this concentration would be used to preserve food products subject to other factors such as maintenance of taste of the product in which the extract is administered.

Table 5.8: The MIC of the common spoilage microorganisms

Test microorganism	MTI:AZA ratio	MIC (mg/ml)	Sodium metabisulphite (mg/ml)
S. aureus	1:1	50	0.20
S. aureus	1: 3	25	-
E. coli	1:1	50	0.20
E. coli	1: 3	3.125	-
C. albicans	1: 1	25	0.39
C. albicans	1: 3	12.5	-

Ziziphus abyssinica (AZA), *Tarmarindus indica* (MTI). Minimum Inhibitory Concentration (MIC)

5.3.4 Preservative effect of the herbal mixture on meat product samples

5.3.4.1 Evaluation of rancidity in the pork sausages

TBARS index was used to evaluate the degree of lipid oxidation during storage. The presence of TBARS reactive substances is caused by the second stage of auto-oxidation, in which peroxides are oxidised to aldehydes and ketones. Lipid oxidation was affected by the time (intrinsic factor) and temperature (environmental factor) (Kaczmarski *et al.*, 2015). In this case four samples of 1800 g of pork sausage mass were each treated with either 4.5 g sodium metabisulphafite which was the control, 6 g, 3 g, or 1.575 g of the herbs mixtures respectively and stored at 4°C, the results obtained were as follows. During the first day, the melondialdehyde present in the initial analysis was found to be 0.26 mg MDA /kg, 0.45 mg MDA/Kg, 0.36 mg MDA/Kg and 0.26 mg MDA/Kg respectively. These initial readings related well with those of the initial TBARS content of 1.06 mg MDA/kg, reported by Kaczmarski *et al*., (2015) and 0.22 mg MDA/kg reported by Wenjiao *et al*. (2014) for dried sausage.

In the third day the analysis indicated an increase in melondialdehyde to 0.94 mg MDA /kg the control, 0.67 mg MDA/Kg, 1.43 mg MDA/Kg and 1.43 mg MDA/Kg respectively. On the fifth day there was further increase to 1.04 mg MDA /kg control, 0.93 mg

MDA/Kg, 1.96 mg MDA/Kg and 1.93 mg MDA/Kg respectively. The malonaldehyde levels continue to increase, the sample with 3 g and 1.575 g of herbs mixture attained the 2 mg MDA/Kg mark sooner than the rest indicating that the control and the sample with 6 g herbs mixture were more effective in controlling oxidation (Fig 5.1). This implies that just like the control, the sausage samples with 6 g of herbs mixture in meat was still edible and would still keep further if TBARS would be the only parameter to be considered to measure edibility (Min *et al*., 2008).

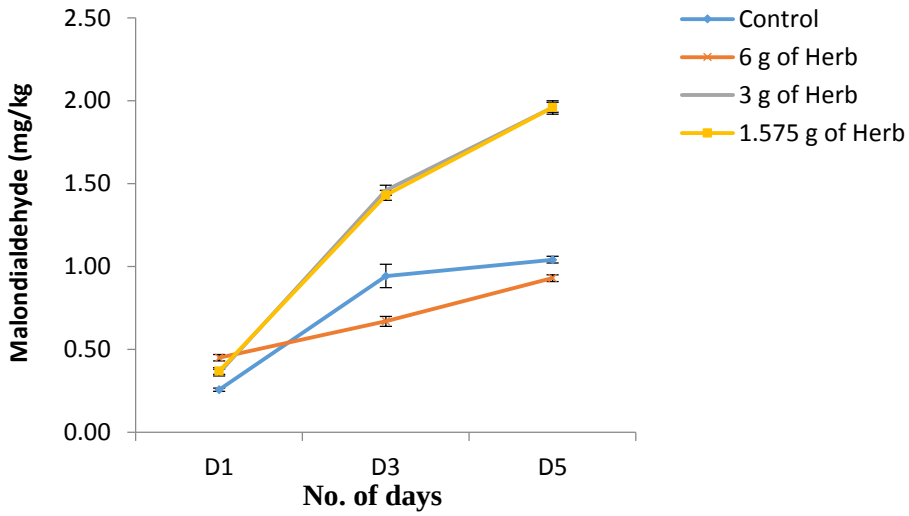


Figure 5.1: Change in TBARS values of pork sausages preserved with herb extract MTI: AZA of 3:1 ratio of different concentration 1.5 g, 3 g and 6 g at 4°C.

5.3.4.2 Determination of total viable count

The TVC of the four samples was determined over 9 days stored at 4°C in order to measure the preservation capacity of the herbs mixture compared to the control (sodium metabisulphite). The preservative would be considered effective if it maintained the colonies below 6 Log CFU/g (Kaczmarski *et al*., 2015). In this case four samples each weighing 1800 g of pork sausage mass were each treated with 4.5 g sodium metabisulphite which was the control, 6 g, 3 g, and 1.575 g of the herbs mixtures respectively and stored at 4°C. The initial colonies present were 4.95 (control), 5.16, 5.54 and 5.23 Log CFU/g

respectively. On the 4th day the increase was slow due to the deterring effect of the preservative and the results were 5.04 for the control, 5.64, 5.25 and5.25 CFU/g respectively. On the 9th day the colonies increased to 6.10 (control), 6.53, 6.37 and 6.27 CFU/g respectively (Fig 5.2). These results indicate that on the 9th day the colonies had slightly crossed the safe limit which 6 CFU/g. considering the fact that in normal practice sausages stored for sale are only kept for a maximum of 5 days at 4°C. The sample with 6 g herbs blend is most effective in inhibiting the growth of the colonies followed by the sample with 1.575 g.

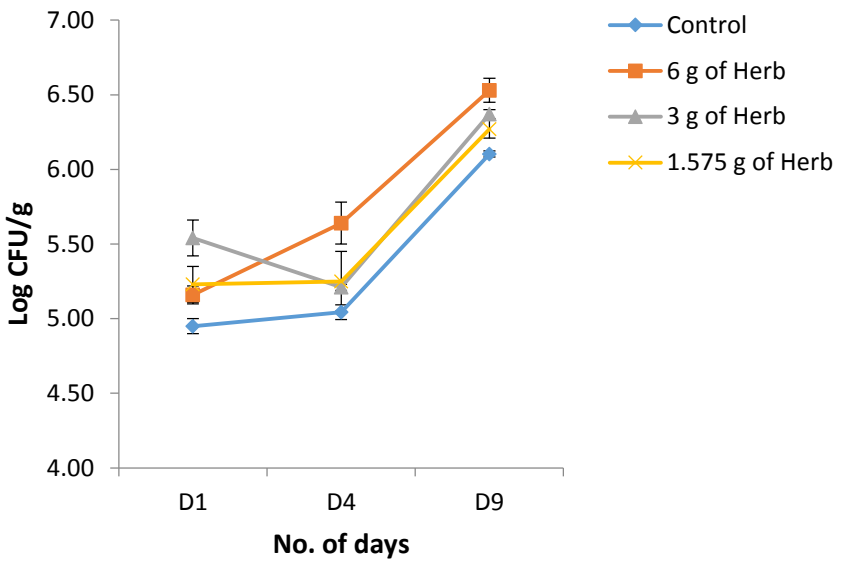


Figure 5.2: The total viable counts in log CFU/g of sausage samples with different amounts of extract and a control taken over a period nine days

5.3.4.3 Changes of *E. coli* in sausage samples preserved for six days

In the analysis on the proliferation of *E. coli* in 1800 g sausage sample mass, the sample inoculated with 4.5 g sodium metabisulphite was the control. The other sausage samples were each inoculated with 1.575 g, 3 g and 6 g herbs blend stored at 4°C and the number of *E. coli* colonies determined on the 1st, 4th and 6th days of storage respectively. Initially

there were 3.64 in the control sample, 3.75, 3.95 and 3.95 CFU/g respectively. On the 4th day the colonies increased to 3.79 (Control), 3.83, 4.1 and 4.1 CFU/g respectively. Consequently on the 6th day the colonies numbered 4.33 (control), 3.92, 4.43 and 4.43 CFU/g respectively. On the 6th day there was a general increase in colonies on all samples apart from the one inoculated with 6 g herbs mixture that showed a decrease. If the samples were kept for 4 days that with 6 g herbs mixture would be the most effective in inhibiting *E. coli* followed with the sample inoculated with the control (Figure 5.3).

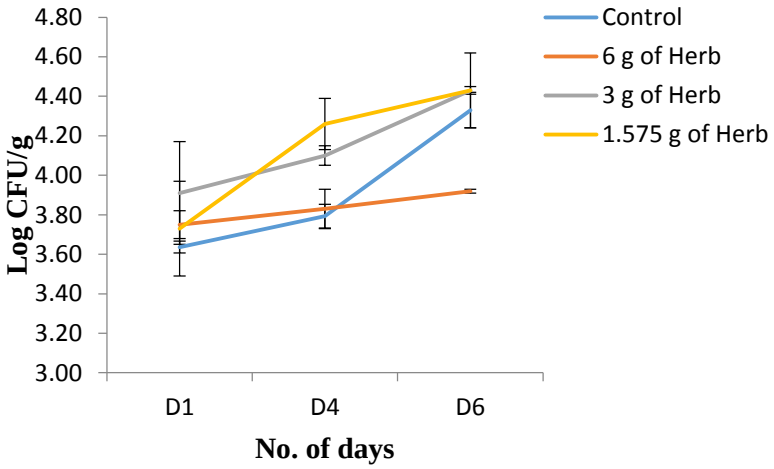


Figure 5.3: Evaluation of *E.coli* in the pork sausage sample

5.3.4.4 Changes of *S. aureus* in sausage samples preserved for six days

S. aureus is a common cause of food poisoning in food products. Once these organisms grow to a certain number, they produce a heat stable enterotoxin which causes vomiting and diarrhea in those who eat the contaminated food. When the four 1800 g samples were inoculated with 4.5 g sodium metabisulphite (control) 6 g, 3 g, 1.575 g herbal mixture preservatives respectively and stored over a period of six days (Figure 5.4) it was noted that; the initial colonies of *S. aureus* in the sausages samples were 4.49 CFU/g (control), 4.63 CFU/g, 4.76 CFU/g and 4.9 CFU/g respectively. This may have been attributed to handlers and the additives added to the sample mass. On the 4th day the analysis yielded the following results, 4.67 CFU/g (control), 4.77 CFU/g, 5.06 CFU/g and 5.18 CFU/g

respectively. On the 6th day of storage of the sausages the results were 4.75 CFU/g (control), 5.06 CFU/g, 5.24 CFU/g and 5.24 CFU/g respectively (Figure 5.4). On the 4th day the sample with 6 g herbs mixture was the most effective in inhibiting the growth of *S. aureus* followed by the sample with the control. When the samples were kept for up to 6 days, the control emerged the most effective in inhibiting the growth of the microorganism followed by the sample with 1.575 g herbs mixture. This comparison was attained when the initial CFU/g was subtracted from the final CFU/g within the same sample.

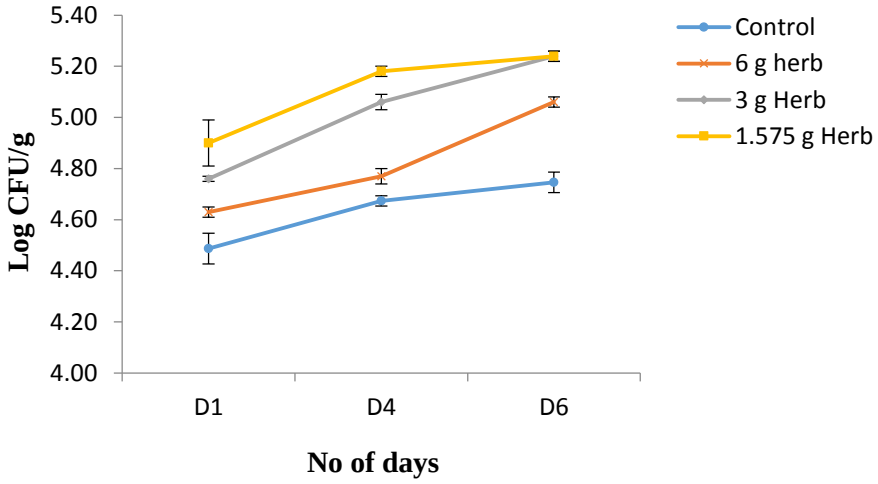


Figure 5.4: Evaluation of *S. aureus* in Pork Sausage samples

5.3.4.5 Changes of C. albicans in sausage samples preserved for six days

When the four 1800 g samples were inoculated with 4.5 g sodium metabisulphite (control) 6 g, 3 g, 1.575 g herbal mixture respectively, and then tested for *C. albicans* the colonies that were in the samples initially were found to be 4.69 CFU/g (control), 4.98 CFU/g, 5.04 CFU/g and 5.11 CFU/g respectively. On the fourth day the colonies found in the samples were 4.83 CFU/g (control), 5.13 CFU/g, 5.22 CFU/g and 5.22 CFU/g respectiively. When each of the samples were analysed individually it was noted that the sample with 1.575 g herbal mix had the highest inhibition effect of *C. albicans* followed by the control, 6 g

herbal mix and lastly the sample with 3 g of the herbal mixture. When the sausages were stored till the 6th day the CFU found were 5.01 CFU/g (control), 5.21 CFU/g, 5.29 CFU/g, and 5.96 CFU/g respectively (Figure 5.5). If the sausages were to be stored for 4 days only, then a concentration of 1.575 g herbal mix would be the most appropriate to control the growth of *C. albicans*, judging from the inhibition effect on *C. albicans.* But since sausages are normally stored for up to 6 days under refrigerator at the grocery stores then a concentration of 6 g in 1800 g of sausage mass would be most appropriate to control the growth of *C. albicans*.

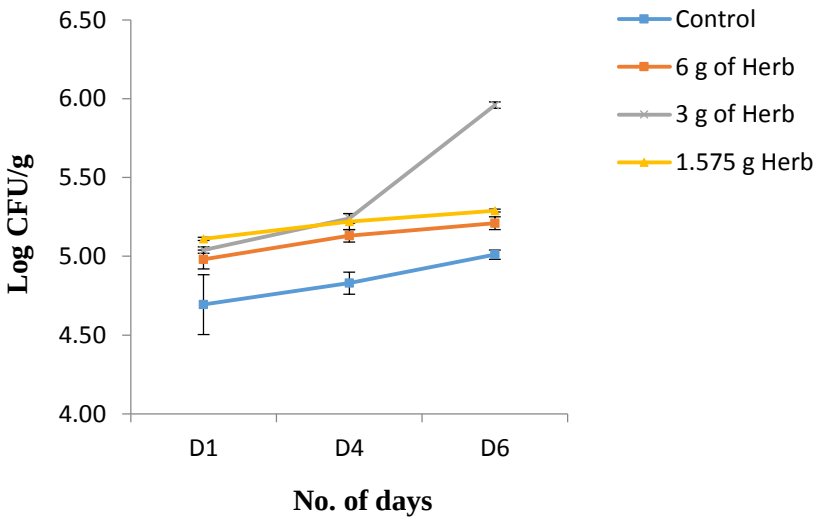


Figure 5.5: Evaluation of *C. albicans* in Pork Sausage samples

5.4 Sensory analysis market data that compares sausages with the herbal preservative and the control

A test was performed to determine the customers rating on sausages with and without herbs on the basis of flavour, texture, hardness of the casing and general acceptability at

different concentrations of the herbs mixture. In the process of analysing data, the initial procedure was testing for the normality of the data using a scatter plot in which it was concluded that the data was not normal. Paired t-test would have been used but owing to the fact that the data was not normally distributed, a non-parametric Wilcoxon Signed-Rank test was used.

5.4.1 Flavour

The sum of the positive ranks were 2 points larger than the sum of the negative ranks when the flavor of the sausages with 1.575 g of herbs mixture were compared with the control that had sodium metabisulfite. This is an indication that flavour rating at 1.575 g concentration of herb was better than for the control variable. The sausages with 3 g of the herbs mixture, had the sum of positive ranks being larger by 6 points compared to the sum of negative ranks. This implies that flavour of the sample with 3 g herbal mixture rated better than flavour for the control sausages. The sausages with 6 g of herbal mixture had the sum of positive ranks being lower than the sum of the negative ranks by 10 points when compared with the flavour of the control sausage. This indicated that flavour rating of the sausage with 6 g herbal mixture was not better than for the control variable. Therefore, the Wilcoxon signed Ranks test indicated that sausages with 3 g herbal extract (mean Rank = 2.67) were rated most favorable of the three other samples (Table 5.10).

Table 5.9: Flavor ranking of the sausages at different concentrations all compared with the control

g/kg of meat	Nature of Ranking	No. of respondents	Mean Rank	Sum of Ranks
1.575	Negative Ranks	1	4.00	4.00
	Positive Ranks	3	2.00	6.00
	Ties	5		
	Total	9		
3	Negative Ranks	1	2.00	2.00
	Positive Ranks	3	2.67	8.00
	Ties	5		
	Total	9		
6	Negative Ranks	4	3.13	12.50
	Positive Ranks	1	2.50	2.50
	Ties	4		
	Total	9		

5.4.2 Texture

In the analysis of texture it was noted that the sausages preserved with 1.575 g herbs mixture extract had the sum of the positive ranks being more than the sum of the negative ranks by 4 points when compared to the control. This indicated that the textural rating was better than for the control variable. The sausage with 3 g herbs mixture had a sum of positive ranks being larger by 10 points compared to the sum of negative ranks. This implied that the texture of sausages with 3 g herbs extract rated better than the control. The sausages that had 6 g herbs mixture had the sum of the positive ranks being larger than the sum of the negative ranks by 9 points when compared with the control. This is an indication that the texture rating at 6 g herbs mixture was better than the control variable. Therefore, the Wilcoxon signed Ranks test gave an indicator that the sausages with 3 g

herbs mixture whose mean rating was 0 was therefore considered more favorable (mean Rank = 2.50) than the control and the other samples (Table 5.10)

Table 5.10: Texture raking of the sausages at different concentrations compared to the control

g/kg of meat		No. of respondents	Mean Rank	Sum of Ranks
1.575	Negative Ranks	3	4.00	12.00
	Positive Ranks	4	4.00	16.00
	Ties	2		
	Total	9		
3	Negative Ranks	0	.00	.00
	Positive Ranks	4	2.50	10.00
	Ties	5		
	Total	9		
6	Negative Ranks	1	3.00	3.00
	Positive Ranks	4	3.00	12.00
	Ties	4		
	Total	9		

5.4.3 Hardness of Casing

When the hardness of casing Ranks were investigated the sausages with 1.575 g herbal extract produced a sum of the negative ranks being larger than the sum of positive ranks by 3 points when compared with that of the control. This was an indication that hardness of casing for the control variable was better than for the sausages with 1.575 g herbal extract. The sausages with 3 g herbal extract had the sum of negative ranks being larger by 9 points compared to the sum of positive ranks. The implication was that hardness of casing for the sausages without the herb was better than that for sausage with 3 g herbal extract. The sausages with 6 g herbal extract had a sum of the positive ranks being smaller than the sum of the negative ranks by 2 points as compared to the control. This was an

indication that hardness of casing of the control was better than that of the sample. A Wilcoxon Signed-Ranks test indicated that sausages with sodium metabisulphite were rated more favorably than those with the herbs extract in terms of the hardness of the casing (Table 5.11)

Table 5.11: Hardness of the casing rakings of sausages at different concentrations compared to the control

g/kg of meat		No. of respondents	Mean Rank	Sum of Ranks
1.5	Negative Ranks	3	2.17	6.50
	Positive Ranks	1	3.50	3.50
	Ties	5		
	Total	9		
3	Negative Ranks	4	3.75	15.00
	Positive Ranks	2	3.00	6.00
	Ties	3		
	Total	9		
6	Negative Ranks	3	2.83	8.50
	Positive Ranks	2	3.25	6.50
	Ties	4		
	Total	9		

5.4.4 General Acceptability

For general acceptability, the sum of the negative ranks and the sum of the positive ranks were equal for a sausage preserved with 1.575 g herbal extract and the control. This is an indication that at this point both the sausages preserved with the herbal extract and the control are equally acceptable at the market. The sausages with 3 g herbal extract had the sum of positive ranks being larger by 3 points compared to the sum of negative ranks. This implied that in general, sausages with 3 g herbal extract were rated better than the control. The sausages with 6 g herbs extract had the sum of the positive ranks being

smaller than the sum of the negative ranks by 7 points when comparing general acceptability of the control. A Wilcoxon Signed-Ranks test indicated that sausages with 3 g herbal extract (mean Rank=1.50) were generally rated more favorable than the control (mean Rank=0.00).

Table 5.12: General acceptability rakings of sausages with different amounts of herbal extract

g/kg of herb	Ranks in relation to the control	No. of respondents	Mean Rank	Sum of Ranks
1.5	Negative Ranks	2	2.50	5.00
	Positive Ranks	2	2.50	5.00
	Ties	5		
	Total	9		
3	Negative Ranks	0	.00	.00
	Positive Ranks	2	1.50	3.00
	Ties	7		
	Total	9		
6	Negative Ranks	4	3.50	14.00
	Positive Ranks	2	3.50	7.00
	Ties	3		
	Total	9		

A Wilcoxon Signed-Ranks test (mean Rank=1.50)(mean Rank of the control=0.00)

5.5 Conclusion

It was evident that the best all round activity of MTI: AZA ratio was 1:3. This was collaborated when the extract was incorporated into the Pork Sausages in various concentrations of 1.575 g, 3 g, 6 g and 4.5 g (control). The sausages were tested for rancidity, TVC, *E. coli*, *S. aureus*, and *C. albicans*. Results indicated that if 6 g was used in the 1800 g of sausage mass, sausages would be preserved at 4°C for more than five days, which is about the same time that Sodium metabisulphite takes to preserve the same product. At this concentration the sausages maintained their flavor, Texture, hardness of casing and general acceptability.

CHAPTER SIX

DEVELOPED PRESERVATIVE PRODUCT FROM THE HERBAL BLEND

6.1 Product Development

The results from the sensory evaluation defines the concentration of the product to be developed. It shall take the following methods to prepare the final product.

6.2 Steps in making the preservative product

1. MTI and AZA was mixed in the predefined ratio.
2. To 10 g of the mixture, 20 g of starch will be added to obtain a solid product
3. The product will be dried at 65°C for 5 hours
4. The dried material in the form of cakes was allowed to cool before breaking into small pieces and milling using a hammer mill.
5. It was then passed through a 1 mm aperture size laboratory test sieve (Endecotts Ltd., London England) to obtain a fine powder (Taylor, 1999)
6. The product was then packaged in a translucent battle for presentation.

6.3 Packaging

From the above findings the product formed is a brown fine powder that is soluble in water. As shown in the plates this powder is soluble in water completely.

Plate 5.1: Photos of the semi refined herbal product processed from the herbs AZA and MTI used to preserve Meat products.

CHAPTER SEVEN

OVERALL CONCLUSION AND RECOMMENDATIONS

7.1 Overall Conclusion

The methods best suited for obtaining extracts from the two herbs MTI and AZA was clearly seen following various test carried out. In the case of AZA the soxhlet and cold methanolic extracts had a very close relationship when targeting flavonoids. When targeting antioxidant and antimicrobial capacities, then cold methanolic extract was the most efficient method.

The findings also show that the activity of herbs result from a combination of many compounds. In our case various compounds may be contributing to the overall antioxidant and antimictobial effects on the food sample. Some of the compounds identified to be responsible for antioxidant effects were Benzenamine, 3,4-dimethyl-, Phenol, 2,5-bis(1,1-dimethylethyl)-, Butylated hydroxytoluene, Methyl decanoate, Hexadecanoic acid / Palmitic acid and Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-ethyl- while the compounds identified to be responsible for antimicrobial effects are 4-Methyl-2-hexanol, Sulfurous acid, cyclohexylmethyl undecyl ester, Decanoic acid (capric acid) and Hydrazinecarboxamide, N,N-diphenyl.

The study also shows that whenever herbs are blended the compounds involved where not antagonistic but add up to the pool of compounds this can be seen when TLC was undertaken on MTI and AZA the various compounds seen individually were all seen when the extracts were mixed. Therefore, the herbs complement each other in both activity and flavor of the food in which it is incorporated.

It can be seen from this study that the herbs MTI and AZA are best suited to make a preservative as compared to the other synthetic preservatives. The preservative can be used to preserve sausages and other foods for about a week under refrigeration, using

about 6 g in 100 ml of water. Increasing its concentration would improve the results though that would be wasteful but not harmful. The processed product is a powder that is soluble in water.

7.2 Recommendations

From this study it is recommended that;

i. Facilitation be availed to identify the general properties of the product, patent the product, and commercialize the product for full exploitation

ii. Appropriate processing steps during extraction and inoculation should be incorporated to avoid wastage of the herbs and attain maximum production.

iii. The product should be used to preserve all meat products.

7.3 Suggestion for Further Research

i. Methods to further improve extraction and preservation of the product should be evaluated

ii. Further trials should be performed on the product to determine its activity on other food products such as Juices and canned products.

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APPENDICES

Appendix I: Questionnaire on use of indigenous plants in preservation of meat

Respondent No. ----------

Location ----------

Interviewer's name ----------

Duration of interview ----------

DEMOGRAPHIC DATA

1. Name----------Male---------- Female----------

2. Age 15---------- 15-20yrs---------- 20-30----------

   30-40yrs-------40-50yrs----------->50yrs----------

3. Head of household----------Marital status----------

4. Number of people in the household----------

5. Occupation of members of the household ----------

6. Level of education: Primary----------Secondary----------

   Tertiary----------Others----------

7. Farm size----------

USE OF HERBS IN FOOD PROCESSING AND PRESERVATION

1. Do you use herbs Oron and Angau in preservation of livestock products?

   □ Yes

   □ No

2. From where do you get the herbs you use?

   ……

3. What method do you use in the collection and processing of the herbs?

   □ Drying
   □ Smoking
   □ Sprinkling with ashes
   □ Using herbs charcoal
   □ Any other comments

4. During which season of the year do you collect the plant and what time of the day do you collect them?

   ……

5. If they are stored how are they preserved (include temperature, method of storage and duration of storage)?

……

6. Once preserved do the foods require any special treatments?

……

7. For how long can the food be preserved using the preservative named above?

□ 1 week
□ 2 weeks
□ 1 month
□ 3 months
□ 6 months
□ Any other

8. Before consuming the meat products are there any post treatments required?

□ Yes
□ No

If your answer to the above is yes, explain?

……

……

9. Does the preservation effect imparted by your treatment affect the quality of the food in any way?

□ Yes
□ No

If your answer to the above is yes, explain?

……

……

10. Any suggestions for improving the preservative affects described in the questionnaire?

……

Appendix II: Sensory evaluation of pock sausages treated with different concentrations of natural preservatives and a control.

Please bite and taste samples of fried pock sausages. Make a judgment of each sample by checking and ticking the appropriate phrase under each of the quality parameters provided. The samples are randomly coded to avoid biased judgment. Rinse between samples.

Name……. Code No.……

1	Extremely bad	6	Slightly good
2	Very bad	7	Moderately good
3	Moderately bad	8	Very good
4	Slightly bad	9	Extremely good
5	Neither good nor bad		

Flavour	
Texture	
Hardness of casing	
General acceptability	

Appendix III: Sensory evaluation of pock sausages treated with different concentrations of natural preservatives and a control

Please bite and taste samples of fried pock sausages. Make a judgment of each sample by checking and ticking the appropriate phrase under each of the quality parameters provided. The samples are randomly coded to avoid biased judgment. Rinse your mouth between samples.

Panelist number ……

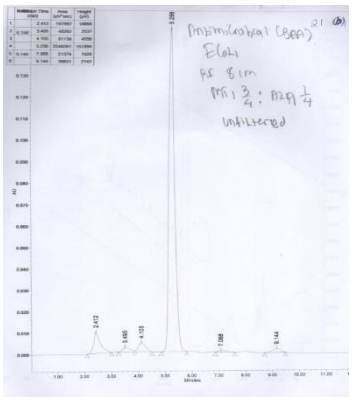
1. Five point deference scale for plate 1, 2 and 3

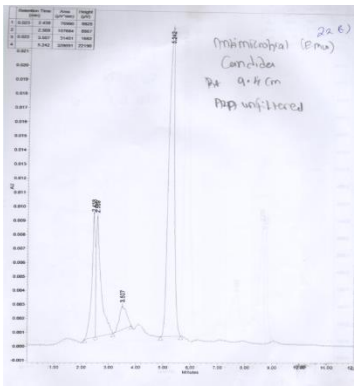
5	**4**	**3**	**2**	**1**
Extremely different	Very different	Moderately different	Slightly different	Neither different nor similar (almost similar)

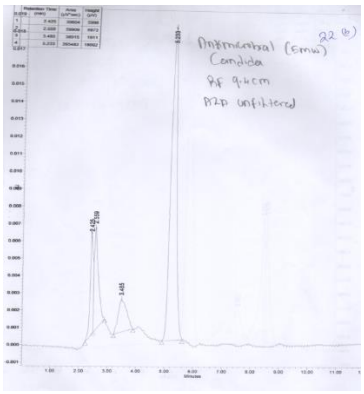
Plate No						
	S	D	S	D	S	D
Indicate which of the 3 samples are similar and which one is different?						
How different is the different one? Rate it using the 5 point deference scale						

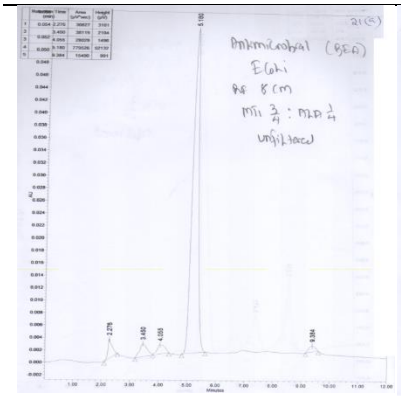
S – Similar, D - Different

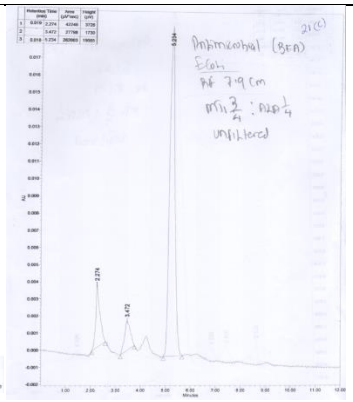
Appendix IV: Images generated by the HPLC machine indicating the main picks of the main compounds in the extracts.


Antimicrobial (EtOH)
Eluent
Rf 8 cm
MPW $\frac{3}{4}$: MPD $\frac{1}{4}$
Unfiltered


Antimicrobial (EtOH)
Candida
Rf 9.4 cm
MPD unfiltered


Antimicrobial (EtOH)
Candida
Rf 9.4 cm
MPD Unfiltered


Antimicrobial (BEA)
Eluent
Rf 8 cm
MPW $\frac{3}{4}$: MPD $\frac{1}{4}$
Unfiltered


Antimicrobial (BEA)
Eluent
Rf 7.9 cm
MPW $\frac{3}{4}$: MPD $\frac{1}{4}$
Unfiltered