

**PHARMACOLOGICAL STUDIES OF *FOENICULUM VULGARE* (MILL.) AND
LIPPIA JAVANICA (BURM. F.) SPRENG. USED AS SPICES IN NKONKOBE
MUNICIPALITY OF THE EASTERN CAPE PROVINCE, SOUTH AFRICA**

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**A thesis submitted to the Department of Botany, Faculty of Science and Agriculture,
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DOCTOR OF PHILOSOPHY (Ph.D.) IN BOTANY**

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DECLARATION

I, Abiola Mojisola Ayodele-Asowata, declare that this thesis is my own work and that all sources of materials used for this thesis have been duly acknowledged.

I declare that this thesis, submitted to the University of Fort Hare for the degree of Doctor of Philosophy (Ph.D.) in Botany in the Faculty of Science and Agriculture, is my original work; and that this work has not been submitted at any other University for the award of any academic degree, diploma, or certificate. I also declare that I am fully aware of the University of Fort Hare policy on plagiarism and have taken every precaution to comply with regulations of the University.

I am fully aware of the University of Fort Hare policy on research ethics and was cleared to conduct my research.

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Signature: -----

INTELLECTUAL PROPERTY AGREEMENT STATEMENT

The ethnobotanical surveys conducted for this thesis were carried out with the full consent of all participants, with further verbal agreement and understanding that this research shall not be used for commercial purposes, but shall serve as enlightenment on the efficacy and safety of *Foeniculum vulgare* and *Lippia javanica* commonly used as spices in Nkonkobe Municipality of the Eastern Cape Province of South Africa.

ETHICAL APPROVAL FOR THE STUDY

The portion of this study involving the Ethnobotanical survey of culinary herbs and spices commonly used in Nkonkobe Municipality of the Eastern Cape Province, South Africa was carried out following the approval of the University of Fort Hare's Ethics Committee (see appendix).

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DEDICATION

I dedicate this project to the Most High God and to my lovely husband; Iyobosa Timothy Asowata, also to our son Justin Asowata.

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GENERAL ABSTRACT

Spices are of great importance in the indigenous culinary and traditional medicine systems of the people of Nkonkobe Municipality of the Eastern Cape, South Africa. The present investigation evaluated the ethnopharmacological potentials of two indigenous South African spices- *Foeniculum vulgare* and *Lippia javanica*. The pharmacological investigations on these two plant species include ultra-morphology, nutrient and mineral analysis, evaluation of the essential oil, phytochemical and antioxidant assays, antimicrobial, anti-inflammatory as well as anti-urolithiatic assay of the acetone and aqueous extracts of the two plants.

The species were selected for study as the most cited plants after an ethnobotanical survey conducted on the indigenous knowledge of plants used as spices and medicine in Nkonkobe Municipality, Eastern Cape of South Africa. Among the plants cited *Foeniculum vulgare* (Apiaceae) and *Lippia javanica* (Verbenaceae) stood out as the most commonly used spices. Others were members of the families; Solanaceae, Apiaceae, Amaryllidaceae, Amaranthaceae and Lamiaceae.

Ultra-morphological studies conducted on the leaves of the two selected plants using scanning electron microscope revealed the presence of non-glandular and glandular trichomes, stomata and crystals. The leaf surfaces of these herbs may serve as secretory sites where aromatic secondary metabolites are produced.

Analyses of the proximate, mineral, vitamin and anti-nutrients contents of these two spices showed that both species are good sources of these phytochemicals and may be used to enrich the human diet. *Lippia javanica* possesses lower lipid (0.50%), fibre (5%) and carbohydrate (64.96%) contents than *Foeniculum vulgare*. On the other hand, protein (20.54%), ash (11.60%) and moisture content (11.69%) were higher in *Foeniculum vulgare* than in *Lippia javanica*. *Foeniculum vulgare* showed higher N (3286 mg/100g), Mg (386.7 mg/100g), K

(3187 mg/100g) and Na (1383 mg/100g) content while *Lippia javanica* was higher in Ca (1833 mg/100g), Zn (4.7 mg/100g), Cu (0.9 mg/100g) and Fe (78.4 mg/100g). Vitamins A and E were also higher in *Lippia javanica* (1.31 mg/100g; 2.52 mg/100g) while *Foeniculum vulgare* (0.45 mg/100g) had higher vitamin C content. No significant differences were observed in the phytate, oxalate and tannin contents of the two spices, but saponin and cyanide were significantly lower in *Lippia javanica* (268.5 mg/100g; 8.45 mg/100g) than in *Foeniculum vulgare* (1855 mg/100g; 10.5 mg/100g)

Evaluation of the essential oil component, cytotoxicity and antimicrobial activities of both fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare* revealed that the dried samples yielded more oil and also contain more chemical than the fresh samples of both plants. The overall antimicrobial activity evaluated using susceptibility and microdilution assays revealed that the oils of *F. vulgare* and *L. javanica* exhibited high antifungal and antibacterial activity, compared to the reference drugs. In addition, essential oil from fresh leaves of both spices was less toxic compared to the oil from the dried leaves.

Polyphenolic evaluation of the aqueous and acetone extracts of the plants revealed that the acetone extract had higher phenol, flavanol, flavonoid and proanthocyanidin contents than the aqueous extracts. The total phenolic content of acetone and aqueous extracts were 4.49 ± 0.411 mg/g and 3.73 ± 0.498 mg/g tannic acid equivalent (TAE) respectively for *Lippia javanica*. The same trend was also observed in *Foeniculum vulgare* with total phenolic content of acetone and aqueous extracts were 4.22 ± 0.325 mg/g and 4.17 ± 0.651 mg/g tannic acid equivalent (TAE) respectively. Further assessment of the antioxidant activity of the solvent extracts revealed that both plants exhibited promising free radical scavenging potentials against 1,1 diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), reducing power, lipid peroxidation, nitric oxide, phosphomolybdate and hydrogen peroxide.

Antimicrobial activities of the acetone and aqueous extracts of the two plants revealed moderate antibacterial and antifungal activities. They inhibited the growth of *Microsporium canis* and *Trichophyton rubrum* that were not susceptible to the standard antifungal drug used as control. The assay revealed that both acetone and aqueous extracts of both plants had the ability to inhibit protein denaturation and maintain membrane stability.

The *in-vitro* anti-urolithiatic assay revealed that *Lippia javanica* and *Foeniculum vulgare* exhibited moderate inhibitory effects. These findings support the folkloric uses of *Foeniculum vulgare* as a good source of anti-urolithiatic drug. This is the first documentation of the *in-vitro* evaluation of anti-urolithiatic potentials of *Foeniculum vulgare* and *Lippia javanica*

This study has revealed that the two species possess high antioxidant, antimicrobial, anti-inflammatory and anti-urolithiatic properties. These could account for the high importance placed on these two plants as spices among the people of Nkonkobe Municipality of the Eastern Cape, South Africa.

CHAPTER ONE

GENERAL INTRODUCTION

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The use of plants as spices

Spices are a group of esoteric food adjuncts that have been in use for thousands of years to enhance the sensory quality of foods (Srinivasan, 2005). The use of plants as spices is rooted from ancient times. Currently, there is a growing consciousness of the importance of spices in diet and health. The role of spices in this current awareness is not only in preparing good tasty meals, but equally the increasing use of spices in all areas of the food industry, pharmacy and medicine (Seidemann, 2005).

Culinary herbs and spices are the edible parts of plants that are traditionally added to foodstuffs for their natural flavours, aroma, preservative or visual properties (European Spice Association, 2014). Spices may be derived from the entire parts of the plant (bark, buds, flowers, fruits, leaves, rhizomes, roots, seeds, stigmas and styles). The term 'herb' is used as a subset of spice and refers to plants with aromatic leaves (European Spice Association, 2014). Spices are often dried and used in a processed, but complete state. Another option is to prepare extracts such as essential oils by distilling the raw spice material (wet or dry), or to use solvents to extract oleoresins and other standardized products.

As natural food preservatives are becoming more popular in the food industry compared to the presently used synthetic preservatives, the combination of flavouring and anti-microbial properties often with antioxidant properties is of great benefit in the health and food industries. The rising interest in the medicinal potential of spices in the last few years is as a result of various factors. These factors include fear over the toxicity, safety of synthetic drugs, as well as easy access and low cost (Weiss, 2002; Kaur and Arora, 2009).

According to Weiss (1997), spices apart from their contribution to taste and flavour, can also act against pathogens such as *Listeria monocytogenes*. So far, many pathogenic microorganisms, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* have been reported as the causal agents of foodborne diseases

and/or food spoilage, also food safety concerns have been focused on pathogens, such as *Listeria* which is recognized as one of the leading causes of foodborne bacterial diseases. The problem of human listeriosis following consumption of contaminated foods has increased worldwide (Kaoutar et al., 2010). Spices can further be used to enhance storage stability by means of active components, including phenols, alcohols, aldehydes, ketones, ethers and hydrocarbons, especially in spices such as cinnamon, clove, garlic, mustard and onion. The first scientific studies of the preservation potential of spices, describing antimicrobial activity of cinnamon oil against spores of *anthrax bacilli* were reported in the 1880s (Burt, 2004). Moreover, clove was used as a preservative to disguise spoilage in meat, syrups, sauces and sweetmeats. In the 1910s, cinnamon and mustard were shown to be effective in preserving apple sauce. Since then, other spices such as allspice, bay leaf, caraway, coriander, cumin, oregano, rosemary, sage and thyme have been reported to have significant bacteriostatic properties (Gutierrez et al., 2008a; Kwon and Lee, 2008).

Spices are also of considerable economic importance, the affluence generated by the spice trade was responsible for several historic voyages and discoveries of new lands (Srinivasan, 2005). Essential oils from spices were used by the early Egyptians and have been in use for centuries in Asian countries such as China and India. Some of the spices, such as clove, cinnamon, mustard, garlic, ginger and mint are still applied as alternative health remedies in India (Weiss, 2002). Essential oil production can be traced back to over 2000 years in the far East, with the beginnings of more modern technologies occurring in Arabia in the 9th century. However, it was also during this period that the medical applications of essential oils became secondary to their use for flavour and aroma (Weiss, 1997).

The most important use of spices is in food to impart flavour, this has not been the case in the olden days when there were no freezers and when livestock were slaughtered for meat once in a year, in the autumn, people had to preserve food with available spices. Spices and

essential oils are used by the food industry as natural agents for extending the shelf life of foods (ESA, 2013). A variety of plant and spice based antimicrobials is used for reducing or eliminating pathogenic bacteria and increasing the overall quality of food products (Angioni et al., 2004). Essential oils in plants generally are mixtures of several components. Some plants like oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, sage and vanilla are used as natural antimicrobial compounds (Holley & Patel, 2005; Gutierrez et al., 2008a), while some other plants such as ginger, black pepper, red pepper, chilli powder, cumin and curry powder, have lower antimicrobial properties (Holley & Patel, 2005). Due to the abundant sources of polyphenolic compounds in spices they have been used as strong antioxidants and could potentially replace the synthetic antioxidants in food systems and offer additional benefits. Consumption of spices has been implicated in the prevention of cardiovascular diseases, carcinogenesis, inflammation, atherosclerosis (Srinivasan, 2005.). There is a need for natural antioxidants at a time when the safety and acceptability of synthetic fractions currently in use are being re-visited. For instance, the currently used synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been reported to be potential carcinogens (Svoboda et al., 1992).

Research Problem

The prevalence of diseases as obesity, diabetes, cardiac arrest, cancers and hypertension constitutes a global public health burden (Cos et al., 2006). Despite the development of new drugs to manage the diseases, it is predicted, for instance, that diabetes mellitus will hit 300 million by 2025. In 2011, 6.5% of the population in South Africa had the diabetes mellitus and it was postulated to rise to 7.2% by 2030 (IDF, 2013). The prevalence of diabetes mellitus has impaired the economy and quality of life in South Africa. Also the overall prevalence of overweight (body mass index >25) and obesity (body mass index >30) in South Africa is high, with more than 29% of men and 56% of women being classified as overweight

or obese. This is higher than that reported in other African countries (Puoane et al., 2002). The prevalence in diabetes mellitus and obesity among South Africans may be due to the food consumed and the uses of food additives.

The food additives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have side effects such as hypoglycaemia, weight gain, edema, abnormal liver function and carcinogenic activities. They are also very expensive and as a result they are inaccessible to the patients living in the rural areas. Owing to this fact, individuals have resorted to the use of medicinal plants with fewer side effects (Kaur & Arora, 2006). Some of these plant remedies have been scientifically authenticated to be used as spices in foods but a large number of them remain unexplored. Further studies on pharmacological, mechanisms of actions and toxicity properties of these spices are necessary; to justify their folkloric usage (Svoboda et al., 1992).

Rationale and justification for choice of plants

In order to authenticate the ethnopharmacological properties of spices used in traditional medicinal system and for culinary purposes among the people of Nkonkobe Municipality in Eastern Cape Province, two indigenous South African spices - *Lippia javanica* and *Foeniculum vulgare* were selected based on an ethnobotanical survey on the medicinal plants commonly used as spices in Nkonkobe Municipality, Eastern Cape of South Africa. Selection of these two spices was also based on the availability of the two plants in Nkonkobe Municipality. Selected pharmacological and chemical composition analysis was done to validate their uses and safety in the folkloric medicine.

Choice of solvent for extraction

In this study, the essential oil, acetone and water (aqueous) extracts were used. The essential oil and water extraction was selected on the basis that traditional practitioners prepare herbal medicine (s) from *Foeniculum vulgare* and *Lippia javanica* as an infusion and decoction. Acetone was also selected as an extractant because it dissolves both hydrophilic and lipophilic components from plants (Eloff, 1998). Acetone is also volatile and has low toxicity for use in microbial bioassays (Eloff, 1998).

Aim and objectives

The overall aim of this research was to collect and document ethnobotanical information on spices that are commonly used in Nkonkobe Municipality, authenticate and validate their ethnomedicinal/pharmacological values and investigate their likely toxicity. Specific objectives are:

- To carry out ultra-morphological assay and elemental composition of *Lippia javanica* and *Foeniculum vulgare*.
- This study aimed at evaluating the proximate and mineral contents of the spices.
- To evaluate the phytochemical constituents of *Lippia javanica* and *Foeniculum vulgare* using current methods.
- To evaluate the free radical scavenging properties of various extracts of the spices using antioxidant assays.
- To evaluate the antimicrobial potentials of *Lippia javanica* and *Foeniculum vulgare*.
- Another objective of this study was to investigate their possible toxicity.

- To evaluate the compounds and bioactivities present in the essential oil of these plants.
- To test the anti-urolithiatic properties of *Foeniculum vulgare* and *Lippia javanica* using an *in-vitro* model.

The structure of this thesis

This thesis is composed of chapters that have been published, accepted or under review in various peer-reviewed and accredited journals. The general introduction is in Chapter 1. Chapter 2 presents the literature review on spices. Chapter 3 is composed of Ethnobotanical survey of culinary herbs and spices commonly used in Nkonkobe Municipality of the Eastern Cape Province, South Africa. The micromorphology of the plant's parts and the probable storage sites of these bioactive compounds are reported in Chapter 4. Chapter 5 is composed of the report of the nutritive value, elemental composition, phytochemical constituents and compounds present in these plants. Chapter 6 presents the results of chemical composition, antimicrobial activity and brine shrimp cytotoxicity of the essential oils of *Lippia javanica* and *Foeniculum vulgare*. Chapter 7 accounts for the polyphenolic content, *in-vitro* antioxidant, antimicrobial and anti-inflammatory activities of aqueous and acetone extracts of these two plants. The evaluation of the *in-vitro* anti-urolithiatic properties of *Lippia javanica* and *Foeniculum vulgare* are reported in Chapter 8. The general discussion, conclusions and recommendations emanating from the entire study are presented in Chapter 9.

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CHAPTER TWO

LITERATURE REVIEW

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Spices and their medicinal uses

Spices are aromatic or pungent vegetable substances used to flavour food such as cloves, pepper, or cumin. Also herbs and spices are traditionally defined as any part of a plant that is used in the diet for their aromatic properties with no or low nutritional value (Hacskeylo 1996; Smith and Winder 1996). Spices and culinary herbs are as important today as they were in ancient times for enhancing the flavour and taste of our food, as well as serving as a source of dietary medicine. They can turn an ordinary meal into a savoury delight, without the addition of calories, fat, salt, or artificial flavours, while providing a rich source of antioxidants and phytonutrients that can improve health and reduce the burden of disease (Davidson, 2010).

The leaf or herbaceous part of a plant, fresh or dried, used for flavouring in food preparation is often referred to as a culinary herb, whereas any other part of a plant, often dried is called a spice (Tapsell, 2006). Examples of spices are buds (cloves), bark (cinnamon/cassia), roots (ginger), berries (peppercorn) and aromatic seeds (cumin). Cookbooks generally distinguish between seasonings (spices used in food preparation) and condiments (spices added after food is served), but not between herbs and spices. In addition to pure spices, other food flavourings are mixed spice blends (henceforth called spices) and condiments (for example mustard paste). However, herbs, which are defined botanically (as plants that do not develop woody, persistent tissue), usually are use in their fresh state, whereas spices generally are dried.

Indeed, spices come from various woody shrubs and vines, trees, aromatic lichens, and the roots, flowers, seeds and fruits of herbaceous plants (Carlsen et al, 2011) but each spice has a unique aroma and flavour which is derived from compounds known as phytochemicals or secondary compounds (because they are secondary to the plant's basic metabolism). These

chemicals evolved in plants to protect them against herbivorous insects and vertebrates, fungi, pathogens and parasites (Walker, 1994; Carlsen et al., 2010).

History of spices

The first authentic records of spices, though fragmentary, belong to the pyramid age of Egypt approximately 2600 to 2100 B.C. There are plenty of historical evidences asserting the significance of South India as a source of high quality spices even from the periods of Babylonian and Assyrian civilizations (Philip, 2010). Until the beginning of the Christian era the source of spices was a mystery to the western world. Oriental spices were popular as priceless assets during the periods of Egyptian civilization. The ancient Egyptians used oils prepared from spices to preserve the dead bodies in 'mummies'. Ebers Papyrus written in B.C 1500 talks about the medicinal values of pepper and cinnamon. Parry (1969) writes that Cinnamon formed part of the aromatics cosmetics used by the Egyptian queen Hatshepsuth. There are evidences proving that Hatshepsuth sent five ships to the east for procuring spices. In the second and third millennium B.C., Arabian traders had the monopoly of carrying goods between east and west among which spices and other aromatic resins were the most important.

Arthasasthra written in the third century B.C. has plenty of remarks about spices including pepper, cardamom, ginger, fenugreek, coriander and mustard. Greek medical science also records the importance of medicinal values of spices. Hippocrates (460 - 377 B.C.), known as the father of modern medicine, the Greek philosopher and scientist Theophrastus (372- 287 B.C), Dioscorides, known as the father of botany (AD 40 - 90) all had mentioned spices in their writings. These all clearly indicate that spices have been an inevitable part of human history (Smith and Winder, 1996). Romans were very lavish in the use of spices, which they used not only for cooking but also as cosmetics. It was customary for them to use cosmetics heavily for which they used spices extensively. Spice flavoured wines were very popular

while scents, balms, and oils made from spices were used as after bath. Spices were expensive during the middle ages and were in great demand among those who could afford them. History says that peppercorns were used as currency in ancient times to pay taxes, tolls, rents and even dowries (Philip, 2010).

Spices used worldwide and their botanical names.

There are about 70 species cultivated in different parts of the world as spices, but nine of them include pepper, ginger, cloves, cinnamon, cassia, mace, nutmeg, pimento and cardamom. These account for as much as 90 per cent of the total world trade, pepper being the most important (Carlsen et al., 2010). In India, the major spices produced are pepper, cardamom, ginger, turmeric and chillies. There are hundreds of different spices and herbs known worldwide with various uses. Table 1 and 2 are lists of some spices that are used worldwide and in South Africa respectively.

Bioactive properties of herbs and spices

Free radicals and antioxidant properties of herbs and spices

Herbs and spices have been identified as sources of various phytochemicals, many of which possess powerful antioxidant activity (Velioglu et al., 1998). Thus, herbs and spices may have a role in antioxidant defense and redox signaling. In the scientific and public literature, antioxidants and oxidative stress are very often presented in a far too simple manner. First, reactive oxygen species (ROS) are lumped together as one functional entity. However, there are many different ROS that have separate and essential roles in normal physiology and are required for a variety of normal processes. These physiological functions are not overlapping, and the different ROS that exist cannot replace each other.

Table 1: Spices used worldwide and their botanical names.

a Spices obtained from flowers, fruits and seeds.			
Name	Botanical names	Family	Uses
Clove	<i>Syzygium aromaticum</i>	Myrtaceae	culinary uses
Capers	<i>Capparis spinose</i>	Capparidaceae	in meat sauce
Saffron	<i>Crocus sativus</i>	Iridaceae	Dried stigmas used as spice
Chillies	<i>Capsicum frutescens</i>	Solanaceae	culinary uses
Long pepper	<i>Piper longum</i>	Piperaceae	in pickles
Vanilla	<i>Vanilla planifolia</i>	Orchidaceae	Confectionary, flavouring
Coriander	<i>Coriandrum sativum</i>	Apiaceae	Flavouring food stuff
Fennel	<i>Foeniculum vulgare</i>	Apiaceae	In perfumery, flavouring
Cumin	<i>Cuminum cyminum</i>	Apiaceae	Curries, prickles
Caraway	<i>Carum carvi</i>	Apiaceae	Condiment
Anise	<i>Pimpinella anisum</i>	Apiaceae	Flavouring food stuff
Ammi	<i>Trachyspermum ammi</i>	Apiaceae	Curry powder
Celery	<i>Apium graveolens</i>	Apiaceae	Flavouring food stuff
Cardamom	<i>Elettaria cardamomum</i>	Zingiberaceae	culinary uses
Cardamom	<i>Amomum aromaticum</i>	Zingiberaceae	culinary uses
Fenugreek	<i>Trigonella foenugraecum</i>	Papilionaceae	Condiment
Black mustard	<i>Brassica nigra</i>	Brassicaceae	Condiment
Nutmeg	<i>Myristica fragrans</i>	Myristicaceae	Flavouring food stuff
b Spices obtained from root and underground stem			
Root			
Angelica	<i>Angelica archangelica</i>	Apiaceae	Flavourings food stuff
Asafoetida	<i>Ferula asafoetida</i>	Apiaceae	Flavourings food stuff
Horse radish	<i>Armoracia lapothifolia</i>	Brassicaceae	used as condiment
Rhizome /Bulb			
Galangal	<i>Alpinia officinarium</i>	Zingiberaceae	sources of essential oil
Ginger	<i>Zingiber officinale</i>	Zingiberaceae	sources of essential oil
Turmeric	<i>Curcuma longa</i>	Zingiberaceae	To flavour and colour
Garlic	<i>Allium sativum</i>	Liliaceae	spice, medicinal
c Spices obtained from bark			
Cinnamon	<i>cinnamomum zeylanicum</i>	Lauraceae	Curry powder
Sassafras	<i>Sassafras albidium</i>	Lauraceae	Flavouring
d Spices obtained from leaves			
Indian cassia	<i>Cinnamomum tamala</i>	Lauraceae	In curries
Marjoram	<i>Marjorana hortensis</i>	Lamiaceae	Flavouring food
Mint	<i>Mentha longifolia</i>	Lamiaceae	Flavouring agent
Peppermint	<i>Mentha piperita</i>	Lamiaceae	Flavouring agent
Peppermint	<i>Mentha arvensis</i>	Lamiaceae	As spice
Savory	<i>Satureja hortensis</i>	Lamiaceae	Culinary uses
Thyme	<i>Thymus vulgaris</i>	Lamiaceae	In perfumery
Sweet Bay	<i>Laurus nobilis</i>	Lauraceae	Flavouring uses
Tarragon	<i>Artemisia dracunculus</i>	Asteraceae	Essential oil
Parsley	<i>Petroselinum crispum</i>	Apiaceae	Flavouring soups
Rosemary	<i>Rosmarinus officinalis</i>	Lamiaceae	Volatile oil for flavouring
Meethi neem	<i>Murraya koengii</i>	Rutaceae	Flavouring

Table 2: Indigenous South African spices and uses

Name	Botanical names	Family	Uses
Buchu	<i>Agathosma betulina</i>	Rutaceae	flavourant
Buchu	<i>Agathosma crenulata</i>	Rutaceae	flavourant
Wild onion	<i>Allium dregeanum</i>	Alliaceae	vegetable, culinary herb
Aloe	<i>Aloe arborescens</i>	Asphodelaceae	herbal tea (tonic)
Bitter aloe	<i>Aloe ferox</i>	Xanthorrhoeaceae	herbal tea (tonic)
Fennel	<i>Foeniculum vulgare</i>	Apiaceae	spice
Rooibos tea	<i>Aspalathus linearis</i>	Fabaceae	tea, ice tea, health drinks
Fever tea	<i>Lippia javanica</i>	Verbenaceae	herbal tea (tonic)
Wild mint	<i>Mentha longifolia</i>	Lamiaceae	tea, drinks, flavourant
Umondi	<i>Mondia whitei</i>	Apocynaceae	spice, fragrance; sweets
Resurrection plant	<i>Myrothamnus flabellifolius</i>	Myrothamnaceae	tea, flavourant, spice; sweets
Sorrel	<i>Oxalis pescaprae</i>	Oxalidaceae	culinary herb
Rose geranium	<i>Pelargonium graveolens</i>	Geraniaceae	Flavourant
Veldtee	<i>Rafnia acuminata</i>	Fabaceae	flavourant, sweetener
Mistletoe	<i>Viscum capense</i>	Viscaceae	health tea, tonic drinks
Peppercorn tree	<i>Warburgia salutaris</i>	Canellaceae	Spice
Indian ginseng	<i>Withania somnifera</i>	Solanaceae	tonic drinks

Source: Adapted from Carlsen et al., 2010.

Different ROS are also strongly implicated in the etiology of diseases such as cancers, atherosclerosis, neurodegenerative diseases, infections, chronic inflammatory diseases, diabetes, and autoimmune diseases (Gutteridge and Halliwell, 2000). Second, the various antioxidants that exist are often viewed as a single functional entity. However, the different endogenous antioxidants that are produced by the body (glutathione, thioredoxins, glutaredoxin, and different antioxidant enzymes) cannot replace each other.

They have specific chemical and physiological characteristics that ensure all parts of the cells and the organs or tissues are protected against oxidative damage. Dietary antioxidants also exist in various forms, with polyphenols and carotenoids being the largest groups of compounds. These have different functions and are produced by plants to protect plant cells against oxidative damage (Lindsay and Astley, 2002).

Based on the complex nature of antioxidants and ROS, it would thus be extremely unlikely that a magic bullet with a high dose of one or a few particular antioxidants such as vitamin C, vitamin E or β -carotene would protect all parts of the cells, organs, and tissues against oxidative damage and oxidative stress, at the same time without destroying any of the numerous normal and beneficial functions of ROS. Indeed, supplementation with antioxidants has often resulted in no effect or even adverse disease outcomes. Recently, several reviews and meta-analyses have concluded that there is now a strong body of evidence indicating that there is no beneficial effect for supplemental vitamin C, vitamin E, or β -carotene (Bjelakovic et al., 2008). An alternative and much more likely antioxidant strategy to test protection against oxidative stress and related diseases would be to test the potential beneficial effects of antioxidant-rich foods, since such foods typically contain a large combination of different antioxidants that are selected, through plant evolution, to protect every part of the plant cells against oxidative damage. This is especially relevant for herbs and spices.

Several phytochemicals found in these spices, such as rosmarinic acid (Lee et al., 2006) in thyme and oregano (Shan et al., 2005), eugenol in clove and allspice (Chainy et al., 2000) and gallic acid in clove, have all been identified as inhibitors of NF- κ B, a transcription factor which is crucial in the orchestration of immune and inflammatory responses. Thyme and oregano essential oils in combination decreased the levels of IL-1 β and IL-6, as well as inflammation related tissue damage in a model of colitis (Bukovska et al., 2007), both of which may also be related to NF- κ B. An extract of clove, oregano, thyme, together with walnuts and coffee were found to inhibit NF- κ B activation in a synergistic manner *in-vitro*, and also *in-vivo* in transgenic mice (Paur et al., 2010). Furthermore, thyme has been found to induce or maintain levels of endogenous cytoprotective proteins in the liver (Sasaki et al., 2005).

Antimicrobial activity of spices

Antimicrobial activity is understood as the ability of some agents to eliminate microorganisms (aiming at different metabolic or structural targets, as nucleic acid synthesis disruption or peptidoglycan synthesis inhibition) or by inhibiting their growth. Numerous studies have been published on the antimicrobial activities of plant extracts against different types of microbes, including foodborne pathogens (Beuchat, 1994). It has been reported that spices owe their antimicrobial properties mostly to the presence of alkaloids, phenols, glycosides, steroids, essential oils, coumarins and tannins (Ebana et al., 1991). As reviewed by López-Malo et al. 2006, some antimicrobial components that have been identified in spices and herbs are: eugenol from cloves, thymol from thyme and oregano, carvacrol from oregano, vanillin from vanilla, allicin from garlic, cinnamic aldehyde from cinnamon, allyl isothiocyanate from mustard.

Antibacterial activity

Spices are used in the food industry as natural agents for extending the shelf life of foods. A variety of plant and spice-based antimicrobials is used for reducing or eliminating pathogenic bacteria, and increasing the overall quality of food products. Plant-origin antimicrobials are obtained by various methods from aromatic and volatile oily liquids from flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots of plants. Essential oil in plants generally are mixtures of several components, its exert antimicrobial effects such as components in oregano, clove, cinnamon, citral, garlic, coriander, rosemary, parsley, lemongrass, sage and vanillin (Kim et al., 2006). Other spices, such as ginger, black pepper, red pepper, chili powder, cumin and curry powder, showed lower antimicrobial properties (Holley & Patel, 2005).

Antifungal activity

The presence and growth of fungi in food may also cause spoilage and result in reduction in quality and quantity (Skrinjar et al., 2000). As reported by a number of authors (Guo et al., 1996; Skrinjar et al., 2000) some *Aspergillus* species are responsible for many cases of food and feed contamination, and *Aspergillus flavus* and *Aspergillus parasiticus* are able to produce aflatoxins in food and feedstuffs, which are known to be potent hepatocarcinogens in animals and humans (Skrinjar et al., 2000). *Aspergillus flavus* and *Aspergillus parasiticus* are major storage fungi found regularly in important cereal grains cultivated and stored throughout the world (Skrinjar et al., 2000). Which produce aflatoxins B1, B2, G1 and G2. The biosynthesis of aflatoxins can be inhibited by extracts and essential oil from certain plants toxic to fungi and can control the fungal growth and mycotoxin production.

Anti-inflammatory properties of herbs and spices

There is a paucity of data concerning the effect of preparative and digestive processes on the anti-inflammatory activity of culinary herbs and spices. Investigations of the impact of cooking and digestion on the anti-inflammatory properties of culinary herbs and spices demonstrate that this property is not diminished by these processes. Chohan et al., (2012) reported that the amounts used in food preparation, uncooked and cooked and digested rosemary, sage and thyme elicited an anti-inflammatory effect via the inhibition of, and also protection against, the action of pro-inflammatory agents' hydrogen peroxide (H₂O₂) and tumor necrosis factor α (TNF α) which resulted in the inhibition of IL-8 release from peripheral blood lymphocytes (PBLs). These decreases were only significant for PBLs exposed to H₂O₂ for the most part which may be indicative of an activity that involves more than the inhibition of a single pro-inflammatory mediator. There was a strong and significant correlation between inhibition of IL-8 release and antioxidant capacity and total phenolic

content irrespective of whether the herbs were uncooked or cooked and digested which indicates that the polyphenols within these foods contribute to this anti-inflammatory activity, and that this activity may be due to their antioxidant properties. However, the findings of Baker et al., (2013) which were focused on culinary spices at levels associated with habitual intake suggest that the contributory role of polyphenols is not so straight forward as indicated by Chohan et al., (2012) and Baker et al., (2013) reported that the spices cinnamon, clove and nutmeg (uncooked, cooked and digested) significantly inhibited the pro-inflammatory enzyme cyclo-oxygenase-2 (COX-2). The study also reported via correlation analysis that the anti-COX-2 activity was only partially associated with the antioxidant capacities and polyphenolic content of these spices. The partial correlation to phenolic content suggests the involvement of non-polyphenolic compounds, which is supported by the literature. For example, cinnamaldehyde, a major constituent of cinnamon and the essential oil responsible for its aroma and flavour has been shown to inhibit COX-2 activity. The partial correlation with antioxidant capacity suggests that other actions may contribute to the anti-inflammatory properties; rosmarinic acid has been shown to inhibit the pro-inflammatory PKC/NF- κ B pathway. Curcumin, a predominant polyphenol in turmeric, also inhibits NF- κ B COX-2 has also been shown to be down-regulated and/or inhibited by eugenol (clove) and apigenin (parsley) (Kim et al., 2003). Culinary herbs, including lemon grass, rosemary and thyme are also reported to enhance the activity of the enzyme superoxide dismutase (SOD), which in addition to being an important antioxidant enzyme has the potential to act as an anti-inflammatory agent as it catalyses the dismutation of the free.

Antiuroolithiasis activities of herbs and spices

Urolithiasis (Urinary calculi) is one of the three prevalent disorders of the urinary system. Many medicinal plants have been used since ages to treat urinary stones. Some of the plants that have been reportedly used for kidney problems are *Allium sativum*, *Apium graveolens*,

Armoracia lopathifolia, *Barbarea vulgaris*, *Capsella bursapastori*, *Citrus japonica* and *Ficus carica*. The genus *Phyllanthus* has also been reported to have a long history of use in the treatment of kidney stones (Cheryl, 2006).

Toxicity effects of herbs and spices

The safety of medicinal and spice plants and their preparations deserves increased scientific attention. One of the main conditions for use of herbal preparations in medicinal conditions is the absence of such risks as mutagenicity, carcinogenicity, and teratogenicity. In general, such products need to have minimal toxicity and side effects. Generally, the vast majority of herbal remedies are recognised as safe and individual hypersensitivity is usually considered as the most common but controllable risk. However, for those individual compounds exhibiting toxic effects in laboratory animals, the question of possible negative effects in humans remains open.

Many spices are toxic when consumed in large doses, for example *Aloe ferox* juice can be toxic by causing abdominal pain, diarrhea, potentially carcinogenic, with other potentiate cardiac glycosides and antiarrhythmic agents, also most herbs and spices that consists of warfarin such as garlic, ginger, cinchona can cause additive effects such as bleeding in pregnant women (Elvin- Lewis, 2001).

Kaefer and Milner (2008) has explain the issue of herb and spice safety and toxicity clearly as follows, herbs and spices are generally recognised as safe (GRAS) by the FDA at least at concentrations commonly found in foods (0.5-1%). However, many herbs, spices and their bioactive components being investigated for potential disease prevention and treatment at concentrations often exceed those used in food preparation. National toxicological program (NTP) has also reviewed the safety of several herbs and spices suggesting some evidence of

carcinogenic activity in spices such as turmeric, when use in very high doses when they are consumed (NTP, 2004).

The German commission E. monographs are probably the most widely known resource on herbal medicines (Blumenthal, 1998). Commission E currently published more than 400 monographs on herbs and spices including pharmacology and toxicology studies. The first category of monographs consists of herbs and spices that are “unapproved”, for instance, where no plausible evidence of efficacy was available or where safety concerns outweighed potential benefits associated with the product use, basil, lemon grass, majoram, nutmeg and saffron are included in this category.

Herbs and spices included in the approved monographs include caraway oil and seed, cardamom seed, cinnamon bark, cloves, coriander seed, dill seed, fennel oil and seed, garlic root, liquorice root, mint oil, onion, paprika, parsley herb and root, peppermint leaf and oil, rosemary, sage, thyme, turmeric root and white mustard seed.

The recommendation for the various essential oils range from 3-6 drops per day for caraway and mint oils to 10-20 drops for rosemary. The recommended dose for seeds and other herb and spices range from 1.5 g/day for caraway and cardamom seeds to 50 g/day of fresh onion or 20 g dried onion (Peter, 2012).

It has been suggested that excessive consumption of garlic can cause a range of problems (Amagese et al., 2001) though extensive testing by FDA in the United States of America supports the commission E. findings that garlic is generally safe (Rahman, 2007).

Ethnobotany of *Foeniculum vulgare*

Hierarchy: Domain-*Eukaryota*, Kingdom-*Plantae*, Phylum-*Spermatophyta*, Subphylum-*Angiospermae*, Class-*Dicotyledonae*, Order-*Apiales*, Family-*Apiaceae*, Genus-*Foeniculum*
Species-*Foeniculum vulgare*

Foeniculum vulgare belongs to the Apiaceae family (cosmopolitan family), Apiaceae family comprises about 446 genera, 3,540 species, these are common in temperate upland regions, about two-third of the species of Apiaceae are native to the Old World. One remarkable feature of umbellifers is the wide range of uses, ranging from food and fodder to spices, poisons and perfumery. They are known as the carrot family, some species in Apiaceae family include fennel (*Foeniculum vulgare*), parsley (*Petroselinum crispum*) and carrot (*Daucus carota*). Some of these are used as flavorings for alcoholic beverages especially anise (Heywoods et al., 2007). Many umbellifers have medicinal uses, for gastrointestinal complaints, cardiovascular ailments, as stimulants and sedatives. Apiaceae are usually herbs, its leaves are alternate with sheathing bases; internodes usually hollow. Plants are aromatic with ethereal oils, terpenoids, saponins and other compounds. Inflorescences are usually involucrate compound umbels (sometimes simple or condensed into a head). Apiaceae flowers are usually small, inconspicuous with 5 distinct sepals, very reduced; 5 petals distinct but developing from a ring-like primordium, usually inflexed; 5 stamens, filaments distinct; 2 connate carpels in an inferior ovary. Styles are basally swollen to form a nectar secreting structure (stylopodium) atop the ovary. Fruit are schizocarp, the 2 dry segments (mericarps) attached to an entire forked central stalk (carpophores) while seeds are with oil glands (Heywoods et al., 2007; Barros et al., 2010).

Description of *Foeniculum vulgare*

A biennial or perennial, fennel sends up four or five smooth stalks, hollow but containing a white pith, and bearing feathery, finely divided linear foliage on clasping leafstalks; blooming



Figure 1: *Foeniculum vulgare* in its natural habit

Plants can reach 6 feet in height, although *F. vulgare* subsp. *vulgare* var. *azoricum*, the vegetable fennel with the bulbous stalk base, is shorter, growing to only 2 feet (Damjanovic et al., 2005).

Habit

Although fennel is a perennial or biennial, it may grow as an annual as far as in many region and *F. vulgare* subsp. *vulgare* var. *azoricum* is almost always grown as an annual (Damjanovic et al., 2005). Plants can also be propagated by division in spring. Fennel prefers moist but well-drained soil with a pH between 4.8 and 8.2. Florence fennel or finocchio (*F. vulgare* subsp. *vulgare* var. *azoricum*) can be hilled with soil as soon as the bottoms of the stalks have formed an egg-sized base; this will blanch the stalks as they grow *F. vulgare* subsp. *vulgare* var. *dulce*, which is grown for its oil, is sometimes confused with *F. vulgare* subsp. *vulgare* var. *azoricum* but does not have finocchio's thick leafstalk base. It is not advisable to plant fennel plant near dill plant because uncertain flavour may result. Fennel will self-seed and spread if seed heads are not cut after flowering (Heywoods et al., 2007; Barros et al., 2010). The leafstalk bases of the vegetable form, finocchio, can be harvested in early autumn or spring. Leaves can be harvested throughout the growing season, and seeds are gathered from the seed head when ripe, for drying.

Economic uses

Fennel has been cultivated and introduced into many regions and grown commercially in many countries such as Russia, India, China and Japan (Damjanovic et al., 2005). Mature fennel fruit and essential oil are used as flavouring agents in food products like liqueurs, bread, pickles, pastries and cheese. They are also used as constituents of cosmetic and pharmaceutical products (Telci et al., 2009). Fennel seeds are anise like in aroma and are used as flavourings in baked goods, meat and fish dishes, ice cream, alcoholic beverages and herb mixtures (Diaaz et al., 2005). The bulb is a crisp, hardy root vegetable and may be sautéed, stewed, braised, grilled or eaten raw. Fennel features predominantly in Mediterranean cuisine, where bulbs and fronds are used, both raw and cooked, in side dishes,

salads, pastas, vegetable dishes. Many cultures in the Indian subcontinent and the Middle East use fennel seeds in their cooking.

Folk remedies and traditional uses

Fennel has a history of medicinal, magical and culinary uses. Fennel was used by the ancient Egyptians as food and medicine, and was used as a remedy for snake bite in ancient China. During the middle ages it was hung over doorways to drive away evil spirits (Philip, 2010).

Ethnobotany of *Lippia javanica*

Hierarchy: Domain-Eucaryota, Kingdom-*Plantae*, Phylum- *Magnoliophyta* , Subphylum- *Magnoliopsida*, Class-*Asteridae*, Order- *Lamiales* , Family-*Verbenaceae*, Genus- *Lippia*, Species- *Lippia javanica*

Description of *Lippia javanica*

The genus *Lippia* (Houst.) of the *verbenaceae* family has approximately 200 species of herbs, small trees and shrubs. Amongst the genera, *Lippia* is differentiated by the two sepals, which are generally, 2-4 toothed. These plants possess three or four petals, four stamens and an ovary, which has two chambers with one ovule per chamber. The flowers are small, short and dense on long stalks that grow on adjacent sides (Figure 2). The *Lippia* species possess a strong smell when their leaves are crushed. The stems have a square appearance when looked at in cross-section. The leaves are hairy with noticeable veins and when crushed gives off a strong lemon-like smell. It is said to be one of the most aromatic of South Africa's indigenous shrubs. The small cream flowers can be found on the shrub from summer to autumn in some areas and others are produced all year. These flowers are arranged in dense, rounded flower heads. The fruit are rather inconspicuous, small and dry (Pascual et al., 2001; Viljeon et al., 2005).



Figure 2: *Lippia javanica* in its natural habit

Distribution

They are distributed all over the tropical and Southern African countries (Pascual et al., 2001; Viljeon et al., 2005). *Lippia javanica* is known as fever tea / lemon bush and has dense creamy white, flower heads. It grows in open veld, bush, and grassland, on hillsides and stream banks and as a constituent of the scrub on the fringes of forest. The plant is widely distributed in Zimbabwe, Ethiopia, East Africa and South Africa (Manenzhe et al., 2004). This species is the largest of the indigenous *Lippia* shrubs, reaching heights of up to 5 m and occurring at altitudes as high as 2000 m above sea level. These plants are widespread throughout large parts of South Africa, with the exception of the Western Cape. *L. javanica* grows from the Eastern Cape northwards extending into tropical Africa including Botswana, Swaziland, Mozambique, Malawi, Tanzania, Zambia, Tanzania and Kenya. It grows in open veld, in the bush as well in the forest margins. Its aromatic leaves protect this plant from animal browsing on it.

Folkloric uses

Lippia javanica is generally known as Izziniba and Umsuzwane among the people of Xhosa and Zulu respectively in South Africa, it is commonly used for the treatment of fever and skin disorders. Different parts (the leaves, twigs and occasionally the roots) of the plant are used for different purposes. The Xhosa people are known to drink the weak infusion as a tea substitute and a stronger infusion for the treatment of coughs, colds and bronchial problems in general (Pascual et al., 2001). It is also excellent for treating skin problems, scabies and scalp infections. Some people inhale the smoke to cure asthma and chronic cough (Viljeon et al., 2005). Preparations are also used as an anti-inflammatory to soothe sore muscles. It is also used traditionally as a charm for protection against dogs, lightning and crocodiles and for ritual cleansing after contact with a corpse (Pascual et al., 2001; Viljeon et al., 2005). The lemony natural oil has many uses in the home, not only medicinally, but also for its insect repelling and pleasant fragrance in linen cupboards and potpourri jars (Pascual et al., 2001; Viljeon et al., 2005).

Economic use

L. javanica is one of the most common and widely used species in the genus *Lippia*. The Xhosa people of the Eastern Cape often use it to disinfect meat contaminated with anthrax and for flavouring drinks (Viljeon et al., 2005).

Cultivation

Despite its popularity for traditional medicine and charms, the lemon-bush is widespread in the wild and locally abundant in some areas. In horticulture the lemon-bush is a prized landscape or herb garden plant. It grows in full sun or partial shade. It is a hardy, drought-resistant plant that grows easily from seed in a variety of soil types. The seeds are small, brown, dry nutlets when ripe. No treatment is necessary as the seeds are easily germinated and grown under most conditions.

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CHAPTER THREE

Ethnobotanical survey of culinary herbs and spices used in the traditional medicinal system of Nkonkobe Municipality, Eastern Cape, South Africa.

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Abstract

This study was conducted to identify and document herbs and spices used for culinary and medicinal purposes in Nkonkobe Municipality, Eastern Cape Province of South Africa. Seventy four community members were interviewed in 7 locations using the rapid appraisal method. Ethnobotanical data was collected for 58 species of plants belonging to 29 families and 50 genera. The use-value and informant consensus factor (ICF) were employed to determine the relative importance of the spices, their culinary and medicinal uses, as well as the homogeneity of the informants' knowledge. The plant family with the highest ICF was Solanaceae with 6 species; this was followed by Apiaceae, Amaryllidaceae, Amaranthaceae and Lamiaceae with five species each, respectively. The spice with the highest therapeutic uses was *Lippia javanica* known locally as Izinzinba. The parts of the plants used as spices are; leaves (52%), rhizome (13%), fruits (12%), seeds (20%) and roots (3%). The plants species enumerated are used for food seasoning (17%), flavouring (12%), as leafy vegetables (6%), preservatives (29%) and traditional medicines (36%). Majority of the spices are prepared for medicinal use as infusions (40%), decoctions (30%), decoctions or tinctures (13%), tinctures (5%) and decoctions or infusions (12%). This survey on herbs and spices used for culinary and medicinal purposes to the best of our knowledge is the first report on plants used as spices in this region. It will therefore serve as a reference as well as document and preserve the indigenous knowledge of these herbs and spices in Nkonkobe Municipality, Eastern Cape and South Africa at large.

Keywords: Culinary, ethnobotanical survey, herbs, medicinal plants, spice

Introduction

Spices are dried seeds, fruits, roots, barks or vegetable substances used primarily to flavour, colour or preserve food in the culinary arts. It is any dried part of a plant used for these three purposes but not as the main ingredient. According to the European Spice Association, culinary herbs and spices are the edible parts of plants that are traditionally added to foodstuff for their natural flavourings, aroma, visual appearance and preservative purposes (ESA, 2013). Common examples of herbs are sage, parsley, basil, oregano, rosemary, dill and thyme, while spices include buds (cloves), bark (cinnamon/cassia), roots (ginger), berries (peppercorn) and seeds (Tapsell et.al., 2006). Herbs and spices have a rich history of traditional use not only for their culinary effect on foods but also to prevent and treat chronic health maladies. Current biomedical efforts are focused on the scientific merits of spices, to provide science-based evidence for their traditional uses and to develop functional foods or nutraceuticals.

In most parts of Southern Africa, there is little evidence of ancient use of spices in food. Information on plant species used as spices and condiments are inadequate or completely lacking. According to Van Wyk (2011), spices are relatively rare in South Africa but some spices such as *Heteropyxis natalensis*, *Mentha longifolia*, *Myrothamnus flabellifolia*, *Pelargonium graveolens*, *Siphonochilus aethiopicus* and *Warburgia salutaris* are of importance as potential sources of new flavours for the food industry. However, while the culinary evidence for the use of common herbs and spices have been scarce or lacking, their beneficial effects in ethnomedicinal applications abound and are generally encouraging.

The Eastern Cape is one of the poorest provinces in South Africa but is well known for its diversity in plant species (Afolayan et al., 2014). The Xhosa people are the major inhabitants of this province and they live primarily in the areas called Ciskei and Transkei. Plants used in traditional medicine by the Xhosas have been extensively documented (Bhat and Jacobs,

1995; Bhat, 2013; Afolayan et al., 2014). A large number of plants of ethnobotanical value indigenous to the Eastern Cape Province have also been reported (Hutchings, 1989; Dold and Cocks, 2000 and Bhat, 2013). Nkonkobe Local Municipality is located within this Province and is home to 15.5% of South Africa's total population. Here the people are more traditional and many aspects of traditional culture are still part of their everyday life (Dold and Cocks 2000). This region abounds in ethnic diversity and has retained traditional knowledge of the value and utility of the native flora. In spite of the previous work reported on South African indigenous plants, there are no documented records of plants used as spices. This study therefore attempts to survey and document (for the first time to our knowledge) the traditional knowledge and use of spices for culinary purposes and their therapeutic relevance for humans in Nkonkobe Municipality. It is expected that this investigation will highlight a few potential spices for possible large scale production both for culinary and medicinal purposes towards economic upliftment of the people.

Materials and Methods

The study area

This study was conducted in the Nkonkobe Municipality within the Amathole district of the Eastern Cape Province, South Africa (Figure 1). The Eastern Cape Province falls within the latitudes 30°00' to 34°15'S and longitudes 22°45' to 30°15'E (Grierson and Afolayan, 1999). It is bounded by the sea on the East and the drier Karroo (semi-desert vegetation) in the West. The altitude ranges from sea level to approximately 2,200 m in the North of the Province. According to the SSA (2014), the population of South Africa is 54 million; of this, 18% (12.6 million) are Xhosa people and live in the Eastern Cape. The province accommodates more women (52.9%) than men (47.1%). The deficit of men is mainly among those in their economically active years (15-59 years). Over one third (37%) of the population were

younger than 15 years, 58% were in their economically active years and 7% were above 60 years.



Figure 1. Map of South Africa showing Alice in Nkonkobe Municipality, Eastern Cape.

Source: Urban-Econ, Eastern Cape, 2011.

Ethnobotanical investigation

The fieldwork was carried out between May 2014 and June 2015. Interviews were conducted using rapid appraisal approach to record the uses of plant species (Martin, 1995). This approach is a bridge between formal surveys and more unstructured methods, such as field observation and interviews, it allows for community participation in a more informal setting and is often considered more effective in ethnobotanical surveys.

Seventy-four community members including women, traditional healers and farmers between the ages of 20 and 80 years participated in the study. This consisted of 35 males (47%) and 39 females (53%). The race distribution is 91% black-African, 3% coloured and 6% white.

An IsiXhosa speaking person was engaged during the study as translator. This allowed us to capture and accurately record information that would normally be lost during interpretation and translation. All information were collected from rural dwellers residing in Tyahli, Fort Cox, Gaga, Ngwenya, Sheshegu, Dyamala, Mhehelo, Chwaru, Alice, Hogsback and Fort Beaufort. Informants were asked to give the local names of the spices, parts used, culinary uses, what ailments they are used to treat and methods of preparation and other uses of the plants. Validation of a plant as a spice, its culinary and medicinal uses were made only when the answers of two or more respondents coincided to the same usage of the plant regardless of the method of preparation.

Preservation and Identification of herbs and spices

Voucher specimens were collected for all the plants mentioned except plants with established voucher specimens. These were pressed and mounted on herbarium sheets and deposited at the Giffen herbarium of the University of Fort Hare, Alice, South Africa. Plants collected were identified and authenticated by Prof. Grierson of the Department of Botany, University of Fort Hare and by Tony Dold of the Selmar Schonland herbarium, Rhodes University also in South Africa.

Statistical analysis

The data collected were summarized on an excel sheet highlighting plant names, families, parts used, preparation and therapeutic applications. The use-value, which shows quantitatively the relative importance of spices known locally, was calculated as

$UV = \sum U / n$, (where UV= use value of a spice / herb; U= number of citations, n= number of informants). Informant consensus factor values were calculated as:

ICF = (nur-nt) / (nur-1), where nur= number of use citations in each category: nt= number of species used. All citation were divided into two major categories (herbs and spices). ICF values range from 0 to 1 and the values will be high if there is a well-defined selection criterion in the community (Afolayan et al., 2014).

Intellectual property agreement / ethical approval

This study was carried out with the full consent of all participants. Further verbal agreement was reached that this research will not be used for commercial purposes but to serve as enlightenment to the community and the entire Eastern Cape, Province on the plants used as spices and their therapeutic usage. Exact dosages of each spice were not mentioned as such knowledge is considered the intellectual property of the people. The University of Fort Hare Ethics Committee granted ethical approval for the study.

Compliance statement

No part of this study in any form will be commercialized, rather it is meant to be used as a tool for information dissemination on the spices used for culinary and medicinal purposes in Nkonkobe Municipality of the Eastern Cape Province of South Africa.

Results and Discussion

Socio-demographic information

Seventy-four informants including traditional healers, herbalists and lay people between the ages of 20-80 years participated in the study. The variability in terms of age, gender and user status (laypeople, traditional healer or herbalist) of the respondents has significant implications with respects to the culinary and medicinal knowledge of spices in Nkonkobe Municipality. In terms of age, 85% of the respondents were above 45 years, while only 15% were below this age. The implication of this is that the indigenous knowledge of the use of

spices is gradually declining and endangered among the younger generation in the study area. Several studies have shown that this decline in indigenous knowledge among youths is not limited to spices or this study area, but rather a trend among indigenous populations whereby most young people do not believe that studying indigenous knowledge has any immediate benefit to their lives (Giday et al., 2009; Shaheen et al., 2014; Hong et al., 2015).

According to gender, 53% of the informants were women who naturally do most of the cooking and culinary activities. This is significant especially for the rural women, as they have to feed and cope with common ailments in the family (De Wet et al., 2013; Afolayan et al., 2014). This is in agreement with the report of d'Avigdor et al. (2015) that women have particular roles in traditional health care delivery especially as mothers, cooks and cultivators of home gardens.

The traditional healers or herbalists are the custodians of medicinal practices among the Xhosas. This implies that the knowledge and use of spices as remedies for major ailments like diabetes, hypertension, cancer, tuberculosis and infertility are still confined to healers, herbalist and diviners while the lay people can conveniently manage minor ailments like constipation, cold, skin disorder and loss of appetite.

Diversity and Use-value of plants utilized as herbs and spices for culinary and medicinal purposes

The botanical names of the spices along with their local names, habits, parts used, culinary and therapeutic applications, frequency, use value and mode of preparation are presented. Table 1 shows the plants mentioned and identified as herbs and spices. These constitute fifty-eight plants species distributed among twenty-nine families (Figure 2). This is an indication of a good diversity of plants used as spices in the study area and could be attributed to the rich diversity of plants in the Eastern Cape (Bhat, 2013). The families contributing the most

taxa were Solanaceae with six species, Apiaceae, Amaryllidaceae, Amaranthaceae and Lamiaceae (five species) each while the other families had one or two species each.

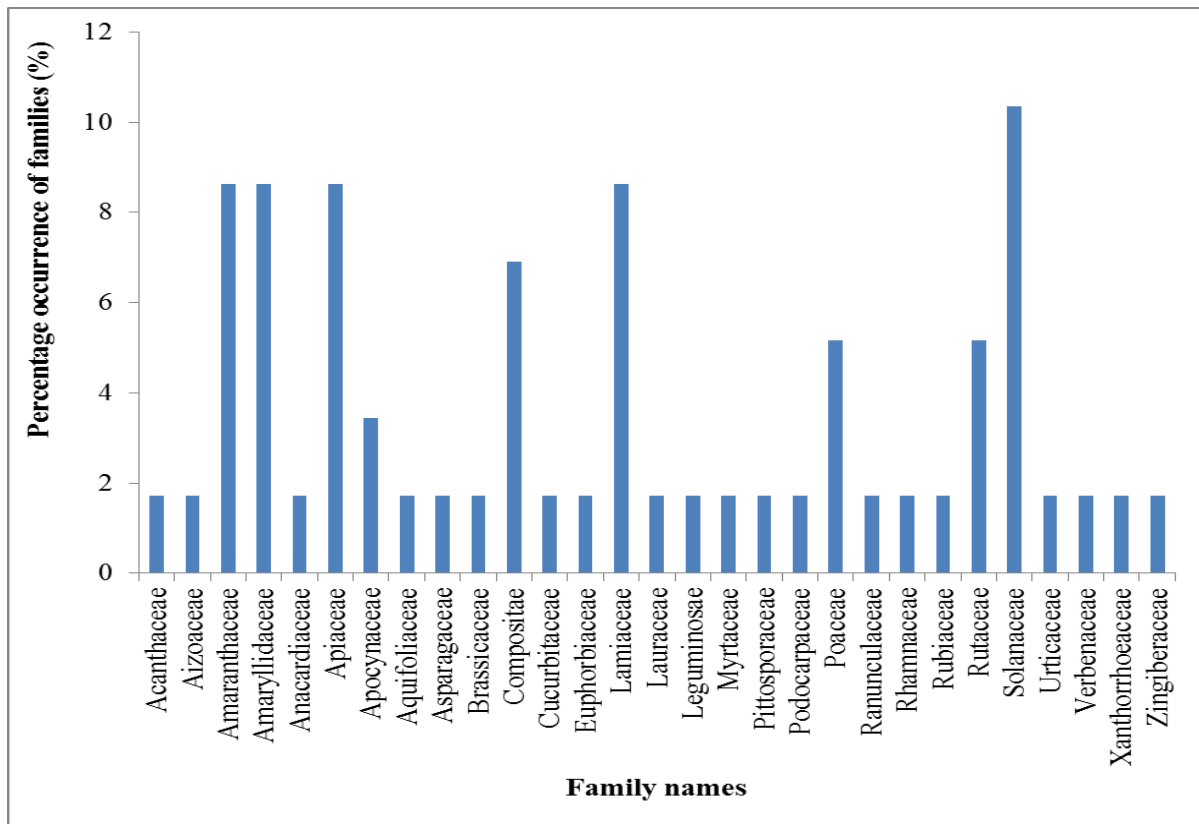


Figure 2: Distribution of plant family used as spices

A large number of spices are used in local ethno-medicinal system. In the present study, the most common ailments treated using spices include colds, coughs, skin diseases, antimicrobial infections, respiratory diseases, worms, immune deficiency, diabetes, ulcers and cancer.

Five of the plant species most cited as spices in the study area had a use value of 6.36. These include *Lippia javanica* (Verbenaceae), *Mentha aquatica* (Lamiaceae), *Mentha longifolia* (Lamiaceae), *Mentha spicata* (Lamiaceae) and *Capsicum annum* (Solanaceae). *Lippia javanica* has been reported for flavouring drinks, treatment of fever and skin disorders (Oliveira et al., 2006). *Mentha aquatica* L., *Mentha longifolia* L. Huds., *Mentha spicata*

L. and *Capsicum annuum* L. mostly used in the management of respiratory related ailments and treatment of other chronic diseases (Punit and Mello, 2004). Others such as garlic, ginger and cayenne pepper have been documented to have hypoglycaemic, hypolipidaemic, antioxidant and antidiabetic properties (Otunola and Afolayan 2013; Otunola et al., 2014; Otunola and Afolayan, 2015).

Plant parts used, culinary/therapeutic uses, methods of preparation and habit

The part of the plants used as spices is shown in Figure 3. The leaves were the most frequently used parts as spices (52%) followed by seeds (20%), rhizomes (13%), fruits (12%) and roots (3%). Their uses for food seasoning (17%), flavourings (12%), leafy vegetables (6%), preservatives (29%) and traditional medicine (36%) are displayed in Figure 4. Spices have been reported to be used for different therapeutic purposes such as immune booster, colds, coughs, rheumatism, stomach ache, throat infection, kidney stone treatment, astringent, antihelminthic, as protection against witchcraft and a number of other therapeutic uses (Grierson and Afolayan, 1999; Corrigan et al., 2011). The methods of preparation for the therapeutic uses of these spices are shown in Figure 5. This includes infusion (40%), decoction (30%), decoction or tincture (13%), tincture (5%) and decoction or infusion (12%). A few of them required a mixture of plant species and preparation for more potency. Of the 58 plant species, herbaceous plants constituted 56%, shrubs 24%, trees 12% and climbers 7% (Figure 6). Most of the spice formulations were administered orally in ailment categories other than dermatological problems. In dermatological ailments, plants were administered topically. Water, alcohol and some additives such as oil, honey and salt were often used in the preparation of remedies. Most of the preparations involved the use of single spices or a single plant part while those mixing different species or plant parts were less encountered in the study area.

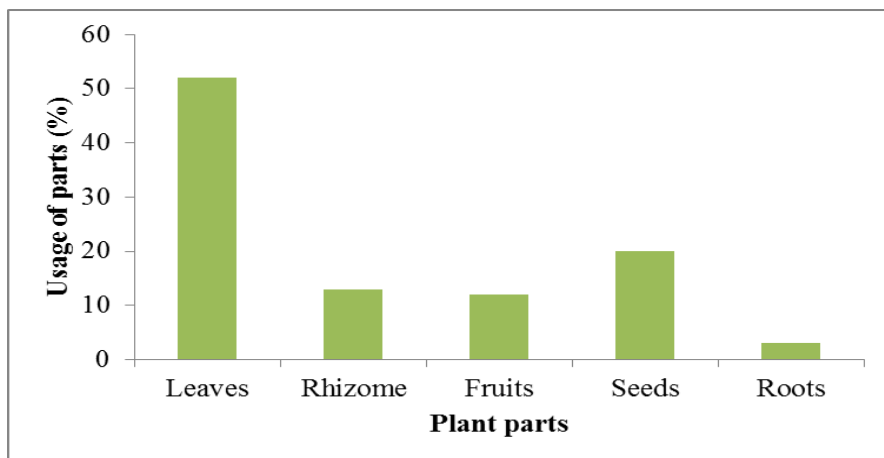


Figure 3: Plant parts used for culinary purposes

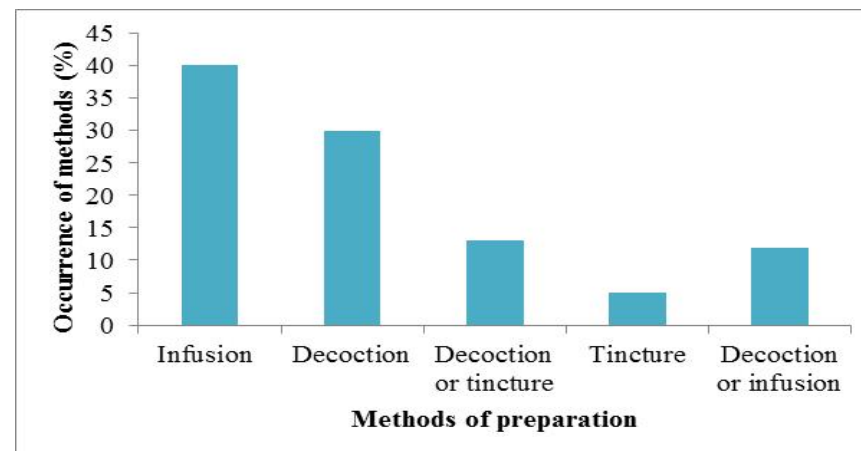


Figure 5: Methods of preparation of spices for therapeutic uses

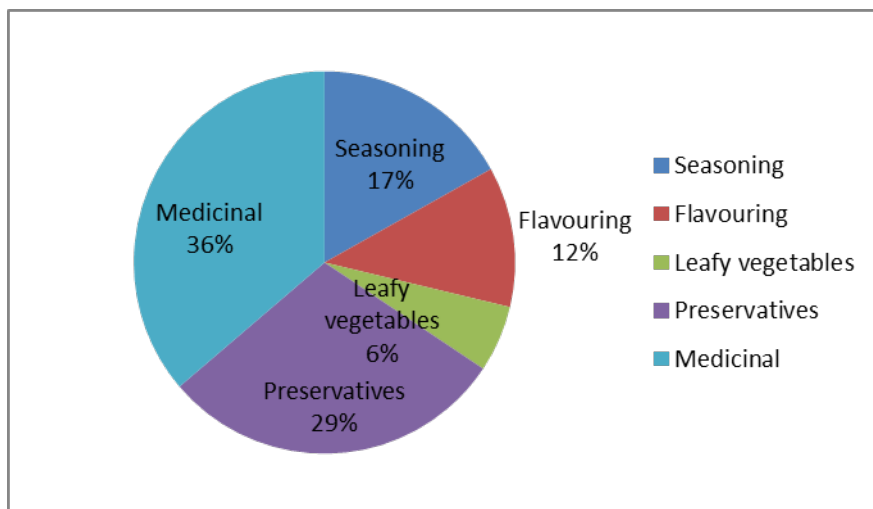


Figure 4: Percentage (%) and distribution of indigenous uses of spices

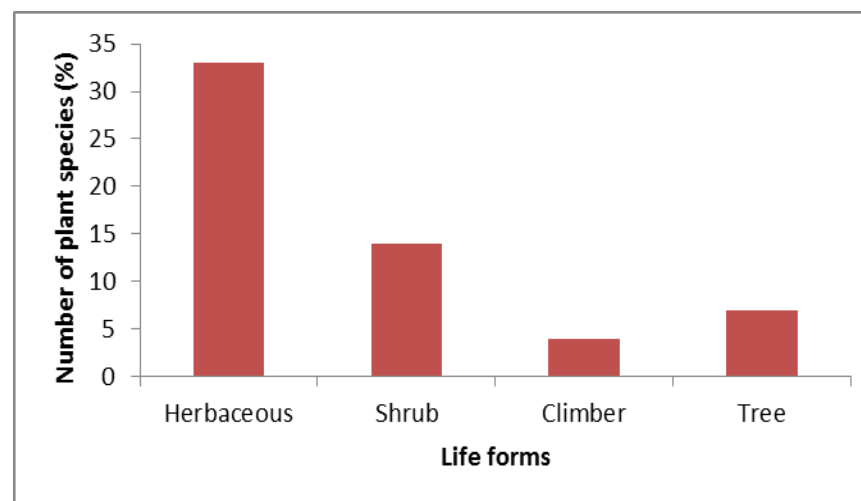


Figure 6: Plant habit of species used as spices

Table 1: Medicinal plants used as spices and herbs in Nkonkobe Municipality

Botanical name/ family name	Local name / common name	Habit	Frequency	*Use- value (UV)	Part(s) used	Culinary uses	Therapeutic uses	Mode of Preparation
<i>Aloe ferox</i> Mill/ Xanthorrhoeaceae	Ikhala (X)/ Bitter aloe (C)	Herbaceous	2	0.85	Leaves	Seasoning and preservative	Purgative	Decoction
<i>Agathosma betulina</i> (P. J. Belgius) Pillans/Rutaceae	Ibhuchu (Z)/ Buchu (C)	Shrub	10	4.24	Leaves and roots	Seasoning and preservative	General immune booster	Infusion
<i>Alepidea amatymbica</i> Eckl. & Zeyh/ Apiaceae	Iqwili (X)/ Larger tinsel flower (C)	Herbaceous	4	1.69	Roots	Seasoning and preservative	Colds, coughs, rheumatism, wounds and to wash divining bones	Infusion
<i>Anethum graveolens</i> L./ Apiaceae	Dille (X)/ Dill (C)	Herbaceous	15	6.36	Leaves and seeds	Seasoning and preservative	Erectile dysfunction, also used for protection against witchcraft.	Decoction or Infusion
<i>Allium cepa</i> L. / Amaryllidaceae	Ikonofile (Z)/ Onions (C)	Herbaceous	2	0.85	Leaves and bulb	Seasoning and preservative	Treat ulcer	Infusion
<i>Allium sativum</i> L./ Amaryllidaceae	Ivimbampunzi (X) Ikonofile(Z)/	Herbaceous	4	1.69	Leaves and bulb	Seasoning and preservative	Stomach ache, throat infections, also used for	Infusion

Garlic (C)								protection against witchcraft	
<i>Arctotis arctotoides</i> (L.F.) O. Hoffm / Compositae	Ubushwa (X)	Herbaceous	2	0.85	Leaves	Flavouring and preservative	To treat fungal infection	Infusion	
<i>Argyrolobium argenteum</i> (Jacq.)Eckl.& Zeyh./Fabaceae	Umfanujale (X)	Herbaceous	1	0.42	Leaves	Flavouring and preservative	Topical (external application to the infected surface)	Decoction	
<i>Artemisia afra</i> Jacq. ex. Willd/ Asteraceae	Umhlonyane (X)/Wild wormwood (C)	Shrub	10	4.24	Leaves	Seasoning and preservative	General immune booster	Infusion	
<i>Amaranthus caudatus</i> L./ Amaranthaceae	Utyuthu(X)/ love-lies-bleeding (C)	Herbaceous	4	1.69	Leaves	Leafy vegetable	Astringent, anthelmintic, diuretic, scrofulous sores.	Infusion	
<i>Amaranthus hybridus</i> L./Amaranthaceae	Imbuya (X)	Herbaceous	1	0.42	Leaves	Leafy vegetable	Anthelmintic, astringent, antidiarrheal	Decoction	
<i>Asparagus africanus</i> L./Asparagaceae	Umathinga (X)	Climber	1	0.42	Leaves	Leafy vegetable	Pro-fertility	Infusion	
<i>Beta vulgaris</i> L. /Amaranthaceae	Beetroot (C)	Shrub	2	0.85	Leaves	Leafy vegetable	Anti-tumour, carminative,	Infusion	

							Emmanagogue		
<i>Bidens pilosa</i> L./ Asteraceae	Umhlabangubo (X)/ Marigold	Herbaceous	3	1.27	Leaves	Leafy vegetable	Blood purifier removes toxins from the body.	Infusion	
<i>Brassica oleracea</i> L./ Brassicaceae	Cabbages (C)	Herbaceous	1	0.42	Bulb / leaves	Seasoning and preservative	slight cooking enhance flavour	Infusion	
<i>Carissa bispinosa</i> (L.) Desf. ex Brenan/ Apocynaceae	Isabetha (Z)	Shrub	1	0.42	Fruits	Flavouring and preservative	Treating chest pain, anti-viral	Infusion	
<i>Carpobrotus edulis</i> L./ Aizoaceae	Unomatyumtyum (X)	Herbaceous	1	0.42	Leaves / Fruits	Flavouring and preservative	Topical /to treat skin infection	Infusion	
<i>Capsicum annum</i> L./ Solanaceae	Ikhanakhana (X) Pelepele(X)/ Chilli pepper	Herbaceous	15	6.36	Fruits	Seasoning and preservative	Deworming, bronchitis, arthritis, diabetes, fatigue, and sore throats	Decoction	
<i>Citrus limon</i> (L.)Burm.F./Rutaceae	Lemon (C)	Herbaceous	5	2.12	Fruits	Flavouring and preservative	Blood cleanser, diuretic	Infusion	
<i>Clausena anisata</i> (Willd).Hook.F.ex Benth /Rutaceae	Umtuto (X)	Shrub	5	2.12	Leaves	Flavouring and preservative	Antimalarial, analgesic	Infusion	
<i>Clematis brachiata</i> Thun./ Ranunculaceae	Ityolo (X)	Climber	1	0.42	Leaves	Flavouring and preservative	To treat sexually transmitted disease	Infusion	
<i>Chenopodium album</i> L./	Imbikicane	Herbaceous		0.42	Leaves	Leafy vegetables	Anthelmintic, toxic	Infusion	

Amaranthaceae	(X)/ Epazote		1							inlarge quantity
<i>Cordia rudis</i> (E.Mey.ex Harv.)Verdc.	Intsinde (X)	Shrub		0.42	Twig, fruits	Flavouring and preservative	Treat stomach ailments			Infusion
/Rubiaceae			1							
<i>Cucurbita pepo</i> L./ Cucurbitaceae	Imithwane (X)/ Pumpkin (C)	Herbaceous	1	0.42	Leaves	Leafy vegetable	To treat arthritis, blood booster			Decoction
<i>Cymbopogon citratus</i> (DC)	Umqungu (X)/ Lemon grass (C)	Herbaceous	2	0.848	Leaves	Flavouring and preservative	Analgesic, anti depressant, antimicrobial			Infusion
Stapf./Poaceae										
<i>Daucus carota</i> L./ Apiaceae	Carrot (C)	Herbaceous	2	0.848	Fruits	Seasoning and preservative	Stimulant, tonic and vermifuge			Infusion
<i>Euphorbia hypericifolia</i> L./ Euphorbiaceae	Umaphipha (X)/ Graceful Spurge (C)	Herbaceous	3	1.27	Leaves	Seasoning and preservative	Used in combination with <i>Bryophyllum pinnatum</i> and <i>Opuntia stricta</i> to treat gonorrhoea			Infusion
<i>Foeniculum vulgare</i> Mill. / Apiaceae	Imbambosi (X)/ Fennel (C)	Herbaceous	1	0.42	Leaves and seeds	Seasoning and preservative	Gastrointestinal pains,digestive			Infusion or Decoction

										aid,carminative, expectorant, mild stimulant and colds
<i>Helichrysum</i> Asteraceae	<i>gymnocomum</i> DC./	Imphepho (X)	Herbaceous	1	0.42	Leaves	Leafy vegetables	Leaves used to treat ulcer	Infusion	
<i>Ilex mitis</i> (L.) Radlk/ Aquifoliaceae		Isidumo(X)/ tree (C)	Water Tree	6	2.54	Leaves	Seasoning and preservative	Bark is chewed as purgative tonic for children, also to protect sick people from being bewitched	Infusion	
<i>Lippia javanica</i> (Burm.F.) Verbenaceae	Spreng/ Fever tea (C)	Izininiba(X)/ Fever tea (C)	Shrub	18	7.63	Leaves	Flavouring and preservative	Coughs, colds, bronchitis, fever, ulcer	Decoction	
<i>Lycopersicon</i> /Solanaceae	<i>esculentum</i> Mill	Tomato (C)	Herbaceous	2	0.85	Fruits	Seasoning and preservative	Topical for skin disorder, to treat asthma, cancer	Tincture or decoction	
<i>Mentha aquatica</i> L./ Lamiaceae		Ityeleba (X)/ (C)	Mint Herbaceous	15	6.36	Leaves	Flavouring and preservative	Anti-anxiety, digestive uses	for Infusion	

<i>Mentha longifolia</i> (L.) L/ Lamiaceae	Inixina(X)/ Peppermint	Herbaceous	15	6.36	Leaves	Flavouring and preservative	Coughs, colds, asthma and other bronchial ailments	Decoction
<i>Mentha spicata</i> L/ Lamiaceae	Imboza (X) Spearmint (C)	Herbaceous	15	6.36	Leaves	Flavouring and preservative	Coughs, colds, asthma	Decoction
<i>Mondia whitei</i> (Hook.F.) Apocynaceae	skeels/ Umindi (X)	Climber	2	0.85	Seeds	Flavouring and preservative	Improve memory	Infusion
<i>Persea americana</i> Mill. /Lauraceae	Avocado tree (C)	Tree	1	0.42	Seeds	Seasoning and preservative	Topical / Face cleanser	Tincture
<i>Podocarpus latifolius</i> (Thunb.) R.Br. ex Mirb. / Podocarpaceae	Umkhomba (Z)	Tree	1	0.42	Leaves	Flavouring and preservative	Topical/to treat skin infection	Tincture
<i>Pittosporum viridiflorum</i> Pittosporaceae	Sims/ (X) umVusamvu (Z) /Cheesewood (C)	Tree	1	0.42	Leaves	Flavouring and preservative	Stomach complaints, abdominal pain and fever erectile dysfunction	Infusion
<i>Petroselinum crispum</i> (Mill) Apiaceae	Fuss/ Parsley(X)/ Parsley (C)	Herbaceous	1	0.42	Leaves and seeds	Seasoning and preservative	Diuretic, can raise blood pressure	Infusion or decoction

<i>Rosmarinus officinalis</i> L./ Lamiaceae	Roseliner (X)/ Rosemary (C)	Shrub	6	2.54	Leaves	Seasoning and preservative	Loss of appetite	Decoction
<i>Schinus molle</i> L. / Anacardiaceae	Umngcunube (Z)/ Pepper tree (C)	Tree	2	0.85	Seeds/fruits	Seasoning and preservative	Heartburn	Decoction
<i>Solanum aculeastrum</i> Dunal./ Solanaceae	Umthuma (X)	Shrub	1	0.42	Fruits	Seasoning and preservative	To treat cancer	Decoction
<i>Solanum nigrum</i> L./ Solanaceae	Umsobosobo (X)/ Black nightshade (C)	Herbaceous	1	0.42	Leaves	Leafy vegetables	Fruit as a treatment for haemorrhoid and dysentery	Decoction
<i>Solanum tuberosum</i> L. /Solanaceae	Amagqabi amatapile (X)/Irish potato (C)	Herbaceous	5	2.12	Tubers	Seasoning and preservative	To treat swollen gums	Infusion
<i>Sorghum bicolor</i> L. Moench/ Poaceae	Imfi, Izimba (X)	Herbaceous	1	0.42	Leaves and seeds	Flavouring and preservative	To treat anaemia	Infusion
<i>Spinacia oleracea</i> L. / Amaranthaceae	Spinach (C)	Herbaceous	2	0.85	Leaves	Seasoning and preservative	Useful in diseases of blood and brain	Decoction
<i>Syzygium cordatum</i> (Hochst.) ex Krauss /Myrtaceae	Umdoni (X)	Tree	1	0.42	Leaves	Seasoning and preservative	Diarrhoea treatment	Decoction

<i>Thymus vulgaris</i> L./ Lamiaceae	Umakhunkula (X)	Shrub	1	0.42	Seeds/Leaves	Seasoning and preservative	Treat infection	Decoction or tincture
	Thyme (C)							
<i>Thunbergia capensis</i> Retz./Acanthaceae	Iyezalehashe (X) /	Climbers	1		Leaves	Flavouring and preservative	Antifungal	Decoction or tincture
	Thunbergia (C)			0.42				
<i>Tulbaghia acutiloba</i> (Harv.)/ Amaryllidaceae	Isivumbampunzi (X)	Shrubs	1	0.42	Corms/bulb/leave s/ Rhizome	Seasoning and preservative	Anticancer, to treat cold	Decoction or Infusion
<i>Tulbaghia alliacea</i> L.F./ Amaryllidaceae	Umwelela (X)	Shrubs	1	0.42	Corms/bulb/leave s/ Rhizome	Seasoning and preservative	Anticancer, to treat cold	Infusion or decoction
<i>Tulbaghia violacea</i> (Harv.)/ Amaryllidaceae	Ivimbampunzi (X)	Shrubs	14	5.93	Corms/bulb/leave s/ Rhizome	Seasoning and preservative	Ulcer, anticancer, treatment for cold	Decoction or tincture
	lomlambo, (Z)/ Wild Garlic (C)							
<i>Urtica dioica</i> L./Urticaceae	Uralijane(X)/ Nettles (C)	Herbaceous	2	0.85	Leaves and Stem	Leafy vegetables	Detoxifier, protein, antiseptic, haemostatic, vitamin, alterative,	Decoction
<i>Withania somnifera</i> (L.)Dunal/ Solanaceae	Ubuvimbha (Z)	Shrub	1	0.42	Fruits	Flavouring and preservative	Infusion /for blood tonic	Decoction

<i>Zea Mays</i> L./ Poaceae	Umbona (X)	Herbaceous	2	0.85	Seeds	Seasoning and preservative	Help to treat constipation	Tincture
<i>Zingiber officinale</i> Zingiberaceae	Roscoe./ Ijinja(X)/Ginger (C)	Herbaceous	14	5.93	Rhizomes	Seasoning and preservative	Treating respiratory disorder	Infusion & decoction
<i>Ziziphus mucronata</i> Willd/Rhamnaceae	Umphafa/ buffalo thorn (C)	Tree	1	0.42	Leaves	Leafy vegetable	To increase longevity and vitality	Decoction

* $UV = \sum U/n$; UV= use value of a species; U=number of citations per species; n=number of informants. (X-Xhosa name, Z-Zulu name, C-Common name)

Consensus factor among informants

The categories are herbs and spices and the informant consensus factors (ICF) are as shown in Table 2. The ICF of the two categories ranged from 0.22 to 0.33 used for traditional medicine and culinary purposes. The highest ICF (0.33) with 86 use citations occurs in spices used for seasoning, flavouring, as leafy vegetables, preservatives and in traditional medicine. The most important plant species in this category is *Anethum graveolens*, followed by *Agathosma betulina* (ibuchu), *Capsicum annuum* (chilli pepper), *Tulbaghia violacea* (wild garlic), *Zingiber officinale* (ginger) while the most cited plants with lowest ICF are *Euphorbia hypericifolia*, *Lippia javanica* and *Mentha aquatica* with 74 citations. Lack of consistency regarding the usage of spices was observed among informants and accounted for the low ICF values. Low ICF values indicate that informants have no agreement on the usage of many of the species mentioned and collected. This low correlation is an indication of the diversity of undocumented indigenous knowledge of spices for culinary and medicinal purposes in the study area.

Table 2: Informant Consensus factors (ICFs) on categories of herbs and spices

Categories of spices	Usage	Number of plant species	Use citation per category	Informant	
				Consensus factor (ICF)	
Herbs	Seasoning	6	74	0.22	8.1
	Flavouring	12			16.2
	Leafy vegetable	8			10.8
	Preservative	19			25.7
	Medicinal	29			39.12
Spices	Seasoning	21	86	0.33	24.4
	Flavouring	7			8.13
	Leafy vegetable	1			1.16
	Preservative	28			32.5
	Medicinal	29			33.7

Conclusion

This study was undertaken to investigate the culinary herbs and spices used in the traditional system of Nkonkobe Municipality, Eastern Cape, South Africa. Fifty-eight species of plants belonging to 29 families and 50 genera were documented. The most frequently cited plant family is the Solanaceae which include the *Lycopersicum esculentum*, *Solanum aculeastrum*, *Solanum nigrum*, *Solanum tuberosum*, *Withania somnifera*. Five of the plant species that are commonly used as herbs and spices which have the highest use value of 6.36 are *Lippia javanica* (Verbenaceae), *Mentha aquatica* (Lamiaceae), *Mentha longifolia* (Lamiaceae), *Mentha spicata* and *Capsicum annuum* (Solanaceae). The usage of these spices is mostly for seasoning, flavouring, as leafy vegetables, preservatives and traditional medicine. This study has shown that despite the uses of these plants for culinary purposes they are also important as remedies for diseases such as diabetes, hypertension, cancer, tuberculosis and infertility. This is a significant contribution to the ethnobotany of the Nkonkobe Municipality because it is the first documented report on plants used as spices, a large number of which are also utilized in the local ethno-medical system among the Xhosa communities. It will therefore serve as a reference for further studies into the uses of these spices.

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CHAPTER FOUR

Foliar micromorphology of *Lippia javanica*

Introduction

Foliar micro-morphological features are useful in the identification and authentication of plants. Advances in light and scanning electron microscopy have increased the capability of microscopy as a veritable means of botanical identification in foliar micro morphological studies. Plants communicate with their external environment, protect and maintain essential internal physiological and biochemical processes through specialized epidermal structures (Da Silva et al., 2009, Otang et al., 2014).

Trichomes are mainly seen on the surfaces of leaves and the scientific interest is based on the economic importance of their products (Koduru et al., 2006). Histochemical studies of some trichomes indicate that secretions from them contain essential oils which are mostly characterized by monoterpenoids (Afolayan and Meyer, 1995; Ascensao et al., 1999; Koduru et al., 2006). To the best of our knowledge, there is little or no information on the morphology and ultrastructure of the leaf appendages of *Lippia javanica*. The objective of this study therefore, was to investigate the structure and distribution of different types of trichomes, stomata, crystals and epidermal cells in *Lippia javanica* as biologically active structures in the plants.

Materials and Methods

Collection and identification of plant materials - The leaves of *Lippia javanica* were collected from their natural habitat in the wild in Alice, Eastern Cape Province of South Africa. These plants grow in abundance in this region, they were identified in Rhodes University Herbarium and Voucher specimen (ASO 2014/1) was prepared and deposited in the Giffen Herbarium at the University of Fort Hare, Alice.

Scanning Electron Microscopy (SEM) - The ultra-structural examination of the leaves of *Lippia javanica* was carried out using the following method. Fresh leaves of *Lippia javanica* were immersed in a fixative solution of 2.5% glutaldehyde in 0.1 M phosphate buffer for 24 hours. Samples were washed for 15-30 minutes with the buffer and dehydrated in graded ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%). Samples were then critically point dried using CO₂ sputter coated with gold under vacuum and viewed using the Scanning Electron Microscope (SEM) equipped with an Energy Dispersive X-ray (EDX) Oxford ISIS 300 micro analytical system and the necessary software for point micro analysis. Images were captured digitally with an image saver connected with an EDX. The major microscopic structures observed in the leaves were: type of stomata, outline of epidermal cells, character of trichomes and type of crystals.

Leaf epidermal studies (Light microscopy) - Microscopic examinations of the epidermal sections of the leaf were carried out according to the procedure of Ogunkunle & Oladele (2008). Leaf samples of 1 to 3 cm were sectioned from the mid portion of the adaxial and abaxial surfaces of a mature leaf using a razor blade. The sections of the leaf were washed with distilled water for 2-3 minutes. Then the sections were placed on clean glass slides with 1-2 drops of sterile distilled water and covered with a cover slip. Prepared slides were observed under a motic light microscope and the microphotographs were taken with a digital camera fitted into the light microscope and the images were captured digitally using Microsoft Image Programme software for windows.

Results and Discussion

The study of the adaxial and abaxial surfaces of the leaves of *Lippia javanica* showed long glandular trichomes, a natural phenomenon in most angiosperms (Koduru et al., 2006).

Appearance and distribution of trichomes - Both the lower and upper epidermis of *Lippia javanica* observed under scanning electron microscopy (SEM) contains unicellular, uniseriate, spine-like, long glandular trichomes with few stomata which are densely distributed on both surfaces. Under the light microscope, spine-like trichomes density was higher on the abaxial relative to the adaxial leaf surface (Figure 3 a, b, c & d). Trichomes are unicellular or multicellular outgrowths originating from the epidermis of plant parts. Their morphological features, location and mode of secretion are varied (Werker, 2000). It has been reported that the type and density of trichomes differ among species and can vary in organs of the same plant (Bhat et al., 2010). Non glandular trichomes are generally classified according to their morphology. They range from unicellular to multicellular structures that can be uniseriate, biseriate or multiseriate, branched or unbranched (Werker et al., 1994). In this study we observed that *Lippia javanica* possessed spine-like, non-glandular, uniseriate trichomes.

According to Werker and Fahn (1981), glandular trichomes limit the transpiration rate and reduce leaf temperature. Withering and falling leaves with intact glandular hairs may also provide a phytotoxic environment for germinating seeds and growing seedlings while non-glandular trichomes often act as a physiological barrier against herbivores and contribute to plant adaptation to environmental conditions, particularly in dry environments (Afolayan and Meyer, 1995; Ascensao et al., 2001; Aneta, 2013). These attributes may explain the wide distribution of *L. javanica* in various climatic environments in Southern Africa. The glandular and non-glandular trichomes observed for *Lippia* species are distributed widely in the Verbenaceae family (Combrinck et al., 2007; Passos et al., 2009).

Histochemical studies of some trichomes indicate that secretions from some species of *Helichrysum aureonitens*, *Helichrysum stioechas* and *Plectranthus ornatus* contain essential oils which are mostly characterized by monoterpenoids (Afolayan and Meyer, 1995; Ascensao et al., 1999; Koduru et al., 2006). Terpenes are stored by the plant in the form of glycosides, when required the aglycones can be mobilized for their respective functionality in the plant (Lalel et al., 2003). Long glandular trichomes (L-GST) are the most abundant trichome type and thus terpenoids isolated through distillation most probably originated from these structures (Combrinck et al., 2007). Thus structures are subjected to rapid loss of volatile terpenoid content after harvest, as described by Combrinck et al. (2006). Terpenoid secretion was found to be restricted to long glandular trichomes (L-GST) while the presence of phenolic compounds in short glandular trichomes was indicated by various colour reactions (Andary et al., 1984). This implies *L.javanica* may contain high terpenoid content due to the presence of long glandular trichomes in the species.

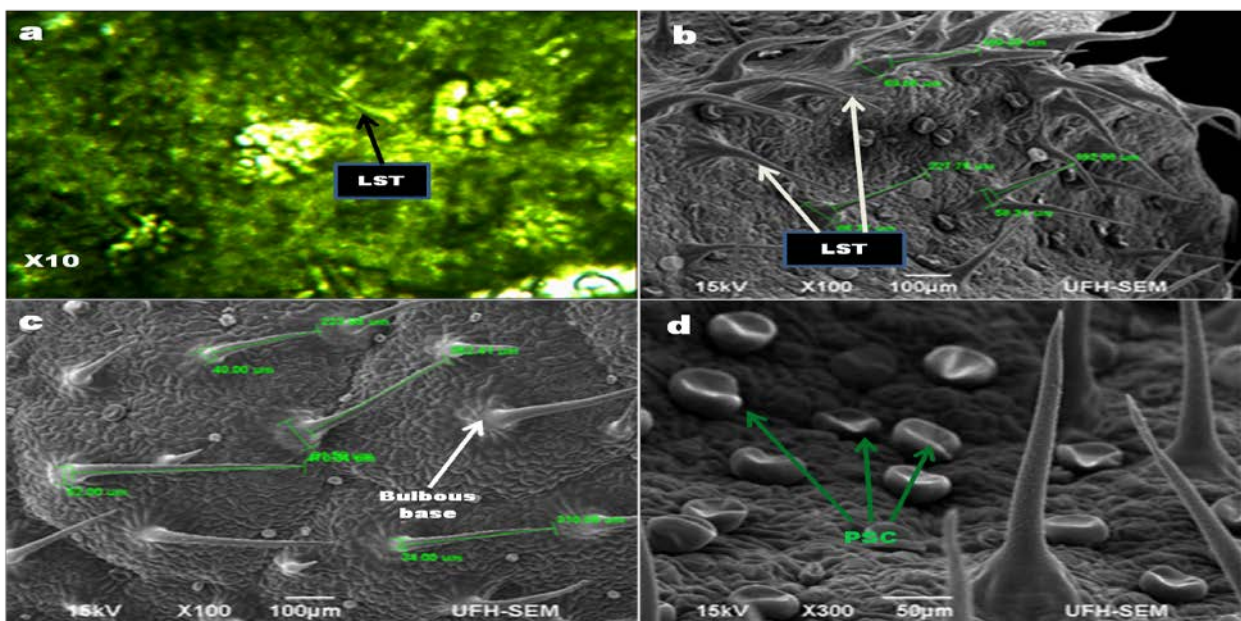


Figure 3: a- Light microscope micrograph showing long stalk trichomes (LST) and guard cells on the abaxial surfaces of *Lippia javanica* leaf (x10); b- SEM micrograph showing long stalk trichomes (LST) (x100); c- bulbous base of trichomes (x100); d- peltate secretory cells (PSC) and conical long stalk (x300) also on the abaxial surfaces of *Lippia javanica* leaf.

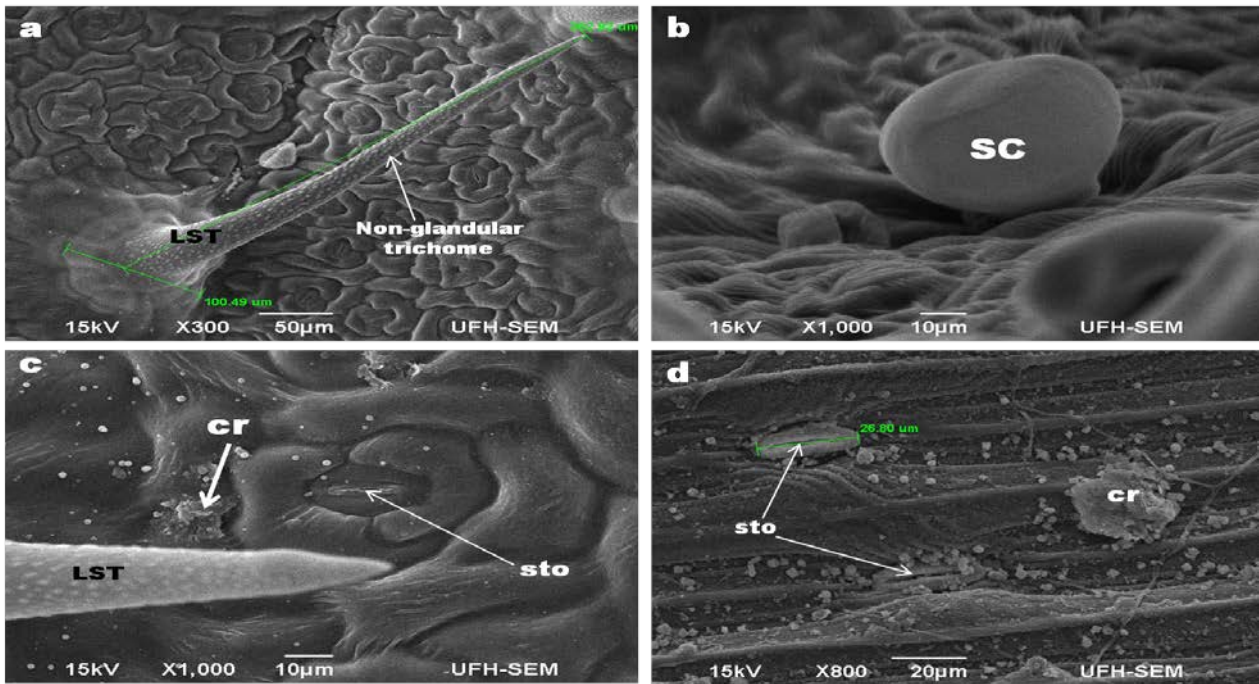


Figure 4: a-SEM photographs shows abaxial surface with a spine like non-glandular trichome (x300); b-SEM photograph shows abaxial surface secretory cells (sc) at higher magnification (x1000); c- stomata-sto and crystals-cr on the abaxial surfaces (cr) at higher magnification (x1000); d): Different shapes of crystals on adaxial surface (x800) present in rectangular, prism, triangular and styloid.

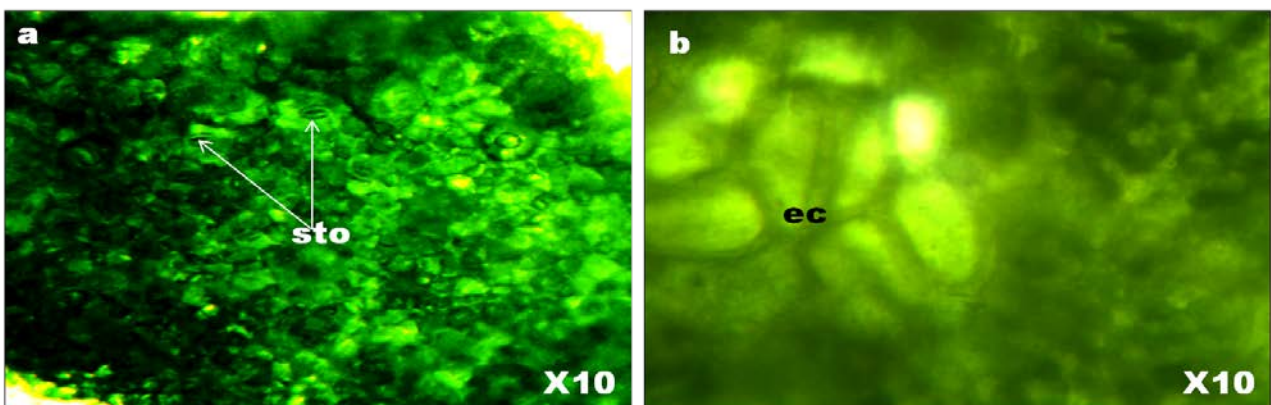


Figure 5: a) light microscope photograph of stomata (st) in the adaxial epidermal surfaces. b) Light microscope micrograph of the adaxial surfaces showing the hexagonal shape of the epidermal guard cells (ec).

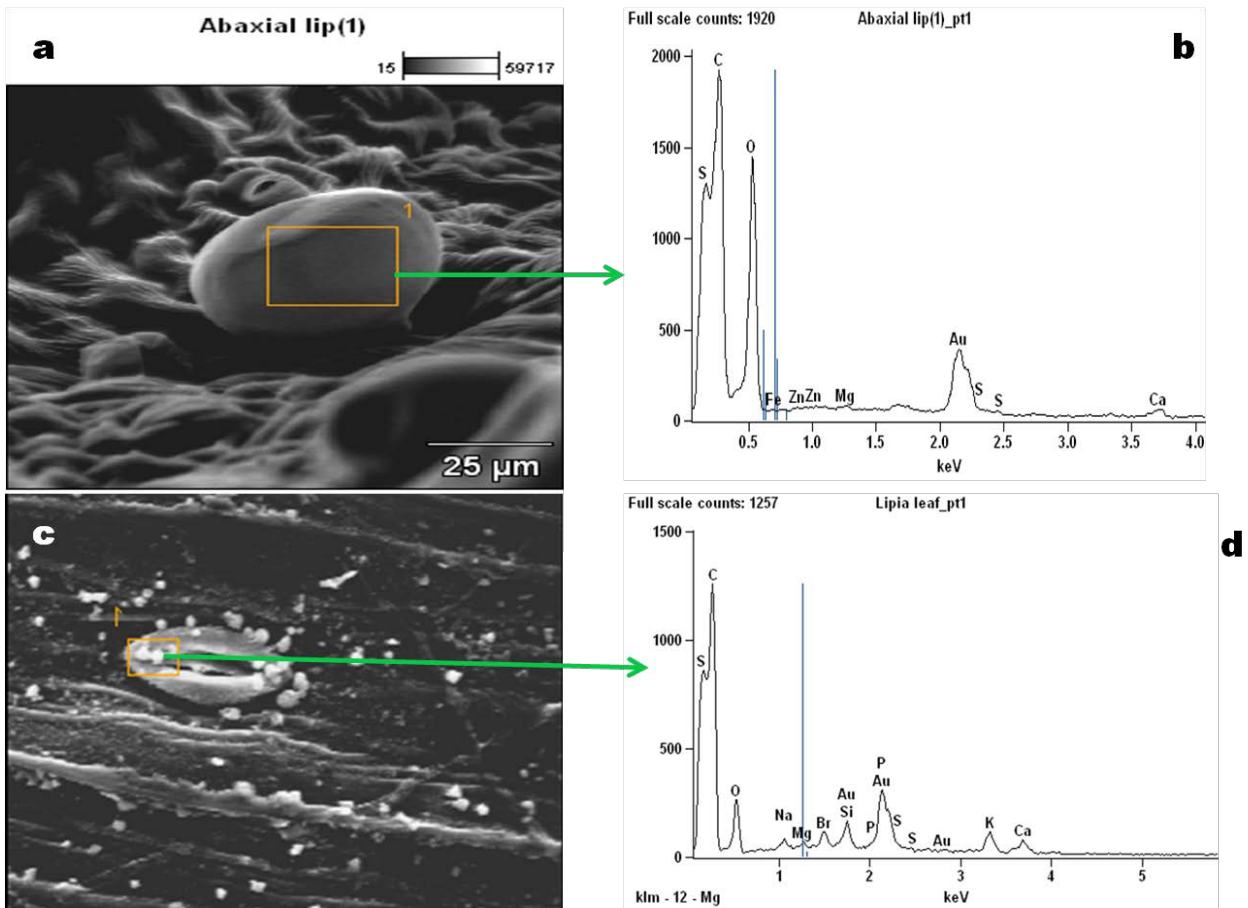


Figure 6. a and c: SEM photograph showing crystal deposits on leaf; b and d: and EDX chromatogram showing elemental composition of crystals in *L. javanica*

Appearance of crystals - In this study, crystals of different sizes and irregular shapes were observed in the leaves of the species investigated (Figure 4 a, b, c & d). This is similar to the observations of Adedeji (2012) on *Stachytarpheta* Vahl. belonging to family Verbenaceae. Rectangular, prism, triangular and spherical shaped crystals in aggregates were confirmed in *Lippia javanica*. According to Adedeji (2012), crystals in the form of small needles or prisms which are widely distributed in all the parenchymatous tissues of leaves axis, of the family Verbenaceae were also observed in *L. javanica* which was characterized with calcium oxalate formation. Crystallization is the most common way by which plants neutralise abundant calcium absorbed in ionic solution. This crystal remains when water vapour transpires. Crystal formation and its distribution in various organs are a common phenomenon in higher plants (Lerstern and

Horner, 2006). The crystals are often classified as drusa, sand, prismatic, raphides or styloid (Maiti et al., 2002; Franceschi and Nakat, 2005; Lerstern and Horner, 2006; Badmus and Afolayan, 2010). They are always formed within cells, which are remarkably diverse among angiosperms (Wintola and Afolayan, 2014).

Stomata distribution - Stomata are minute apertures bounded by two guard cells. They can be interpreted as intercellular spaces between the highly specialised guard cells. Regarding the position of guard cells in relation to the epidermal cells, they are found to be at the same level. The two guard cells of a stoma are almost equal in size, bean-shaped and surrounded by 2 to 5 subsidiary cells (Akhil and Subhan, 1997). The diacytic, anisocytic, anomocytic, circular to elliptic are different shapes of stomata in plants. Leaves of *Lippia javanica* investigated are characterized by amphistomatic, stomata which are more or less randomly distributed over the epidermis lying almost close to each other and are fewer in number in between the veins and over finer veins (Figure 4 a, b, c & d and Figure 5 a & b).

Elemental composition - The chemical nature of the crystal deposits on *L. javanica* leaves as observed using EDX showed that they were predominantly composed of Ca, Na, S, Al, S, P, Cl, K, Mg and Fe (Table 3). These elements (micro and macro) are very essential for plant growth. Various functions have been attributed to their presence. These include regulation of Calcium (Ca) levels in plant tissues and organs, involvement in photosynthetic processes and detoxification of heavy metals or oxalic acid in plants (Franceschi and Nakata, 2005; Badmus and Afolayan, 2010). The high percentage of oxygen observed in the plant confirmed the use of oxygen in the transport of other nutrients into plants but calcium is required to make nutrients mobile within the plant tissue (Figure 6 a & b). In humans, the presence of calcium is essential for healthy bones, teeth, blood and regulation of skeletal, heart and tissue muscles, while magnesium has been reported to be helpful in fighting heart disease, stroke and in cell repair. Iron may increase packed cell volume, boost the immune system and prevent anaemia in humans (Larson and Wolk, 2007;

Agunbiade et al., 2012; Afolayan and Otunola, 2014). Therefore, these elements and their various functions as well as the bioactives present in the secretory trichomes, could account for the therapeutic actions of *L. javanica*.

Table 3: Elemental composition (%) of *Lippia javanica* leaves as shown by Energy Dispersive X-ray (EDX) analysis.

Elemental composition	<i>Lippia javanica</i> leaf
Calcium (Ca)	6.98
Oxygen (O)	36.46
Sodium (Na)	0.56
Aluminium (Al)	0.74
Silicon (Si)	25.81
Phosphorus (P)	0.27
Sulphur (S)	0.11
Chlorine (Cl)	0.23
Potassium (K)	1.89
Iron (Fe)	1.49
Bromine (Br)	1.37
Carbon (C)	-
Magnesium (Mg)	-

Conclusion

Lippia javanica (Verbenaceae) is an aromatic indigenous South African plant with medicinal values. The foliar epidermal surfaces and trichomes of this plant species were investigated using light and scanning electron microscopy (SEM) connected with energy dispersive x-ray spectroscopy (EDX). Results showed that the leaves of *Lippia javanica* was amphitrichomic while the stomata distribution was characterized by amphistomatic, these were more or less randomly distributed over the epidermis lying almost close to each other and were fewer in number in between the veins and over finer veins. The major constituents of crystals found in the plant were Ca, Na, S, Al, S, P, Cl, K, Mg and Fe. The presence of non-glandular trichomes on the leaf surfaces of this plant may serve as secretory sites where the aromatic secondary metabolites are produced.

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The micromorphology of *Foeniculum vulgare* (Apiaceae)

Introduction

There is no information regarding the leaf, stem, shoots, flowers and seed anatomy and micromorphology of *Foeniculum vulgare*. The aim of this study therefore is to examine the ultrastructural morphology and elemental compositions of the leaves, stems and seeds of *Foeniculum vulgare* using scanning electron microscope (SEM) and to relate our findings to their possible functional role in the production of therapeutic compounds and nutritional values.

Materials and Methods

Collection and identification of plant materials

The leaves of *Foeniculum vulgare* were collected from their natural habitat where it grows in abundance in the wild in Alice, Eastern Cape Province of South Africa. The plant was identified in Rhodes University by Tony Dold and voucher specimen (ASO 2014/2) was prepared and deposited in the Giffen Herbarium of the University of Fort Hare, Alice.

Scanning Electron Microscopy (SEM)

The SEM ultra-structural examination of the leaf, stem and seed of *Foeniculum vulgare* was carried out using the following method. Fresh leaves of *Foeniculum vulgare* were immersed in a fixative solution of 2.5% glutaldehyde in 0.1 M phosphate buffer for 24 hours. Samples were washed for 15-30 minutes with the buffer and dehydrated in graded ethanol series (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%). Samples were then critically point dried using CO₂ sputter coated with gold under vacuum and viewed using the Scanning Electron Microscope (SEM) equipped with an Energy Dispersive X-ray (EDX) Oxford ISIS 300 micro analytical system and the necessary software for point micro analysis. Images were captured digitally with

an image saver connected with an EDX. The major microscopic structures observed in the leaf, stem and seed were: type of stomata, outline of epidermal cells and type of crystals if any.

Results and Discussion

Appearance of the epidermal cells and stomata distribution

The leaf and stem of *Foeniculum vulgare* as observed under SEM showed considerable amount of amphistomatous (equal distribution of stomata on both adaxial and abaxial surfaces) stomata densely distributed on the leaf and stem surface (Figures 7 & 8) on the epidermal surfaces. Stomata function primarily for gaseous exchange during respiration and photosynthesis in the leaves of plants. It absorbs CO₂ thereby reducing the amount of carbon in the atmosphere which is a major driver for global warming and releases O₂ for human and animal use. Water is also released through the stomata hence the amount of transpiration (release of water from plant into the atmosphere) is highly mediated through the stomata; hence there is a correlation of the number of stomata and transpiration (water loss) in plant. The densely amphistomatous stomata in *F. vulgare* suggest its habitat which is in the open with adequate amount of water intake because plants growing in shady and drought prone environments have sparse distribution of stomata (Pavina et al., 2013). The stomata is diacytic while the guard cells are elliptical in shape, this is in agreement with the report of Annal et al., (2006) on taxonomic significance of leaf characters in some species of Apiaceae.

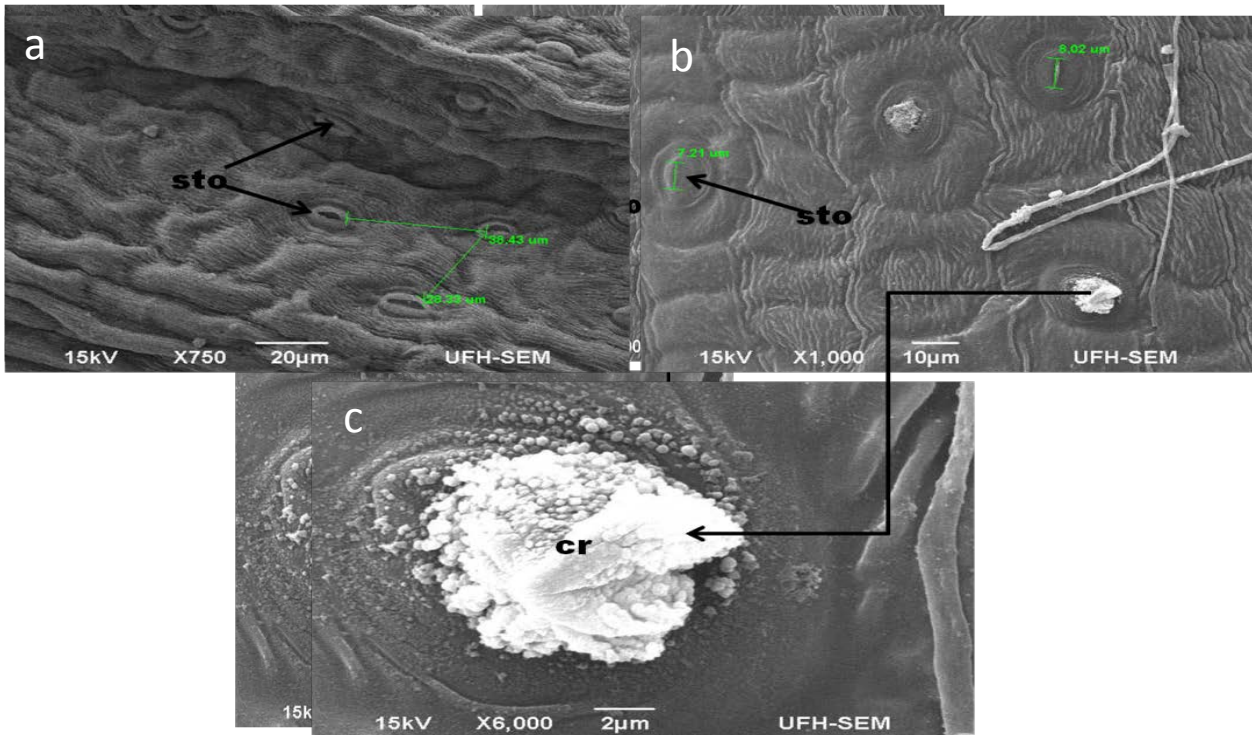


Figure 7: SEM photograph of a- epidermal surfaces and stomata distribution (x750) showing the distance between stomata b- crystal deposit from a stoma (x 1000) c- shows styloid crystals at higher magnification x 6000 in *Foeniculum vulgare*

The structure of the stem in figure 8 shows a longitudinal form of lenticels (a type of pore) which serve as a site of gas exchange on the plant (place where oxygen and carbon dioxide can enter and exit). Lenticels are also, unfortunately, a site where pathogens such as fungi, bacteria and viruses can enter the plant. Figure 9 shows a cross section of the vascular bundle of the xylem (arranged in ringed shapes) and phloem tissues of the stem. The xylem tissue serves as a vessel for transporting water, dissolved minerals and fibres which support the plant (Heywood et al., 2007). The phloem transports organic substances through the stem. It also shows the presence of droplets of mineral sediments on the phloem tissue which are translocated to all parts of the plant. It also shows the structure of pith which help to store water and starch, and also help in exchange of gases through the intercellular air spaces. These features indicate that *Foeniculum vulgare* contain a lot of mineral deposit and can be of significance to microorganisms and man.

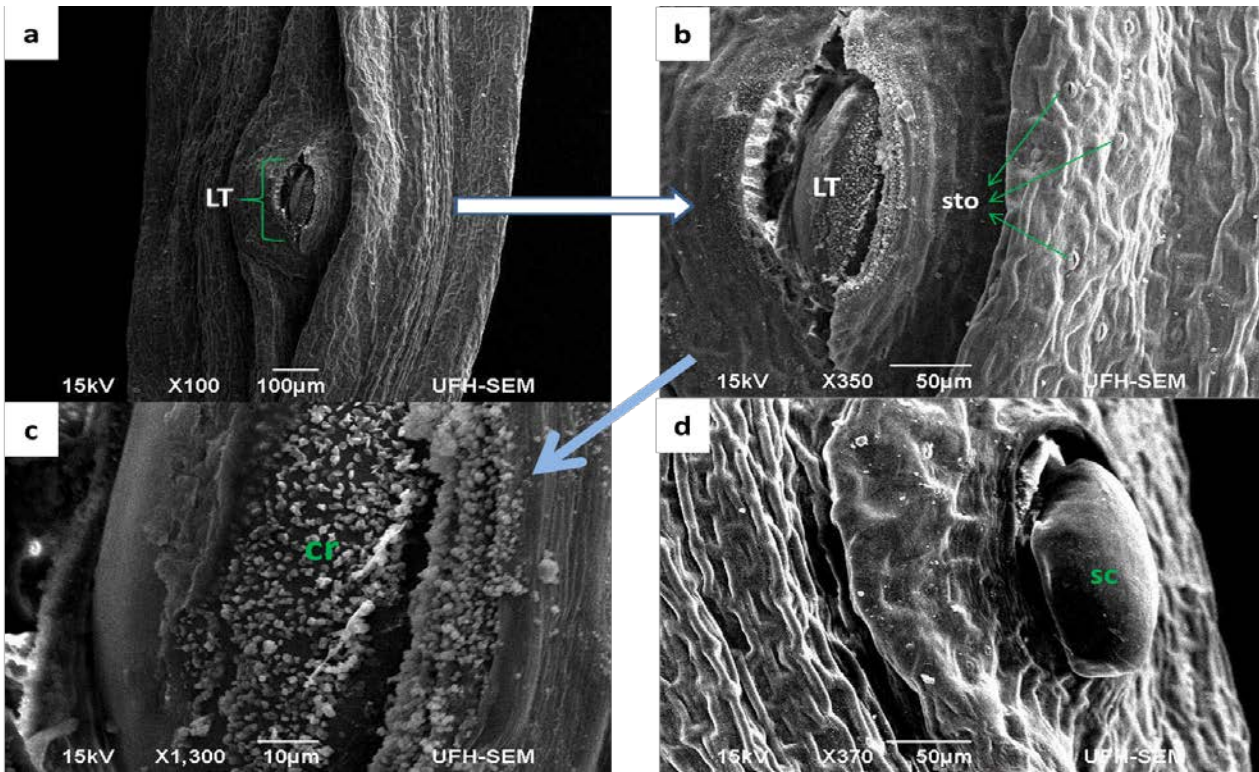


Figure 8: Scanning Electron Micrograph of *Foeniculum vulgare* stem (a & b) transverse sectioning of the stem showing the lenticels (LT); (c & d) stem having mineral sediments.

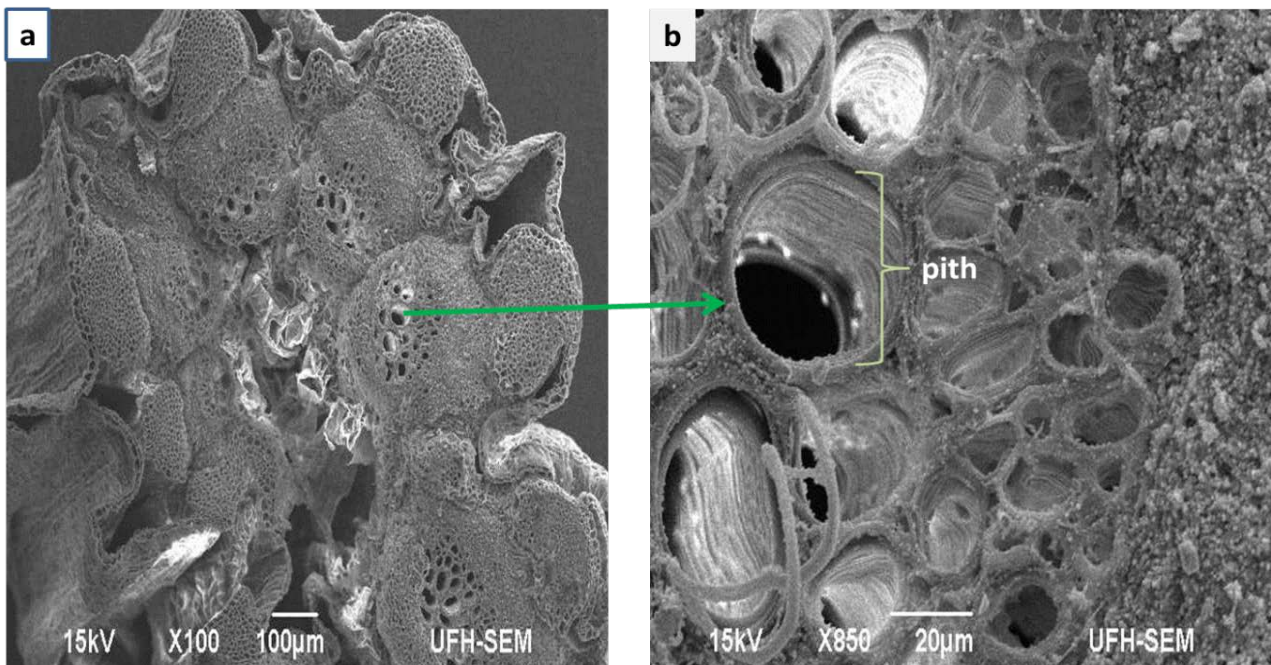


Figure 9: Scanning Electron Micrograph of *Foeniculum vulgare* stem (a) cross sections showing the inner rings of xylem tissues; (b) showing the pith.

Appearance of crystals and elemental composition

Crystal formation and its distribution in various organs are a common phenomenon in higher plants (Lerstern and Horner, 2006). The crystals are often classified as drusa, sand, prismatic, raphides or styloid (Maiti et al., 2002; Franceschi and Nakat, 2005; Lerstern and Horner, 2006; Badmus and Afolayan, 2010). In this study, styloid crystals were observed in *Foeniculum vulgare* (Figure 7 & 8c). Although, the significance of crystals and their formation in plants is largely unknown, various functions have been attributed to their presence (Nakata and McConn, 2000).

The X-ray microanalysis of the leaf, stem and seed of *F.vulgare* generated spectra of the following micro and macro mineral elements; Calcium (Ca), Oxygen (O), Sodium (Na), Aluminium (Al), Silicon (Si), Phosphorus (P), Sulphur (S), Chlorine (Cl), Potassium (K), Iron (Fe), Bromine (Br), Carbon (C), Magnesium (Mg) (Figures 10, 11 & 12). The major elemental constituents of *Foeniculum vulgare* are Ca, Na and S while Al, K and Mg were present in minute quantity (Table 4). Calcium is essential for muscle contraction, oocyte activation, building strong bones and teeth blood clotting, nerve impulse regulating heart beat and fluid balance within cells (Pavina et al., 2013). The high percentage of calcium observed may be required to make nutrients mobile within the plant tissue while sulphur is involved in cellular respiration, protein synthesis, cell regeneration and blood cleansing which helps the cells to use oxygen efficiently. Magnesium has been reported to be helpful in fighting heart disease, stroke and in cell repair in humans. The body needs a small amount of sodium to help maintain blood pressure and normal function of muscle and nerves (Pavina et al. 2013). Therefore, these elements and their various functions as well as the bioactivities present in the secretory cells, crystals and vascular bundles, could account for the therapeutic actions of *Foeniculum vulgare*. A further study to evaluate the metabolites present in this plant is in progress.

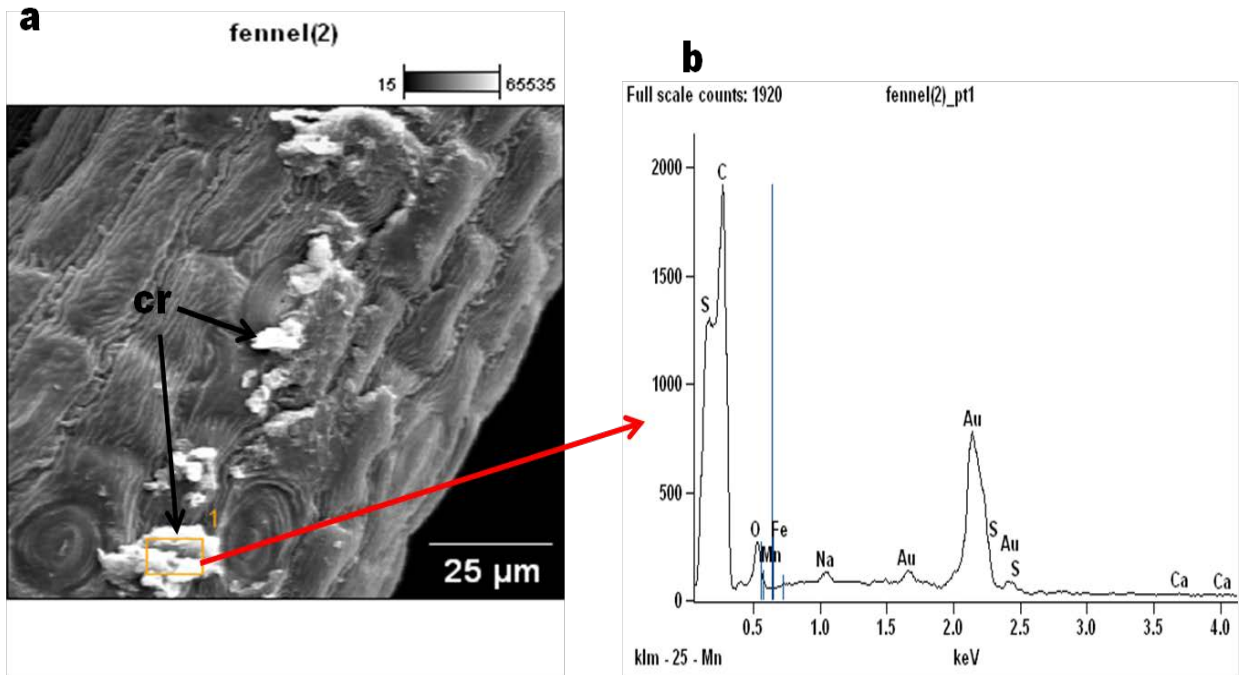


Figure 10: Energy Dispersive X-ray spectroscopy of the leaf surface of *Foeniculum vulgare*.

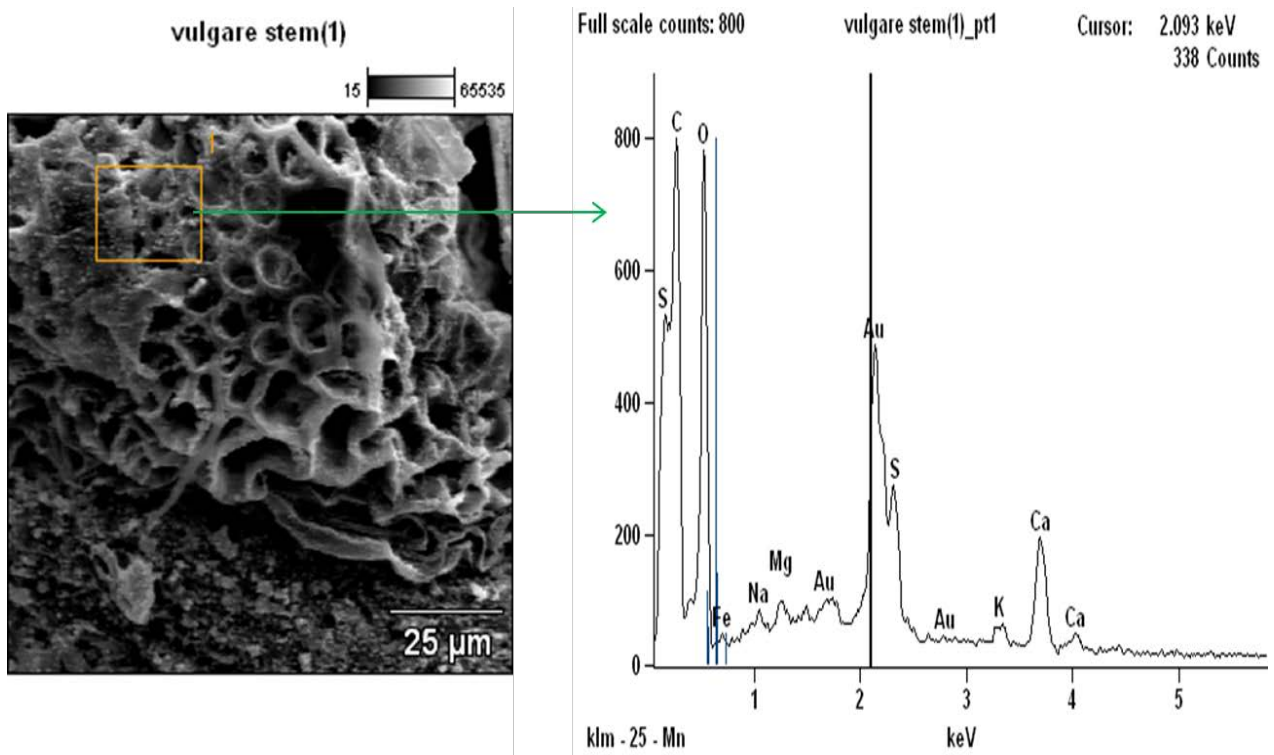


Figure 11: Energy Dispersive X-ray spectroscopy of the stem surface of *Foeniculum vulgare*.

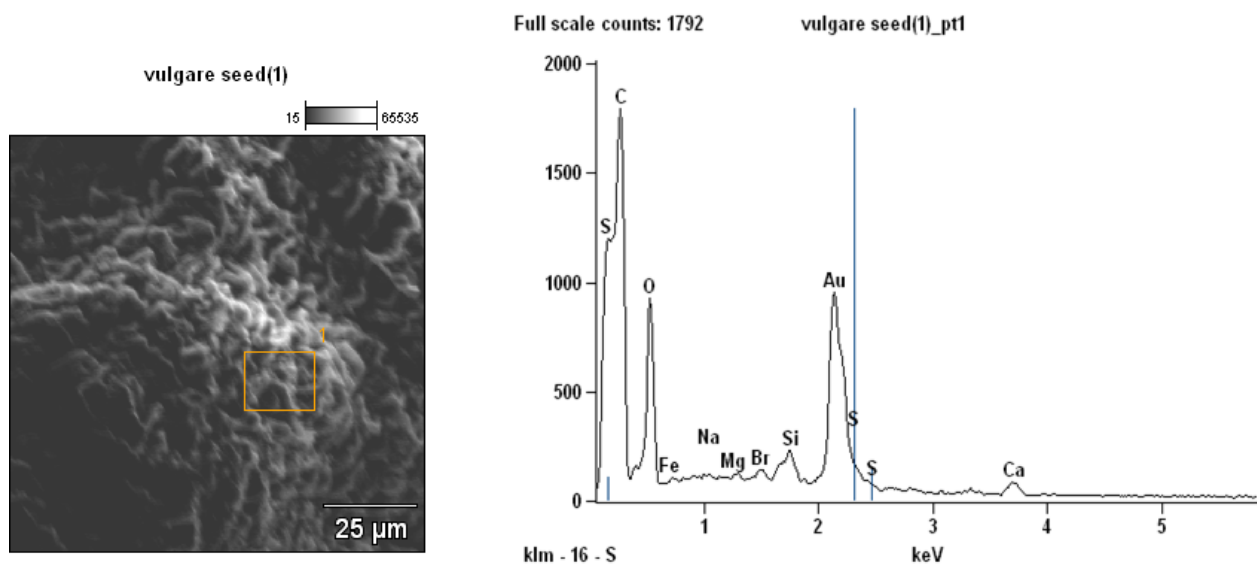


Figure 12: Energy Dispersive X-ray spectroscopy of the seed surface of *Foeniculum vulgare*.

Table 4: Elemental composition (%) of *Foeniculum vulgare* leaves as shown by Energy Dispersive X-ray (EDX) analysis.

Elemental composition	Leaf (%)	Stem (%)	Seed (%)
Calcium (Ca)	3.96	5.54	2.30
Oxygen (O)	41.6	28.09	37.84
Sodium (Na)	0.47	0.42	0.16
Aluminium (Al)	-	-	-
Silicon (Si)	-	-	2.36
Phosphorus (P)	-	-	-
Sulphur (S)	0.7	4.58	2.07
Chlorine (Cl)	-	-	-
Potassium (K)	0.72	0.83	-
Iron (Fe)	-	0.28	0.44
Bromine (Br)	-	-	1.27
Carbon (C)	32.09	16.19	39.86
Magnesium (Mg)	0.76	0.54	0.24

Conclusion

Foeniculum vulgare (Apiaceae) is an aromatic indigenous South African plant with culinary and medicinal values. The epidermal surfaces of leaf and stem of this plant species was investigated using light and scanning electron microscopy (SEM) connected with energy dispersive x-ray (EDX) spectroscopy. Stoma is well distributed over the surfaces of both leaf and stem with no evidence of a trichome. Styloid crystals were observed on the leaf surface. The major elemental constituents of *Foeniculum vulgare* were Ca, Na and S while Al, K and Mg were also present in minute quantity. The absence of trichomes which is a storage and secretory structure (for major phytochemicals) of leaf suggests that other specialized structures may be responsible for their storage of the phytochemicals.

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CHAPTER FIVE

Comparative nutrient, mineral, vitamin and antinutrient composition of *Lippia javanica* and *Foeniculum vulgare*.

Introduction

Spices are known to add flavour to foods and beverages, but they also help to stimulate the appetite (Bassey et al., 2001). Spices play important roles in the diet and traditional medicine such as aids digestion (cardamom), speed up of metabolism (cayenne pepper), anti-hypertensive (celery) and antimicrobial (basil). In recent times, the use of spices has gained much attention because of their roles in food. The place of spices in the diet is usually not considered to be important from the nutritional point of view, though they are widely used throughout the world. According to Pradeep et al., (1993), spices are not normally included in diet surveys nor are they suggested or recommended in what is known as balanced diets, probably because it was thought that the intake of these spices was so small that their contribution to body nutrients is insignificant. However, spices have been observed to be good sources of many nutrients and the percentage contribution of spices to the total intake of various nutrients could range from 1.5 to 14.5% depending on the nutrients (Pradeep et al., 1993). Many spices are rich in health promoting components, including vitamins, minerals, low / unsaturated fats, high fibre and other bioactive factors. Apart from their nutritive values, reports have also shown that spices possess natural compounds known as antinutrients such as saponins, phytic acid, tannin and oxalate. Phytic acids are used as a unique and versatile food preservative. Also, the addition of phytic acids into food increase nutritive value, prolong shelf life and prevent discolouration of foods (Egbuna & Ifemeje, 2015). Dietary saponins from different plants were found to reduce weight gain in salmon fish, reduce fertility and cause ruminant bloat and photosensitization. It affects protein digestibility by inhibiting various digestive enzymes such as trypsin and chymotrypsin (Shimoyamada et al.,

1998). In general, phytic acids, flavonoids and tannins are anti-nutrients that interfere with the absorption of minerals from the diet (Beecher, 2003).

Despite various usage of spices, there is little information about the nutritional value of indigenous spices. Although there are some information on the nutrient composition of other spices, the data available so far is not adequate, in particular, there is a paucity of information on the nutrient content of *Lippia javanica* and *Foeniculum vulgare*. Therefore, this study is aimed at rectifying this. Nutrient composition data for these spices are necessary to increase the knowledge of biodiversity in food composition, facilitate health intervention research, develop nutrient databases that will support both practical and research applications (Philips et al., 2014) as well as to encourage their increased cultivation and consumption.

Materials and Methods

Preparation of Plant Materials

Each spice was individually oven-dried at 45°C, pulverized, and stored in airtight glass containers at 4°C prior to analysis.

Proximate Analysis

Moisture content

This was determined by weighing out approximately 5 g of ground sample into preweighed petri dishes and placed in an oven set at 105°C for 12 h. The sample was allowed to cool in a desiccator, weighed again, until constant weight was obtained. Moisture content was calculated as described previously (Antia et al., 2006):

$$\text{Moisture content \%} = 100 \times \frac{(B-A) - (C-A)}{(B-A)}$$

where: A= Weight of clean, dry scale pans (g), B= weight of scale pan+ wet sample (g), C= weight of the scale pan + dry sample (g)

Ash content

2 g of powdered sample was placed in pre-weighed crucibles and incinerated in an E-range muffle furnace with the TOHO program at 550°C for 12 h, Ash content of the sample was calculated as:

$$\text{Ash content \%} = \frac{\text{Weight after ashing-weight of empty crucibles}}{\text{Weight of crucible and sample-weight of empty crucible}} \times 100$$

Crude lipid

5 g of the powdered sample was weighed into 250 ml conical flask, 100 mL of diethyl ether was added, covered with aluminium foil and shaken in an orbital shaker for 24 h, filtered and the supernatant decanted. Another 100 mL of diethyl ether was added to the residue and shaken for another 24 h, residue obtained after filtration was the lipid free sample and it was calculated as:

$$\text{Crude lipid} = \frac{\text{Weight of diethyl ether extract}}{\text{Initial weight of the sample}} \times 100$$

Crude fibre

5 g of the powdered sample was weighed and digested in 100 mL of 1.25% sulphuric acid for 30 minutes, acid digested sample was allowed to cool and then filtered. The residue was collected for further digestion with 100 mL of 1.25% sodium hydroxide and was filtered. The residue was dried in an oven at 100°C to a constant weight and the dried residue was incinerated in a muffle furnace for 24 h at 550°C. The crude fibre was calculated from the loss in weight on ignition of dried residue after digestion of fat free samples.

$$\% \text{ fibre} = \frac{\text{loss of weight on ignition}}{\text{Weight of sample used}} \times 100$$

Crude protein (%)

2 g of powdered sample was digested in a Kjeldahl digestion flask by boiling with 20 mL of concentrated H₂SO₄ and a Kjeldahl digestion tablet (catalyst) until the mixture was clear. The digest was filtered into a 250 mL volumetric flask and the solution made up to mark with distilled water and connected for distillation. Ammonia was steam distilled from the digest to which had been added 50 mL of 45% sodium hydroxide solution. 150 mL of the distillate was collected in a conical flask containing 100 mL 0.1N HCl and methyl red indicator. The ammonia that was distilled into the receiving conical flask reacted with the acid and the excess acid in the flask was estimated by back titration against 2.0M NaOH with colour change from red to yellow (end point). Determinations were made on all reagents alone (blank determinations).

% Nitrogen was calculated as follows:

$$\frac{[(\text{ml standard acid} \times \text{N of acid}) - (\text{ml blank} \times \text{N of base})] - (\text{ml std base} \times \text{N of base}) \times 1.4007}{\text{Weight of sample in grams}}$$

where, N = normality

Percentage crude protein was obtained by multiplying the nitrogen value by a factor of 6.25.

% crude protein = Nitrogen in sample x 6.25

Digestible carbohydrate by (difference)

The total carbohydrate content was determined by adding together the sum of the percentage moisture, ash, crude lipid, crude protein and crude fibre, then subtracting this value from 100 as shown (Muller & Tobin, 1980)

% Total carbohydrate = 100 - (% moisture + % Ash + % fat + % Protein + % Fibre)

The percentage contribution to energy

The percentage contribution to energy due to protein (PEP), total fat (PEF) and carbohydrate (PEC) respectively were calculated. The percentage utilizable energy due to protein (UEDP %) was also calculated (Hathcock, 1985).

PEF was calculated as follows

$$\text{PEF} = \text{Fat} \times 37 \text{ (constant value for fat)} \dots\dots\dots \text{A}$$

$$\text{PEP} = \text{Protein} \times 17 \text{ (constant value for protein)} \dots\dots\dots \text{B}$$

$$\text{PEC} = \text{CHO} \times 17 \text{ (constant value for protein)} \dots\dots\dots \text{C}$$

$$\% \text{ PEF} = \text{A} / \text{total (A+B+C)} \times 100 \dots\dots\dots \text{D}$$

$$\% \text{ PEP} = \text{B} / \text{total (A+B+C)} \times 100 \dots\dots\dots \text{E}$$

$$\% \text{ PEC} = \text{C} / \text{total (A+B+C)} \times 100 \dots\dots\dots \text{F}$$

$$\text{UEDP} = \text{B} / \text{total (D +E + F)} \times 60 \text{ (constant for UEDP)}$$

$$\text{Total energy} = \text{D} + \text{E} + \text{F} \text{ (KJ/100g)}$$

Mineral Analysis

Mineral content of the spices were determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP/OES) (Bvenura & Afolayan, 2012). Ca/P, Na/K, Ca/Mg and the millequivalent ratio of $[\text{K}/(\text{Ca} + \text{Mg})]$; the mineral safety index (MSI) of Na, Mg, P, Ca, Fe and Zn were also calculated. Selenium powder, sulphuric acid and salicylic acid were used as the digestion mixture. The finely ground material was divided into samples of 0.3 g, which were placed in dry, clean digestion tubes. A volume of 2.5 mL of the digestion mixture was added to each tube and allowed to react at room temperature for 2 h. The tubes were heated in a block digester at 110°C for 60 min. After removal from the digester, the tubes were allowed to cool and three successive portions of 1 mL hydrogen peroxide were added, allowing at least 10 s between

each addition because of the volatility of the reaction. The tubes were returned to the block digester at a temperature of 330°C and were removed from the block digester when the digest was colourless. The tubes were allowed to cool to room temperature before their contents were transferred to 50 mL volumetric flasks and deionised water was added to attain volumes of 50 mL. Standards were prepared for all the elements. The samples were then analysed for the various elements by an inductively coupled plasma optical emission spectrometer (ICP-OES; Varian 710–ES series, SMM Instruments, Cape Town, South Africa).

Vitamins determination

Vitamin C

The Vitamin C (Ascorbic acid) content of the plant was determined by a modified spectrophotometric method as described by Simeon et al., 2014. Briefly, 2.5 g of coarsely powdered sample was weighed and 12 mL of glacial acetic acid added. The mixture was stirred for about 20 min and filtered. The filtered solution was made up to 100 mL using distilled water. From this, 50 mL of the sample solution was mixed with 10 µL of methylene blue solution (0.4 mmol/L) and diluted to 10 mL with distilled water. Absorption was measured at 665 nm using a spectrophotometer (AJ-IC03). Stock solution of ascorbic acid (1M) was prepared by dissolving 10 g of ascorbic acid in 56.76 mL of distilled water. The different concentrations were prepared by diluting the stock standard solution in water before use and absorption was also measured at 665 nm. The calibration graph was drawn by plotting the absorbance against concentration of ascorbic acid. The obtained calibration curve was linear in a concentration range of 0.1 to 1M with the linear regression equation as;

$y = 0.7535x$, $R^2 = 0.749$, where y is the absorbance and x is the concentration of Vit C.

Vitamin A

One gram (1 g) of ground sample was macerated with 20 mL of petroleum ether. This was decanted into a test tube and then evaporated to dryness. About 0.2 mL of chloroform-acetic anhydride (1:1 v/v) was added to the residue. A 2 mL of Trichloroacetic acid-chloroform in like (1:1 v/v) was added to the resulting solution and absorbance was measured at 620 nm. Vitamin A standard was prepared in the same procedure and the absorbance taken at 620 nm. The concentration of vitamin A in the sample was extrapolated similarly as in vitamin C from the standard curve using the calibration curve equation;

$y = 0.7365x$, $R^2 = 0.8244$, where y is the absorbance and x is the concentration of Vit A.

Vitamin E

One gram (1 g) of the sample was macerated with 20 mL of ethanol and then filtered. Then, 0.2% ferric chloride in ethanol and 1 mL of 0.5% α - α -dipyridine was prepared and added to 1 mL of the filtrate. This was diluted to 5 mL with distilled water. Absorbance was taken at 520 nm. The standard solutions were prepared similarly as in vitamin C and the concentration of vitamin E extrapolated from the standard curve using the calibration curve equation;

$y = 0.5544x$, $R^2 = 0.6769$, where y is the absorbance and x is the concentration of Vit E.

Correlation coefficients (R^2) are expressed as values between +1 and -1. A coefficient of +1 indicates a perfect positive correlation: A change in the value of one variable will predict a change in the same direction in the second variable. A coefficient of -1 indicates a perfect negative correlation. A change in the value of one variable predicts a change in the opposite direction in the second variable. Lesser degrees of correlation are expressed as non-zero decimals. A coefficient of zero indicates there is no discernable relationship between fluctuations of the variables (Weinsstein, 2016). See appendix c

Antinutrients

Total Tannin

0.2 g of sample was measured into a 50 mL beaker; 20 mL of 50% methanol was added, covered with foil paper and placed in a water bath for 1 h. It was shaken thoroughly to ensure uniform mixing. The extract was filtered using a double layered Whatman No 41 filter paper into a 100 mL volumetric flask. To this, 20 mL of distilled water, 2.5 mL Folin –Denis reagent and 10 mL of 17% Na₂CO₃ were added and mixed thoroughly. The mixture was made up to mark with distilled water and allowed to stand for 20 minutes to develop the bluish green colour. Absorbance of tannic acid, the standard solution and samples were read on a Spectronic 21D at a wavelength of 760 nm. The average gradient factor content was extrapolated from the standard curve (Awe & Sodipo, 2001). Percentage tannin was calculated as:

$$\% \text{ Tannin} = \frac{\text{Absorbance of sample} \times \text{average gradient factor} \times \text{Dilution factor}}{\text{Weight of sample} \times 10000}$$

Total Saponin

20 g of the plant sample was added to 100 mL of 20% ethanol and kept on a shaker for 30 minutes. The aliquot was then heated over a water bath at 55°C for 4 h. The resulting mixture was filtered and the residue re-extracted with 200 mL of 20 % aqueous ethanol. The mixture was reduced to 40 mL over a water bath at 90°C. The concentrate was transferred into 250 ml separatory funnel and extracted twice with 20 mL diethyl ether. The ether layer was discarded while the aqueous layer was retained, followed by the addition of 60 mL of n-butanol. The butanol extract was washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution was heated over a water bath and evaporated to dryness to constant weight at 40°C. The saponin content was calculated using the equation (Obadoni & Ochuko, 2002).

$$\% \text{ Saponin content} = \frac{\text{Weight of residue} \times 100\%}{\text{Weight of sample}}$$

Determination of phytic acid

2 g of each sample were weighed into 250 mL conical flask. 100 mL of 2% conc HCl was used to soak the samples in the conical flask for 3 h and then filtered through a double layer filter paper. 50 mL of each of the sample filtrate were placed in a 250 mL beaker and 107 mL of distilled water was added to give/ improve proper acidity. 10 mL of 0.3% ammonium thiocyanate solution was added to each sample solution as indicator and titrated with standard iron chloride solution which contained 0.00195 g iron/mL and the end point was signified by brownish-yellow colouration that persisted for 5 min (Aina et al., 2012). The percentage phytic acid was calculated as: % Phytic acid = $y \times 1.19 \times 100$, where, y = titre value \times 0.00195 g.

Determination of cyanogenic glycosides content

The alkaline picrate method was employed for the determination of cyanogenic glycosides. A 5 g portion of each sample was dissolved in 50 mL distilled water in a corked conical flask and extracted for 12 h. It was then filtered and the filtrate was used for the cyanide content determination. To 1 ml of the filtrate in a corked test tube, 4 mL of alkaline picrate was added and incubated in a water bath for 5 minutes. The absorbance was read at 490 nm. The absorbance of the blank, which contained only 1 mL distilled water and 4 mL alkaline picrate solution was read and used to stabilize the spectrophotometer before taking the absorbance of the samples (Jonathan & Funmilola, 2014). The cyanide content was extrapolated from a cyanide standard curve and calculated using the formula:

$$\text{Cyanide} = \frac{\text{Absorbance} \times \text{Gradient factor} \times \text{Dilution factor}}{\text{Weight of sample}}$$

Determination of Oxalate

0.2 g of sample was put in a 20 mL conical flask, 15 mL of 3M H₂SO₄ was added and stirred for 1 h with a magnetic stirrer then filtered using a Whatman No 1 filter paper. 5 mL of the filtrate was titrated against hot 0.05M KMnO₄ solution till a faint pink colour persists for 30 seconds. Oxalate content was then calculated by taking 1 mL of 0.05 M KMnO₄ as equivalent to 2.2 mg oxalate (Dauda et al., 2014).

Statistical analysis

All experiments were done in triplicates and the results were expressed as Mean \pm SD. Where applicable, the data were subjected to student t-test to compare the difference between the acetone and aqueous extracts using the Minitab program (version 12 for windows) (Minitab Inc., Pennsylvania, USA). $p < 0.05$ were considered significant.

Results and Discussion

The proximate analysis of *Lippia javanica* and *Foeniculum vulgare* showed that there was no significant difference in the moisture content of both spices (Table 5). Moisture content of a food sample is an index of water activity and is used as a measure of stability and susceptibility to microbial attack. Therefore, the low moisture content of the spices is an indication that they can be preserved for a reasonable length of time without the risk of microbial deterioration and spoilage (Mcneely, 1990). Ash content in *Lippia javanica* (7%) was less than in *Foeniculum vulgare* (12%). Ash content is an indication of the levels of minerals or inorganic component of the samples. *Foeniculum vulgare* had a higher level of crude fibre (7%) compared to *Lippia javanica* (5%). The fibre content of both spices was quite appreciable and indicates that *Lippia javanica* and *Foeniculum vulgare* could help the body to maintain an internal distention for proper peristaltic movement of the intestinal tract. A diet with high fibre content have been used for

weight control and fat reduction as they give a sense of satiety even when small food is eaten (COMA, 1984).

The daily energy requirement for an adult is between 2500-3000 kCal (10455-12548 KJ) depending on the physiological state while that of infants is 740 kCal (3094.68 KJ) (Jonathan & Funmilola, 2014). This means that diets with higher energy will require lower quantity of the spices to satisfy the percentage utilizable energy due to protein (UEDP %) assuming 60% utilization, 9% and 1% for *Lippia javanica* and *Foeniculum vulgare* respectively. The UEDP % compared favourably with the recommended safe level of 8% for an adult who requires about 55g protein per day with 60% utilization. The proportion of total energy due to fat (PEF %) values were similar in *Lippia javanica* (1%) and *Foeniculum vulgare* (2%).

Table 5: Proximate composition of *Lippia javanica* and *Foeniculum vulgare* (%)

Nutrient	<i>Lippia javanica</i>	<i>Foeniculum vulgare</i>
Ash	6.75 ± 2.81 ^a	11.60 ± 2.33 ^a
Moisture	10.73 ± 1.10 ^a	11.69 ± 2.94 ^a
Lipid	0.50 ± 0.31 ^a	0.67 ± 0.29 ^a
Crude Fibre	5.00 ± 0.00 ^a	6.67 ± 5.77 ^b
Crude protein	12.06 ± 1.19 ^a	20.54 ± 0.51 ^b
Digestible carbohydrate	64.96 ± 0.88 ^a	48.83 ± 0.30 ^a
PEF	1.39	2.06
PEP	15.44	68.94
PEC	83.17	28.99
UEDP	9.26	1.02
Energy KJ/100g	1327.84	1204.08

Note: Moisture content, Ash, lipid and carbohydrate shows no significant different in *Lippia javanica* and *Foeniculum vulgare* ($p > 0.05$) while the crude fibre and crude protein were significantly different ($p < 0.05$), values are Means ± SD of triplicate samples; means with different superscript are significantly different (PEP=Proportion of total energy due to protein, PEF=Proportion of total energy due to fat, PEC=Proportion of total energy due to protein, UEDP = Utilizable energy due to protein).

This is far below the recommended level of 30% and 35% for total fat intake, which can also be used for weight control and fat reduction; this can be helpful for people trying to maintain weight through a healthy diet (Nkafamiya, 2010).

Minerals act as inorganic co-factors in metabolic processes which mean that the absence of these inorganic co-factors could lead to impaired metabolism (Dauda et al., 2014). Table 6 shows the mineral contents and ratios of the two spices. Ca, Mg, K, P, Na and Fe were also comparably high in the two species, although *Foeniculum vulgare* had higher N, Mg and K content, while *Lippia javanica* was higher in calcium. Potassium is very important in maintaining the blood fluid volume and osmotic equilibrium, the high level of potassium in both *Lippia javanica* (1907 mg/100g) and *Foeniculum vulgare* (3187 mg/100g) implies that the two spices could be a source of potassium and could help in maintaining the blood fluid volume and osmotic equilibrium. Phosphorus level in the two plants were the same (400 mg/100g), both spices had a higher phosphorus content compared to other plants reported (Nkafamiya et al., 2010).

Calcium is essential for healthy bones, teeth, blood and regulation of skeletal, heart and tissue muscles, while magnesium has been reported to be helpful in fighting heart disease, stroke and in cell repair. Iron may increase packed cell volume, boost the immune system and prevent anaemia in humans (Agunbiade et al., 2012; Afolayan & Otunola, 2014). The Ca/P in *Lippia javanica* (4.58 mg/100g) and *Foeniculum vulgare* (4.17 mg/100g) is higher than the minimum ratio (0.5) required for favourable calcium absorption in the intestine for bone formation (Adeyeye & Adesina, 2012; Dauda et al., 2014). Also for normal retention of protein during growth and for balancing fluid a K/Na ratio of 1.0 is recommended, the high value of K/Na ratio in *Lippia javanica* (5.32 mg/100g) and *Foeniculum vulgare* (2.30 mg/100g) obtained in the present report suggests that they can help in modulating or adjusting diets rich in Na. The high value of Ca/Mg ratio obtained for *Lippia javanica* (7.132 mg/100g) and *Foeniculum vulgare* (4.309 mg/100g) was higher than the minimum recommended daily allowance of 1.00 mg. This means both spices are

good dietary sources of minerals needed for maintenance of normal health. The milliequivalent ratio of [K/Ca+Mg] in *Lippia javanica* (0.91) and *Foeniculum vulgare* (1.55) is the normal adult value for magnesium of 1.5-2.5 mEq/L recommended in the blood, (Adeyeye & Adesina, 2012; Dauda et al., 2014) is an indication that both spices could be good sources of magnesium in the human body. Copper stimulates the immune system to fight infections, repair injured tissues as well as to promote healing. Severe deficiency of Cu in pregnant women increases the risk of health problems in both foetus and infants (Mcneely, 1990). The copper content reported in the two spices (*Lippia javanica* and *Foeniculum vulgare*) suggest that they may be used to enrich the diet of pregnant women.

Table 6: Elemental contents of *Lippia javanica* and *Foeniculum vulgare* (mg/100g)

Elemental analysis	<i>Lippia javanica</i>	<i>Foeniculum vulgare</i>
N	1930 ± 0.60 ^a	3286 ± 0.09 ^b
Ca	1833 ± 0.80 ^a	1666 ± 0.85 ^b
Mg	257 ± 0.45 ^a	386.67 ± 0.78 ^b
K	1907 ± 0.7 ^a	3187 ± 0.18 ^b
P	400 ± 0.40 ^a	400 ± 0.45 ^a
Na	358 ± 0.05 ^a	1383 ± 0.45 ^b
Zn	4.7 ± 0.39 ^a	3.6 ± 0.17 ^b
Cu	1.4 ± 0.15 ^a	0.9 ± 0.73 ^b
Mn	7.5 ± 0.68 ^a	7.3 ± 0.15 ^a
Fe	78.4 ± 0.95 ^a	17.7 ± 2.37 ^b
Na/K	0.188	0.434
K/Na	5.327	2.304
Ca/P	4.582	4.165
Ca/Mg	7.132	4.309
[K/(Ca + Mg)]	0.91	1.55

*values with different letters within the same row (between the two species) are significantly different (p<0.05).

Zinc is very important because it is found in every tissue of the human body and is directly involved in cell division, a good antioxidant and helps to prevent cancer (Eleazu et al., 2012). It is also involved in proper endocrine function and the maintenance of ideal hormone level. Both spices have a high content of zinc (4.7 mg/100 g & 3.6 mg/100 g) for *Lippia javanica* & *Foeniculum vulgare* respectively, and would be good sources of zinc in the diet.

The iron content was very high (78.4 mg/100 g) in *Lippia javanica* compared to *Foeniculum vulgare* (17.7 mg/100 g). This is an indication that *L. javanica* can help to increase packed cell volume, boost the immune system and prevent anaemia in humans (Agunbiade et al., 2012; Adeyeye & Adesina, 2012). The high mineral content and the ratio in which they occur relative to each other could mean that addition of these spices to food will adjust the content of diets rich in Na, K and Mg. This could account for their medicinal uses in the treatment of hypertension.

Vitamin A and E were significantly higher in *L. javanica* compared to *F. vulgare* while the vitamin C content was higher in *F. vulgare* (Figure 13). Vitamin C promotes absorption of soluble non-haemolytic iron possibly by chelation or simply by maintaining the iron in the reducing Fe²⁺ form (Hallberg et al., 1987). The effect can be achieved with the amounts of vitamin C obtained in foods. However, the amount of dietary vitamin C required to increase iron absorption ranges from 25 mg upwards and depends largely on the amount of inhibitors such as phytates and polyphenols present in the meal (Hallberg et al., 1987). Vitamin A is an essential nutrient needed in small amounts by humans for normal functioning of the visual system, growth and development and care of epithelial cellular integrity, immune function and reproduction, Vitamin E is the most effective, fat-soluble antioxidant known to occur in the human body. It maintains the integrity of the body's intracellular membranes and provides a defence line against tissue damage caused by oxidation. The presence of these vitamins in *Lippia javanica* and *Foeniculum vulgare* makes them good sources of these vitamins in food.

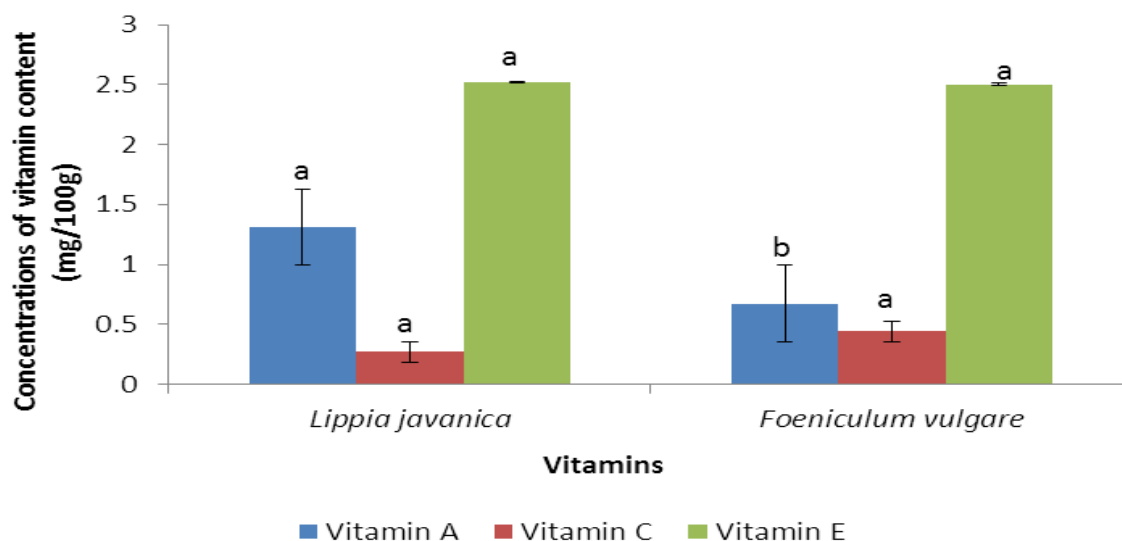


Figure 13: Vitamin A, E, and C contents of *Lippia javanica* and *Foeniculum vulgare* (mg/100g)
 Note: Vitamin A, C and E were significantly different ($p < 0.05$) in *Lippia javanica* and *Foeniculum vulgare*, values are Means \pm SD of triplicate samples; means with superscript are significantly different between species.

The antinutrient and phytochemical content of *L.javanica* and *F.vulgare* is given in Table 7. The low amount of oxalate in both spices is an indication that the availability of minerals like calcium to the body will not be affected. However, high amount of saponin in *L. javanica* and *F. vulgare* may lead to coagulation of red blood cells (Eleazu et al., 2012), the fact that saponins is heat labile means that the negative antinutrient effect will be nullified since most spices are added to food during cooking.

Table 7: Antinutrient content of *Lippia javanica* and *Foeniculum vulgare* (mg/100g)

Anti-nutrient	<i>Lippia javanica</i>	<i>Foeniculum vulgare</i>
Oxalate	0.73 \pm 0.54 ^a	1.114 \pm 0.034 ^a
Saponin	268.5 \pm 0.8 ^a	1855 \pm 0.7 ^b
Phytate	0.012 \pm 0 ^a	0.012 \pm 0 ^a
Tannin	0.003 \pm 0.001 ^a	0.002 \pm 0.000 ^a
HCN	8.45 \pm 0.600 ^a	10.5 \pm 3.421 ^a

Note: Oxalate, phytate, tannin and hydrogen cyanide shows no significant in *Lippia javanica* and *Foeniculum vulgare* ($p > 0.05$) while saponin were significantly different ($p < 0.05$), values are Means \pm SD of triplicate samples; means with different superscript are significantly different.

High amount of saponin in *Lippia javanica* and *Foeniculum vulgare* may also been associated with haemolytic activity, cholesterol binding properties and bitterness (Okwu, 2001; Aliyu et al., 2012). It has been reported that saponin contain properties that participate in precipitating and coagulating blood cells. Tannins are dietary anti-nutrients that are responsible for the astringent taste of foods and drinks (Chikezie et al., 2008). Tannins bind both proteins and carbohydrates which have several implications for commodities containing tannins. Their presence can cause browning or other pigmentation problems in both fresh processed food products. The presence of tannin in these spices implies they may have astringent properties and in addition could quicken the healing of wounds and burns (Eleazu et al., 2012). High level of cyanide in food has been implicated with cerebral damage and lethargy in human and animal, cyanide has also been linked to an increased incidence of goitre, the mean daily ingestion of cyanide ion was calculated to be 0.61 mg/kg of body weight (Akyildiz et al., 2010). Since spices are adjuncts to food which are required in little quantity, the consumption of *Lippia javanica* and *Foeniculum vulgare* may not be detrimental to brain function. It is evident that antinutrients and phytochemicals have both adverse and beneficial effects in humans. For example, it has been reported that phytates limit the availability of some notable minerals like zinc, magnesium, iron and calcium by forming complexes that are indigestible thereby decreasing their bioavailability (Mcneely, 1990). On the other hand when phytates, tannins, saponins and other antinutrients were used at low levels they exhibited hypoglycaemic, hypocholesterolomic, antioxidant, anti-inflammatory, antihypertensive and anticancer properties. In addition, since most of these antinutrients are heat labile, they may not be limiting factors to the use of these spices. In addition, saponins and tannins are useful for their antioxidant properties and in the treatment of several diseases including cancer. Therefore, the presence of these compounds in the spices under investigation could account for their folkloric and therapeutic uses.

Conclusion

The study revealed that *Lippia javanica* and *Foeniculum vulgare* can contribute useful amount of nutrients to human and animal diets. The anti-nutrients present were of negligible amount and lower than most found in some already established spices, and hence will not interfere with its nutrients absorption. Most of these antinutrients are heat labile, and they may not be limiting factors to the use of these spices. Preparation techniques can also reduce the anti-nutrient effects, but can also inadvertently reduce the amount of nutrients also present in the plants. These plants can serve as supplement to many mineral deficiencies and also serve as sources of vitamins; it can also provide meaningful level of bioactive compounds when consumed in a variety of foods. Further studies on its toxicity are ongoing to ascertain its possible adverse effects.

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CHAPTER SIX

Variations in chemical composition, antimicrobial activity and cytotoxicity of the essential oil of *Lippia javanica* and *Foeniculum vulgare*.

Introduction

Essential oils are naturally volatile, complex plant compounds that are oily or lipid in nature usually characterized by a strong aromatic fragrance (Burts, 2004). Due to their pungent and aromatic nature, there is an increasing interest in the use of essential oils in food and cosmetic industries. Many essential oil producing plants are spices such as rosemary, parsley, fennel, dill, basil (Burts, 2004). Although the food industry primarily uses essential oils as flavourings, they represent an interesting source of natural antimicrobials for food preservation. In addition, essential oils are considered to be secondary metabolites and important for plant defence as they often possess antimicrobial properties (Grayer and Harborne, 1994).

There has been no report to compare variation in chemical composition, antimicrobial activity and cytotoxicity of the essential oil from both fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare*. The present study is therefore aimed at investigating the variation in chemical components, toxicity of essential oil obtained from fresh and dried leaves of the two plants as well as the potential antimicrobial properties of essential oil obtained from the dried samples.

The evaluation from both fresh and dried leaves reveals that the dried sample yielded more oil than the fresh samples and more chemical compounds were found in dried than in fresh samples of both plants.

Materials and Methods

Extraction of the Essential oils

One hundred and sixty grams (160 g) of each plant material was oven dried to constant weights at 40°C. The fresh and dried leaves of each plant were separately subjected to hydro-distillation for 3 h using a Clavenger unit as described by the British Pharmacopoeia (1980).

Chemical analysis of the essential oils

GC-MS analysis of the oil was carried out using a Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column. Initial temperature was 70°C, maximum temperature 325°C, equilibration time 3 min, ramp 4°C/min, final temperature 240°C; inlet: split less, initial temperature 220°C, pressure 8.27 psi, purge flow 30ml/min, purge time 0.02 min, gas type helium; column: capillary, 30 m × 0.25 mm i.d., film thickness 0.25 µm, initial flow 0.7 ml/min, average velocity 32 cm/s; MS: EI method at 70eV.

Identification of components

The individual constituents of the oil were identified by matching their mass spectra and retention indices with those of Wiley 275 library (Wiley, New York) (Joulain and Konig, 1998). The yield of each component was calculated per gram of the plant material while the composition was calculated from the summation of the peak areas of the total oil composition.

Brine shrimp lethality test

The brine shrimp lethality test was used to assess the toxicity of the oils and was conducted as described by Okoh and Afolayan (2011) using brine shrimp eggs obtained from Ocean Star International, Inc. Company USA.

The Shrimp eggs were hatched in sea water over 48 h at room temperature in a glass vials. The naupili (newly hatched shrimps) were attracted to one side of the vials with a light source. Solutions of the oils were made in dimethyl sulfoxide (DMSO), at varying concentrations (100, 80, 60, 40, 20 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Twenty brine shrimp larvae were placed in a mixture of sea water and amphotericin as a control. After 48hrs, the vials were examined against a lighted background and the average number of larvae that survived in each vial was counted.

Antibacterial activity

The reference strains used in this study were chosen based on their pathological effects on human and deterioration of food products: two gram positive (*Staphylococcus aureus* and *L. monocytogenes*) and two gram negative (*Salmonella typhimurium* and *Escherichia coli*) bacteria were obtained from the Department of Microbiology, University of Fort Hare.

The agar well diffusion based method of Deans and Ritchie (1987) modified by Oyedeji et al., (2009) was used to determine the susceptibility of bacteria to the essential oil. A 100 µL of 18 h bacterial cultures were used to spread a bacterial lawn on nutrient agar. The cultures were adjusted to approximately 10^5 CFU/mL using McFarland standard. Twenty five microliters (25 µL) of various concentrations of plant extracts was added to each well (diameter of 4 mm) bored on nutrient agar plates under aseptic condition. The plates were left for 30 min at room temperature for the diffusion of the extracts and incubated at 37 °C for 18 hours. The diameters of zones of

inhibition (mm) were measured after 18 hours using a ruler. Each concentration of the extract was repeated three times. The minimum inhibitory concentration of *Lippia javanica* and *Foeniculum vulgare* extract against the test bacteria was determined by agar dilution method as described by the National Committee for Clinical Laboratory Standards (2004). The MICs were determined as the lowest concentrations of the extract resulting to complete inhibition of visible growth of the test organisms.

Antifungal assay

Pathogens and media

The fungi used in this study were chosen primarily on the basis of their importance as pathogens of humans. Strains from the American Type Culture Collection (ATCC) were used: *Aspergillus fumigatus* ATCC 204305, *Aspergillus niger* ATCC 16888, *Microsporium canis* ATCC 36299, *Microsporium gypseum* ATCC 24102, *Trichophyton tonsurans* ATCC 28942, *Trichophyton rubrum* ATCC 28188, *Trichophyton mucoides* ATCC 201382, *Pennicullin aurantiogrieseum* ATCC 16025 and *Pennicullin chrysogenum* ATCC 1010. Both Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were prepared according to the manufacturer's instructions. The fungi were maintained at 4°C on SDA plates and the inoculum for the assays was prepared by diluting scraped cell mass in 0.85% NaCl solution, adjusted to 0.5 McFarland standards and confirmed by spectrophotometric reading at 580 nm (Afolayan, 2003; Duarte et al., 2005). Cell suspensions were finally diluted to 10^4 CFU mL⁻¹ for the use in the assays.

Antifungal susceptibility assays

The agar diffusion and microdilution methods were used to determine the antifungal activities of the plant extracts against the fungi (Shad et al., 2008; Otang et al, 2012).

Agar well diffusion assay

The agar diffusion assay was carried out with slight modifications (Samie et al., 2010). Using the micropipette, 100 μL of 0.5 Mcfarland solution of each fungus culture in 0.85% sterile distilled water (SDW) was placed over the surface of an agar plate, and spread using a sterile inoculation loop. The same procedure was followed for the other fungi. Using a sterile cork borer, four holes (5 mm in diameter) were punched in each of the culture plates. In the first hole, 50 μL of a positive control drug was added (Nystatin); 50 μL of the corresponding extract solvent was added as a negative control in the second hole; and 50 μL of the plant extract was added in the third and last holes at concentrations of 25 and 50 mg/mL respectively. Each test was duplicated. The culture plates were then incubated at 37°C and the results were observed after 24 hours to 6 days depending on each fungal growth. The clear zone around each well was measured in mm, indicating the activity of the plant extracts against the fungal organisms.

Microdilution assay

The microdilution method was employed to determine the minimum inhibitory concentration (MIC) of the plant extracts using 96 well microtitre plates (Samie et al., 2010). Initially, 120 μL of sterile distilled water was added into each well of the first (A) and last (H) rows and also into all the wells of the last column (Dagne et al., 1996). Then, 120 μL of Sabouraud dextrose broth was added into each well of the second row (B) and 150 μL of Sabouraud dextrose broth was added into the remaining wells of the first column and 100 μL into the rest of the wells from the second column rightward. Fifty microliters of the plant extract were then added into the third well of the first column while 50 μL of the positive and negative control were separately added into the remaining wells of the first column. A two-fold serial dilution was done by mixing the contents in each well of the first column (starting from the third row) and transferring 100 μL into the second well of the same row and the procedure was repeated up to the 11th well of the same row and the

last 100 μ L from the 11th well was discarded. Hence various concentrations of the plant extracts ranging from 0.005 mg/mL to 5 mg/mL were prepared in the wells, following the two-fold dilution method. Thereafter, 20 μ L of 0.5 McFarland fungal suspensions was inoculated into the wells. The growth of the fungi was measured by determining the absorbance at 620 nm with a microtitre plate reader before and after incubation. The plates were incubated at 37°C at various durations (24 hours to 48 hours). The lowest concentration which inhibited the growth of the fungi was considered as the MIC of the extract.

Determination of the minimum fungicidal concentration (MFC).

The MFC was determined by inoculating the contents from the MIC plates onto Sabouraud dextrose agar plates, and the results were observed after incubation at 37°C at various durations depending on the fungi. The presence of the fungal colonies on the agar plates was an indication that the plant extracts only inhibited the growth of the fungi without killing them and the absence indicated that the plant extracts were able to kill the fungal organisms (Jones et al., 2007). The smallest concentration of the plant extracts that was able to kill the microorganisms was considered as the minimum fungicidal concentration (MFC).

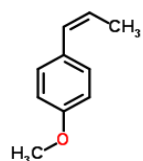
Statistical analysis

The experimental data was expressed as mean \pm standard deviation (SD) of the three replicates. Statistical analysis was done by using MINITAB program (version 12 for Windows) (Minitab Inc., Pennsylvania, USA). One-way analysis of variance (ANOVA) was used to compare the data among the plant fractions with the controls. $p < 0.05$ was considered statistically significant.

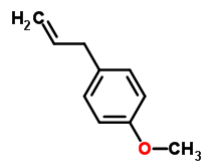
Results and Discussion

The fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare* yielded 0.7%, 2.7%, 0.4%, and 2.4% oil respectively. The dried sample yielded more oil compared with the fresh sample of

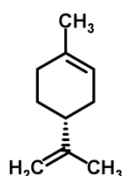
the plants, and more chemical compounds were found in dried samples than in fresh samples, Asekun et al., (2006) reported similar findings. Sixteen chemical compounds were found in the oils of dried samples of *Lippia javanica* while only six were found in the oils of fresh samples. Likewise, in *Foeniculum vulgare*, dried sample contained 17 components while its fresh leaves oil only has 10 compounds (Table 8). The major chemical in the oil extracted from fresh leaves of *Lippia javanica* include myrcene (2.86%), bicyclo (3,1,1) hept-3-en-2-one,4,6,6,-trimethyl- (5.37%), 3-methyl-2-butenic acid, tridec-2-ynyl ester (6.18%) while the major component of oil extracted from dried leaves of *Lippia javanica* were bicyclo (3,1,1) hept-3-en-2-one,4,6,6-trimethyl- (21.46%), Isophorone (2.70%), Caryophyllene (3.28%), 1,6-cyclodecadiene and 1-methyl-5-methylene-8-(1-methylethyl)-(3.74%). Bicyclo (3.1.1) hept-3-en-2-one, 4,6,6-trimethyl, 3-methyl-2-butenic acid and tridec-2-ynyl ester were found to be common to both fresh and dried *Lippia javanica*. Figure 14 and 15 show some of the bioactive compounds such as bicyclo (3.1.1) heptan-2-one and myrcene that have been identified as major constituents of *Lippia javanica* by other studies (Viljeon et al., 2005). Alpha-phelladrene (3.35%, 1.61%), o-cymene (0.91%, 1.20%), D-limonene (18.2%, 15.86%), bicyclo (2.2.1) heptan-2-one,1, 3,3- trimethyl- (1.48%, 1.66%), estragole (3.01%, 2.75%), anethole (62.20%, 54.51%) were identified in both oil extracted from fresh and dried leaves of *Foeniculum vulgare*. Cymene was the only chemical component found in both *Lippia javanica* and *Foeniculum vulgare*. The other components were generally monoterpenes which include anethole, α -pinene, o-cymene, D-limonene, verbenone, β -myrcene, 2-thujene, fenchone, estragole. Sesquiterpenes such as pentadecane, germacrene D, copaene and caryophyllene were also present in trace amount (Figures 14 & 15).



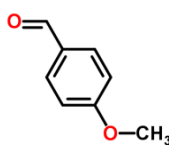
Anethole



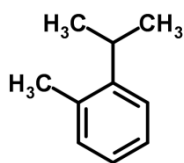
estragole



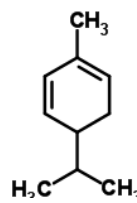
D-limonene



Benzaldehyde,4-methoxy-

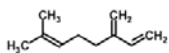


o-cymene

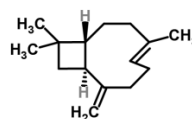


phellandrene

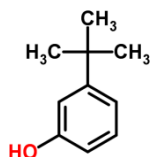
Figure 14: Some of the prominent compounds in the essential oil of *Foeniculum vulgare*



myrcene



caryophyllene



Phenol,m-tert-butyl

Figure 15: Some of the prominent compounds in the essential oil of *Lippia javanica*

Table 8: Percentage composition of essential oil extracted from both fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare*.

Component	<i>L. javanica</i>		<i>F. vulgare</i>	
	Fresh	Dried	Fresh	Dried
α -pinene	-	-	7.37	4.42
Verbenone	5.37	21.46	-	-
Nonyne	-	1.4	-	-
beta . -Myrcene	2.86	-	-	-
Isophorone	-	2.7	-	-
Copaene	-	0.41	-	-
Caryophyllene	-	3.28	-	-
germacrene D	-	3.74	-	-
Thujene	-	9.31	0.9	-
Methyl-2-butenic acid, tridec-2-ynyl ester	6.18	1.04	-	-
Methyl m-tolyl carbinol	-	6.31	-	-
Bicyclo[4.2.0] oct-1-ene, 7 – exo-ethenyl-	-	1.08	-	-
α .-phelladrene	-	-	3.35	1.61
β .-phellandrene	-	3.09	-	-
o-cymene	-	1.76	0.91	1.2
D-limonene	-	-	18.2	15.86
9,10-Diazatricyclo[4.4.0.(2,8)] dec-9-ene	1.64	4.55	-	-
p-linalool	-	3.6	-	-
butane-1,1-dicarbonitrile, 1-cyclo hexyl-3-methyl-	-	26.4	-	-
Fenchone	-	-	1.48	1.66
Tetradecane	-	-	-	2.41
Estragole	-	-	3.01	2.75
Borneol	-	-	1.74	2.34
p-anisaldehyde	-	-	0.78	2.22
Anethole	-	-	62.2	54.51
formic acid, 2-isopropylphenyl ester	-	-	-	1.32
Pentadecane	-	-	-	0.88
2,4,-di-tert-butylphenol	-	-	-	3.55
p-tert-butylphenol	2.6	8.26	-	-
Eicosane	-	-	-	0.97
pentadecanoic acid, triethylsilylester	-	-	-	1.16
Total (%)		90.13	99.9	100
Unknown (%)	0	9.87	0.1	0
Yield of oil (%)	0.7%	2.7%	0.4%	2.4%

*-Compound not identified

The essential oils extracted from dried leaves of *Foeniculum vulgare* and *Lippia javanica* were tested against four human pathogenic bacterial and both plants' oil gave varying activities on all the bacteria tested with mean zones of inhibition (ZI) ranging from 14.3 mm to 32 mm (Table 9). Based on the zones of inhibition, *Lippia javanica* oil had better activity than *Foeniculum vulgare* oil against *S. typhimurum* (30 mm and 25 mm respectively) and *E. coli* (32 mm and 23.3 mm respectively), but *Foeniculum vulgare* oil had a better activity than *Lippia javanica* oil against *L. monocytogenes* (21.4 mm and 14.3 mm respectively) and *S. aureus* (24 mm and 14.3 mm respectively). Essential oils from both plants gave better antibacterial inhibition than the control drug used (Table 9). According to Manenzhe et al., (2004), essential oil of *L. javanica* had low activity against *S. aureus* and *E. coli* with ZI of 18 mm and 16 mm at 10 mg/mL respectively. The same trend was observed in this study for *S. aureus* (14.3 mm). However, the ZI for *E. coli* was high (32 mm) at 50 mg/mL.

The low MIC values recorded in both plants showed that they are very active against the test organisms. The MIC results obtained for the essential oils from *L. javanica* and *F. vulgare* ranged from 0.625 to 1.25 mg/mL. *F. vulgare* oil inhibited the growth of all four test bacteria at a concentration value of 0.625 mg/mL, while *L. javanica* oil inhibited the growth of *S. typhimurum* and *E. coil* at a concentration of 0.625mg/mL but required 1.25 mg/mL to completely inhibit *L. monocytogenes* and *S. aureus*. Essential oils from both plants had higher MIC values than the tested drug.

The essential oils from both *Foeniculum vulgare* and *Lippia javanica* were also tested against nine human pathogenic fungi. The result revealed that the oil from both plants were active against eight of the tested fungi except for *A. niger* that was resistant, with zones of inhibition ranging from 6.75 to 35 mm (Table 10). The reference drug, nystacin, was also active on seven of the tested fungi except for *M. canis* and *T. rubrum* which were resistant to it (Table 10). The most active was the essential oil extracted from *Foeniculum vulgare* oil. The inhibitory activity of the oils on

the overall mean inhibition diameters was in the order *L. javanica* oil > nystacin > *F. vulgare* oil. The most susceptible fungi based on the overall mean diameter of growth inhibited were *Tricophyton mucoides* (30mm), *Tricophyton rubrum* (30mm), *Microsporum gypseum* (27.5 mm) and *Tricophyton tonsurans* (27.5 mm) while *Aspergillus niger* was not susceptible to the two oil tested.

The Minimum Fungicidal Concentration (MFC) recorded for the essential oils of both plants ranged from 1.25 to >5 mg/mL (Table 11). The high MFC values recorded indicate a low activity level of both essential oil on the tested fungi as compared to the low MIC values recorded in bacterial which indicated a high activity level of both essential oils on bacteria. The two essential oils gave better activity against the fungi studied than the control drug except for *T. mucoides* where the control drug had the greatest MFC (0.005 mg/mL) activity than the plants.

Although, moderate antifungal activity was observed with fungi isolates tested, these plants species could serve as sources of antifungal agents against susceptible fungi isolates especially the *Microsporum* and *Trichophyton* species which are known to be resistant to antibiotics and cause deleterious gastrointestinal, central nervous system, skin and foot infections in humans (Drozdowska and Drzewoski, 2008; Martinez-Rossi et al., 2008).

According to Viljeon et al., (2005), *L. javanica* essential oil is mainly used traditionally to treat respiratory disorders such as coughs, colds and bronchitis. The moderate antimicrobial activity recorded in this study justifies its use in African traditional medicine for the treatment of colds and flus associated with microbial infections.

Table 9: Zone of Inhibition (mm) and minimum inhibitory concentration for antibacterial activity of *Lippia javanica* and *Foeniculum vulgare*

Extracts (Essential oil extracts from dried samples)	<i>Samonella typhimurum</i>		<i>Escherichia coli</i>		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>	
	ZI (mm)	MIC	ZI (mm)	MIC	ZI (mm)	MIC	ZI (mm)	MIC
<i>L. javanica</i> oil extract	30.0±1.0	0.625 ^a	32.0±1.4	0.625 ^a	14.3±1.5	1.25 ^b	14.3 ± 0.6	1.25 ^b
<i>F. vulgare</i> oil extract	25.0±1.0	0.625 ^a	23.3±2.2	0.625 ^a	21.4±1.5	0.625 ^a	24.0 ± 2.0	0.625 ^a
Control (Amoxycillin)	18.3±1.5	<1.25 ^a	13.3±1.2	1.25 ^b	16.0±0.0	1.25 ^b	13.7 ± 1.5	1.25 ^b

Inhibition diameters with the same superscript letters in the same column are not significantly different from each other (P > 0.05).

Table 10: Zone of Inhibition (mm) for antifungal activity of oil extracts of *Lippia javanica* and *Foeniculum vulgare* from dried samples

	Zone of Inhibition (mm)								
	<i>M.gypseum</i>	<i>P.aurantiogriseum</i>	<i>A.niger</i>	<i>T.tonsurans</i>	<i>A.fumigatus</i>	<i>P.chrysogenum</i>	<i>T.mucoides</i>	<i>M.canis</i>	<i>T.rubrum</i>
<i>F.vulgare</i> oil	30 ±0.7 ^a	6.75 ±2.12 ^a	NA	25 ±0.71 ^a	10 ± 14.1 ^a	17.5 ± 10.6 ^a	35 ±14.14 ^a	15 ± 21.2 ^a	30 ± 8.49 ^a
<i>L.javanica</i> oil	25 ±14.1 ^b	22.5 ±3.53 ^b	NA	30 ±7.1 ^b	NA	25 ± 7.1 ^b	25 ± 0.71 ^b	29.5 ± 0.71 ^b	30 ± 14.1 ^a
Positive Control	27.5 ±3.5 ^d	25.5 ±0.71 ^c	25 ±7.1	27.5 ±10.6 ^a	25 ±0.71 ^b	22.5 ± 3.54 ^c	30 ± 0.7 ^d	NA	NA

*NA = not active. Af = *Aspergillus fumigatus*, Mc = *Microsporium canis*, Mg = *Microsporium gypseum*, Pa= *Penicillium aurantiogriseum*, Pc= *Penicillium chrysogenum*, Tm = *Tricophyton mucoides*, Tr- *Tricophyton rubrum*, Tt- *Tricophyton tonsuran*, An = *Aspergillus niger* * Concentration of positive control drug = 0.03mg/mL.

Table 11: Minimum fungicidal concentration of oil extracts from *Lippia javanica* and *Foeniculum vulgare* from dried samples (mg/mL)

	Af	Mc	Mg	Pa	Pc	Tm	Tr	Tt	An
<i>F. vulgare</i> oil	2.5 ^a	2.5 ^a	2.5 ^a	2.5 ^a	2.5 ^a	2.5 ^a	>5 ^b	2.5 ^a	NT
<i>L. javanica</i> oil	2.5 ^a	2.5 ^a	2.5 ^a	2.5 ^a	2.5 ^a	1.25 ^b	2.5 ^a	2.5 ^a	NT
Control	>5 ^a	2.5 ^b	>5 ^a	>5 ^a	>5 ^a	<0.005 ^c	>5 ^a	2.5 ^b	>5 ^a

Inhibition diameters with the different superscript letters in the same row are significantly different from each other ($P > 0.05$). (NT = not tested, Af = *Aspergillus fumigatus*, Mc = *Microsporum canis*, Mg = *Microsporum gypseum*, Pa = *Penicillium aurantiogriseum*, Pc = *Penicillium chrysogenum*, Tm = *Tricophyton mucoides*, Tr = *T. rubrum*, Tt = *Tricophyton tonsuran*, An = *Aspergillus niger*).

The brine shrimp assay of the essential oils extracted from both fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare* showed LC₅₀ values of 35.7, 48.7, 64.6 and 17.53 µg/mL respectively (Figure 16). The oils exhibited a concentration dependent toxicity against brine shrimp, the least concentration of 20 µg/mL used for the cytotoxicity test for both the essential oil extracts and control (Amphotericin) was toxic on the brine shrimps except for sea water which was used as the negative control. These result supported reports that the essential oils from *Lippia javanica* contains triterpenoids and iridoid glycosides (Olivier et al., 2010). As *Lippia javanica* is also administered in the form of tinctures and teas, it is most probably that the non- volatile compounds could be acting in a synergistic /additive manor to produce enhanced medicinal properties (Viljeon et al., 2005). This may be due to the presence of some monoterpenes in the oil, which are medicinally active to brine shrimp larvae (Okoh and Afolayan, 2011). The low LC₅₀ values of the essential oils extracted from fresh leaves of *Lippia javanica* (35.7 µg/mL) and dried leaves of *Foeniculum vulgare* (17.53

µg/mL) probably means that the fresh leaves of *L. javanica* and dried leaves of *F. vulgare* may be more active.

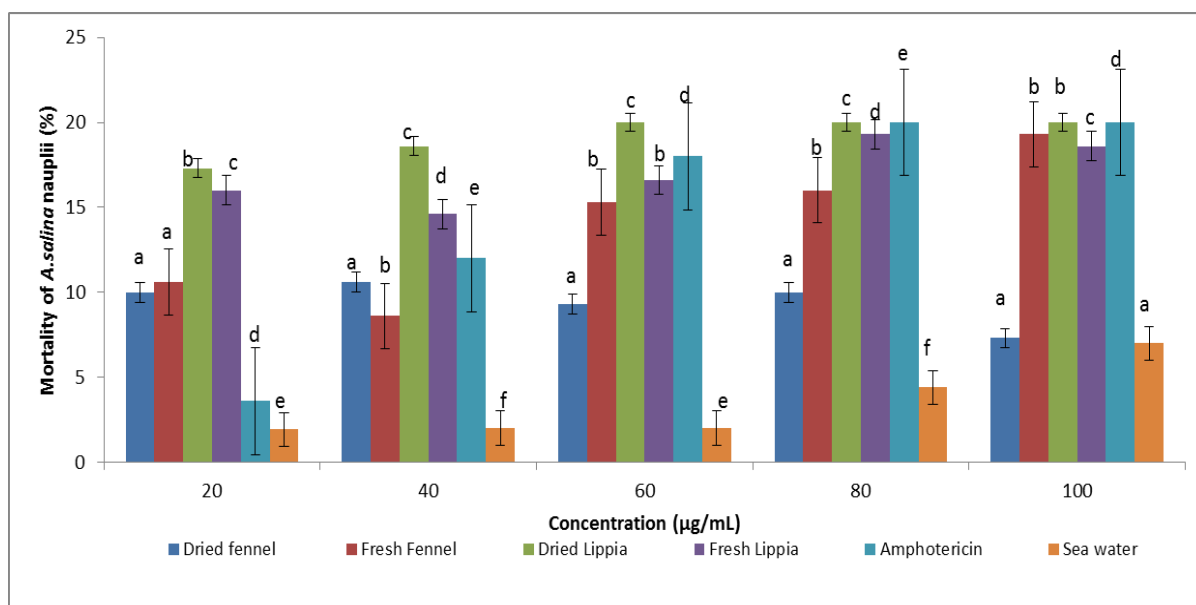


Figure 16: Toxicity (%) of essential oil extracted from both fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare* on brine shrimp larvae. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel–*Foeniculum vulgare*

Conclusion

The chemical composition, antimicrobial activity and cytotoxicity of the essential oil of fresh and dried leaves of *Lippia javanica* and *Foeniculum vulgare* were evaluated. The dried sample produced more oil compared to the fresh sample of the plants. The overall antimicrobial activity indicates that the oil of *F.vulgare* exhibited a higher antifungal and antibacterial activity compared to the reference drugs. This study also showed that the oil from fresh leaves of *Lippia javanica* and dried leaves of *Foeniculum vulgare* probably means that the fresh leaves of *L. javanica* and dried leaves of *F. vulgare* may be more active.

Therefore, it may be safer to use dried leaves of *L. javanica* and fresh leaves of *F. vulgare* for medicinal and pharmaceutical purposes. This implies that essential oil from the two plants can be a potential source of raw materials for drug development and pesticides.

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CHAPTER SEVEN

Comparative polyphenolic contents, *in-vitro* antioxidant, anti inflammatory and antimicrobial activities of *Lippia javanica* and *Foeniculum vulgare*

Introduction

Biologically active compounds from natural sources have always been of great interest to scientists. It has been documented severally that some naturally occurring substances in plants possess antioxidant activity (Feher & Schmidt, 2003; Galm & Shen, 2007; Ejele et al., 2012). These naturally occurring substances include a wide variety of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins and endogenous metabolites (Cao et al., 1996). A large number of phytochemicals belonging to several chemical classes have been reported to show some biological activities such as antioxidants, antimicrobial, anti-inflammatory, antiviral, antitumor, antimalarial, anti-uro lithiatic and analgesic (Raina et al., 2014). Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs have recently been shown to have radical scavenging activity as part of their mechanism of action (Conforti et al., 2008). Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack, different secondary metabolites exhibiting antimicrobial activities have been isolated from plants and it has been revealed that antimicrobial activity is mainly due to alkaloids, flavonoids, phenolic compounds, terpenoids and tannins. Also bioactive compounds are consider to be part of preformed defence system of higher plants, it is assumed that all families of higher plants possess more or less bioactive secondary metabolites involved in the comprehensive plant defence system (Grayer and Harborne, 1994).

Free radicals are molecules with an unpaired electron and are important intermediates in natural processes involving cytotoxicity, control of vascular tone and neurotransmission. Free radicals are very unstable and react quickly with other compounds and try to capture the needed electron to gain stability (Abheri et al., 2010). A powerful method to generate specific free radicals and measure their reactivity is known as radiolysis (Oakley, 1998). There are two common forms of free radicals namely reactive oxygen species (ROS) and reactive nitrogen species (RNS) for example ROS are the superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), the extremely reactive hydroxyl radical (OH^\cdot) and the peroxy radical (HO^{2-}).

Many pathological disorders have been associated with oxidative stress and inflammation. Reactive oxygen species (ROS) are involved in a diversity of important pathological processes such as inflammatory and neurodegenerative diseases, atherosclerosis, cancer and reperfusion injury. Inflammation is the foundation of most chronic diseases; the presence of inflammation makes most disease perceptible to an individual. It often does occur for years before it exists at levels sufficient to be apparent or clinically significant (Raina et al., 2014). How long it has been smoldering really determines the degree of severity of a disease and often the prognosis assuming the inflammation can be controlled. Hence one could also argue that without inflammation most disease would not even exist (Libby, 2007).

The mechanism of inflammatory injury is attributed in part to the release of reactive oxygen species from activated neutrophils and macrophages. This over production leads to tissue injury by damaging macromolecules and lipid peroxidation of membranes (Conforti et al., 2008). Free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can reduce inflammation (Geronikaki, 2006).

The phenolic constituents found in plants have attracted considerable attention for being the main components of antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential, the antioxidant activities of phenolics play an important role in the adsorption or neutralization of free radicals (Basile et al., 2005). Free radicals, particularly reactive oxygen species (ROS) have a greater impact on humans both from within the body and the environment. Radical scavenging action is dependent on both the reactivity and concentration of the antioxidant (Selvakumar et.al, 2011). Scientist has defined biological antioxidant as “any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate” (Halliwell and Gutteridge, 1995). Therefore, this study was to evaluate the free radical scavenging properties and antimicrobial activities of various extracts used.

Materials and Methods

Preparation of the plant

The aerial parts of *Foeniculum vulgare* and *Lippia javanica* were dried in the oven at 37°C for 48 h. The plants were milled into fine powder, packed into airtight plastic bottles and stored at 4°C until needed. Air dried powder (100 g each) was then extracted with 70% acetone by shaking for 24 h in an orbital shaker. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was then concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heidolph, Germany). For aqueous extracts, 50 g of *Foeniculum vulgare* was extracted with 1000 mL of distilled water and boiled for 10 min at 100 °C. It was allowed to cool, filtered and then

freeze-dried (Vir Tis bench top K, Vir Tis Co. Gardiner, NY). The freeze-dried sample was reconstituted with distilled water to give desired concentrations used in the study.

Chemicals used

The following chemicals were used for the various experiments: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)(ABTS), vanillin, aluminium chloride (AlCl_3), potassium acetate ($\text{CH}_3\text{CO}_2\text{K}$), ferric chloride (FeCl_2), BHT, ascorbic acid, rutin, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), phosphate buffer, potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$], trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), glacial acetic acid (CH_3COOH), sodium nitroprusside ($\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}]_2\text{H}_2\text{O}$). They were purchased from Merck, Gauteng, South Africa. All other chemicals used were of analytical grade.

Determination of polyphenols

Total phenols

The amount of phenol in the whole plant extracts of *Lippia javanica* and *Foeniculum vulgare* was determined spectrophotometrically using the modified method of Wolfe et al., (2003) with Folin-Ciocalteu reagent. An aliquot of the extract was mixed with 5 mL Folin-Ciocalteu reagent (previously diluted with water at a concentration of 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 s and left to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm using the AJI-C03 UV-VIS spectrophotometer. Results were expressed as mg/g of tannic acid equivalent using the calibration curve:

$Y = 2.0573x + 2.635$, $R^2 = 0.9985$, where y is the absorbance and x is the tannic acid equivalent

All the experiments were done in triplicates.

Total flavonoid

Total flavonoid content was determined using the method of Ordon Ez et al. (2006). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of the sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. Plant extracts were evaluated at a final concentration of 0.1 mg/mL. Total flavonoid content was calculated as mg/g of quercetin using the following equation based on the calibration curve:

$Y = 0.3705x + 1.1779$, $R^2 = 0.9812$; where y is the absorbance and x is the quercetin equivalent.

Total flavonols

Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran (2007). The reaction mixture consisted of 2.0 mL of the sample, 2.0 mL of $AlCl_3$ prepared in ethanol and 3.0 mL of (50 g/l) sodium acetate solution. The absorbance at 440 nm was measured after 2.5 h at 20°C. Total flavonol content was calculated as mg/g of quercetin equivalent from the calibration curve using the equation:

$Y = 0.3705x + 1.1779$, $R^2 = 0.9812$; where y is the absorbance and x is the quercetin equivalent.

Total proanthocyanidin

Total proanthocyanidin was determined based on the procedure of Oyedemi et al. (2010). To 0.5 mL of 1 mg/mL of the extract solution was added 3 mL of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature and the absorbance was measured at 500 nm. Total proanthocyanidin content was evaluated at a concentration of 0.1 mg/mL and expressed as catechin equivalent (mg/g) using the calibration curve equation:

$Y = 0.6845x + 0.7147$, $R^2 = 0.9277$, where y is the absorbance and x is the catechin equivalent.

Antioxidant assay

The antioxidant activities of the whole plant extracts of *Lippia javanica* and *Foeniculum vulgare* were determined using DPPH, ABTS, reducing power, lipid peroxidation, nitric oxide and phosphomolybdate.

ABTS radical scavenging activity

The method described by Adedapo et al (2008) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1 ml ABTS+ solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min using a spectrophotometer. The percentage inhibition of ABTS+ by the extract was calculated from the following equation:

$$\% \text{ inhibition} = [(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100.$$

DPPH radical scavenging assay

The method of Liyana-Pathiana and Shahidi (2005) was used for the determination of scavenging activity of DPPH free radical. DPPH (1 mL, 0.135 mM) prepared in methanol was mixed with 1.0 mL of aqueous extract ranging from 0.025 to 0.5 mg/mL. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant extract was calculated using the equation:

$$\text{DPPH scavenging activity (\%)} = [(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100,$$

where Abs control is the absorbance of DPPH + methanol and Abs sample is the absorbance of DPPH radical + sample (sample or standard).

Ferric reducing power of the extracts

The reducing power of the whole plant extracts of *Lippia javanica* and *Foeniculum vulgare* was evaluated according to the method described by Aiyegoro and Okoh (2010). The mixture containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $\text{K}_3\text{Fe}(\text{CN})_6$ (1% w/v) was added to 1.0 mL of the extracts and standards (0.025–0.5 mg/mL) prepared in distilled water. The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 ml of TCA (10% w/v), which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl_3 (0.1% w/v). The absorbance was then measured at 700 nm against blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

Nitric oxide scavenging activity

The modified method described by Oyedemi et al. (2010) was used to determine the nitric oxide radical scavenging activity of aqueous and acetone extracts of the whole plant extracts of *Lippia javanica* and *Foeniculum vulgare*. A volume of 2 mL of 10 mM of sodium nitroprusside prepared in 0.5 mM phosphate buffered saline (pH 7.4) was mixed with 0.5 ml of plant extracts, gallic acid and BHT individually at 0.025–0.5 mg/mL. The mixture was incubated at 25°C for 150 min. 0.5 mL of the incubated solution was mixed with 0.5 mL of Griess reagent [1.0 mL sulfanilic acid reagent (0.33% prepared in 20% glacial acetic) acid at room temperature for 5 min with 1 mL of naphthylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min, followed by the measurement of the absorbance at 540 nm. The amount of nitric oxide radicals inhibited by the extract was calculated using the following equation:

NO radical scavenging activity (%) = $[(\text{Abs control} - \text{Abs sample})/(\text{Abs control})] \times 100$,
where Abs control is the absorbance of NO radicals + methanol and Abs sample is the absorbance of NO radical + extract or standard.

Lipid peroxidation

A modified thiobarbituric acid-reactive species (TBARS) assay described by Dasgupta and De (2004) was used to measure the lipid peroxide formed, using liver homogenates as lipid-rich media. Liver homogenate (0.5 mL, 10% in distilled water, v/v) and 0.1 mL of the whole plant extracts of *Lippia javanica* and *Foeniculum vulgare* were mixed in a test tube and the volume was made up to 1 mL with distilled water. Finally, 0.05 mL FeSO₄ (0.07 M) was added to the mixture and incubated for 30 min to induce lipid peroxidation. Thereafter, 1.5 mL of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5 mL of 0.8% TBA (w/v)

(prepared in 1.1% sodium dodecyl sulfate) and 0.05 ml 20% TCA were added, vortexed and heated in a boiling water bath for 60 min. After cooling, 5.0 ml of n-butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. For the blank, 0.1 mL of distilled water was used in place of the extract.

Phosphomolybdate assay

The total antioxidant activity of plant sample was determined by phosphomolybdenum method according to the protocol of Kanwal (2015). 0.1 mL of plant sample solution was taken in test tubes and dissolved in 1 mL of reagent solution containing 0.6 M sulphuric acid, 4m M ammonium molybdate and 28m M sodium phosphate. Then the test tubes were covered with silver foil and incubated in a water bath at 95°C for 95 minutes. It was allowed cooling at room temperature after that the absorbance of the mixture was measured at 765 nm against a blank. Ascorbic acid was used as standard. Higher absorbance indicates higher total antioxidant potential.

Anti-inflammatory test

Protein denaturation method

The reaction mixture (0.5 mL, pH 6.3) consisted of 0.45 mL of bovine serum albumin (5% aqueous solution) and 0.05 mL of distilled water, pH was adjusted at 6.3 using a small amount of 1 N HCl. 1000 µg of *Lippia javanica* and *Foeniculum vulgare* of aqueous and acetone extracts (mg/mL of respective organic solvents) was added to the reaction mixture and were incubated at 37°C for 30 min and then heated at 57°C for 5mins after cooling the samples. 2.5 mL of phosphate buffer was added. Turbidity was measured spectrophotometrically at 600nm. For negative control 0.05 mL distilled water and 0.45 mL

of bovine serum albumin were used. The percentage inhibition of protein denaturation was calculated as follows (Sakat et.al., 2010).

$$\text{Percentage inhibition} = \frac{\text{Control} - \text{Treated Sample}}{\text{Control}} \times 100$$

Membrane stabilizing activity

Preparation of erythrocyte suspension

Whole blood was obtained with heparinised syringes from a rat through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 minutes at 3000g.

Hypotonic solution induced rat erythrocyte haemolysis

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte haemolysis (Alam et al., 2008). The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10m M sodium phosphate buffered saline (pH 7.4) containing the extract (0.25- 2.0 mg/mL) or diclofenac (0.1 mg/mL). The control sample consisted of 0.5 mL of RBC mixed with hypotonic buffered saline solution alone. The mixtures were incubated for 10min at room temperature and centrifuged for 10 min at 3000 g (13200 rpm) and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated as follows:

$$\% \text{ Inhibition of haemolysis} = 100 \times (\text{OD1} - \text{OD2} / \text{OD1})$$

Where: OD1= optical density of hypotonic buffered saline solution alone

OD₂= Optical density of test sample in hypotonic solution.

The activity was expressed as 50% inhibitory concentration (IC₅₀). IC₅₀ values were estimated from the best-fit line obtained by linear regression analysis of the percentage scavenging activity/ inflammation inhibitory activity versus the concentration. The lower the IC₅₀ value, the higher the antioxidant or anti-inflammatory activity (Park et al., 2014).

Results and Discussion

Polyphenolic constituents

The flavonoid and flavanol contents of *Lippia javanica* and *Foeniculum vulgare* extracts were expressed as quercetin equivalents while total phenol and proanthocyanidin were expressed as tannic acid and catechin equivalents respectively. The acetone extract of *Lippia javanica* and *Foeniculum vulgare* showed a higher content of phenol, flavonoid and proanthocyanidin compared to the aqueous extract (Table 12). This could be attributed to the better extracting power of acetone over water. The total phenolic content of acetone and aqueous extracts were 4.49 ± 0.411 mg/g and 3.73 ± 0.498 mg/g tannic acid equivalent (TAE) respectively for *Lippia javanica*. The same trend was also observed in *Foeniculum vulgare* with total phenolic content of acetone and aqueous extracts were 4.22 ± 0.325 mg/g and 4.17 ± 0.651 mg/g tannic acid equivalent (TAE) respectively.

Phenolic compounds aid in the preservation of food, fresh flavour, taste, colour and help in prevention of oxidative deterioration. In particular, many phenolic compounds are attracting the attention of food and medical scientists because of their antioxidative, antiinflammatory, antimutagenic, and anticarcinogenic properties and their capacity to modulate some key cellular enzyme functions (Jimoh et al., 2008). Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or

preventing decomposition of hydrogen peroxides into free radicals (Yanishlieva et al., 2001). In addition, flavonoids and phenolic compounds are effective in preventing the formation of reactive oxygen species and protecting low density lipoprotein (LDL) from iron and copper mediated free radical production (Owen & John, 2002). Flavonoids are hydroxylated phenolics and are potent water soluble antioxidants which help in radical scavenging and prevention of oxidative cell damage. Flavonoids have been shown to be utilized by plants for specific physiological functions. They are also shown to be essential for the formation of pollen tubes in the pistil containing germinating pollen, they are naturally occurring auxin transport regulators and they may be involved in the signal transduction process mediating the translation of externally perceived signals into new protein synthesis. They have been reported to possess strong antioxidants effects (Wintola & Afolayan, 2011) while proanthocyanidins are group of polyphenolic bioflavonoid which have a protective effect in eliminating hydroxyl radicals. The concentration of flavonoids and proanthocyanidin was higher in the acetone extracts compared with the aqueous extracts in this study which agree with several studies that acetone extracts had higher concentration of phenolic compound (Wintola & Afolayan, 2011). The presence of these polyphenols could have accounted for the therapeutic effects of *Lippia javanica* and *Foeniculum vulgare*.

Table 12: Polyphenolic contents of *Lippia javanica* and *Foeniculum vulgare*.

	<i>F. vulgare</i> H ₂ O	<i>F. vulgare</i> acetone	<i>L. javanica</i> H ₂ O	<i>L. javanica</i> acetone
Phenol	4.17±0.651 ^a	4.22±0.325 ^a	3.73±0.498 ^b	4.49± 0.411 ^a
Flavanol	1.32±0.002 ^a	1.47±0.061 ^b	1.21±0.002 ^c	1.53±0.060 ^d
Flavonoid	1.37±0.16 ^a	1.65±0.006 ^b	1.30±0.002 ^c	1.67±0.002 ^b

Proanthocyanidin	0.72±0.001 ^a	0.78±0.026 ^b	0.73±0.002 ^a	0.76±0.045 ^c
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*Values represented by the same subscript show no significant difference along the row at P<0.05.

Free radical scavenging activity

ABTS

The percentage inhibition of ABTS by *Lippia javanica* and *Foeniculum vulgare* extracts are shown in Figure 17. All the tested samples inhibited the radical, but the acetone extracts exhibited a higher inhibitory activity compared to the aqueous extracts. The IC₅₀ values (Table 13) confirmed that the acetone extracts exhibited a greater inhibition than the aqueous extract, as well as comparable activity with the standards. The ABTS radical scavenging ability of the samples can be ranked as rutin > acetone extracts > BHT > aqueous extracts.

The scavenging activity of ABTS by the aqueous and acetone extracts was found to vary with concentration used and IC₅₀ values, the acetone extracts exhibited better scavenging effect with ABTS compared to the aqueous extracts. The solubility of extracts in different testing systems and radical reactivity confirming the removal of odd electron are believed to be responsible for the higher scavenging activity of ABTS. The scavenging activity of ABTS radical by the plant extracts justifies the presence of compounds with free radical scavenging activity as well as the possibility of the extracts being used for treating radical related pathological ailments (Wintola & Afolayan, 2011).

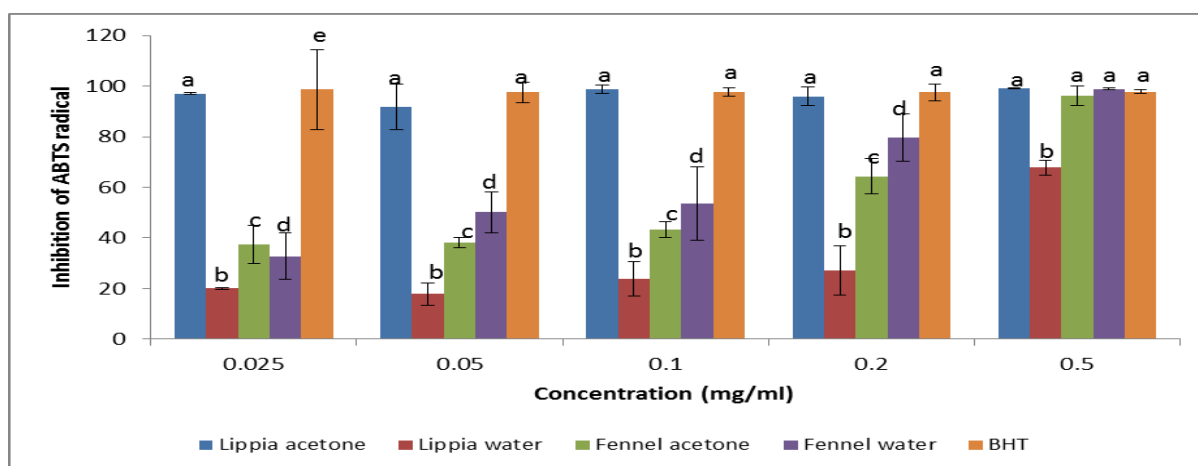


Figure 17: Inhibition of ABTS radical (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare*

DPPH

Figure 18 shows the DPPH radical scavenging capacity of the extracts and standards. The DPPH molecule is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals (Awah & Verla, 2010). The delocalization of electron also gives rise to the deep violet color. When a solution of DPPH is mixed with that of a substrate that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour measurable spectrometrically (Awah & Verla, 2010). The results from a series of concentration ranging from 0.025 to 0.5 mg/mL of the plants' fractions and the standards showed different strengths of violet colour during the study. The observed inhibitory potential of the plants' fractions against the DPPH radical was dose dependent indicating its potential in relieving oxidative stress, also the IC_{50} revealed similar result (Table 13).

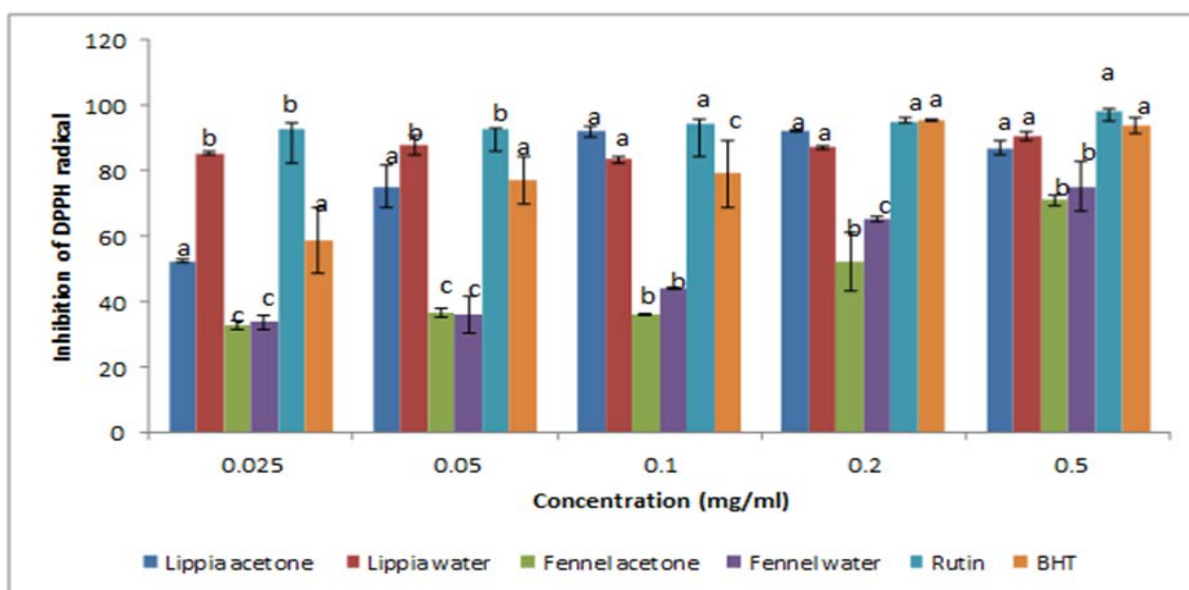


Figure 18: Inhibition of DPPH radical (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare* * y axis -%

Reducing power

The potentials of the plant extracts to reduce Fe^{3+} to Fe^{2+} by electron transfer is an indication of their antioxidant ability. The reducing power of the extracts in comparison with the standards (BHT, rutin and vitamin C) is presented in Figure 19. The BHT and vitamin C exhibited better reducing power compared to the extracts, while the aqueous extracts exhibited a higher reducing power compared with the acetone extracts. The result obtained showed that the plant fractions possessed antioxidant activity which was dose dependent. This was characterized by the formation of Perl's Prussian blue coloration after ionic reduction, to produce a reduction in the ferric ion/ ferricyanide complex to ferrous form across all the concentration assayed. This activity could be ascribed to electron transfer capability by the metabolites present (Sharma et al., 2012).

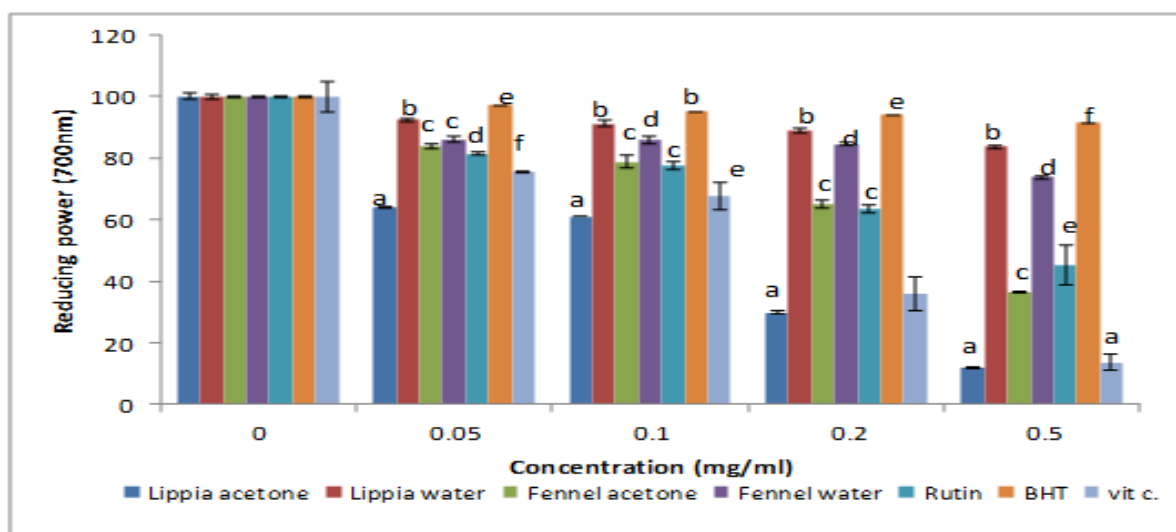


Figure 19: Inhibition of reducing power (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, *Fennel-*Foeniculum vulgare* * y axis -%

Nitric oxide

Nitric oxide radical scavenging was in a concentration dependent manner and was in the order of Rutin > aqueous extracts > BHT > acetone. The IC_{50} values obtained for the aqueous extracts and acetone extracts (<0.025 mg/mL) showed a comparable activity with the standards, rutin (< 0.025 mg/mL) and BHT (< 0.025 mg/mL). The data demonstrated that both the aqueous and acetone extracts are potent scavengers of nitric oxide, since the IC_{50} values of the extracts are less than the range of concentration used (Figure 20).

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages and neurons. It is involved in the regulation of various physiological processes. Excess concentration of nitric oxide is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals.

Though the standard (gallic acid) exhibited greater nitric oxide inhibition, both the aqueous and acetone extracts of both plants species were potent scavengers of nitric oxide and could be useful in the management of inflammatory reactions that are detrimental to human health (Ynishlieva et al., 2001).

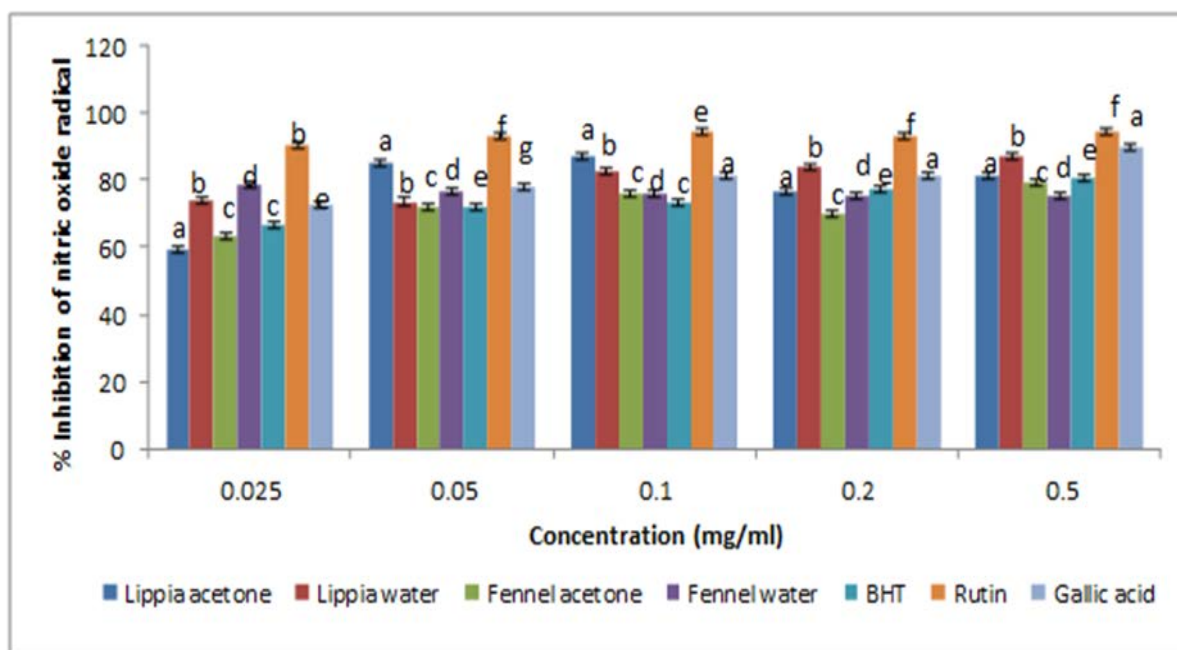


Figure 20: Inhibition of nitric oxide radical (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare*

Lipid peroxidation

The liver homogenate from a rat was used for this assay. The extracts exhibited significant ($P < 0.05$) lipid peroxidation quenching activities in a concentration dependent manner, reaching the peak at concentrations of 0.5 mg/mL (Figure 21). The results from IC_{50} values (Table 13) showed that the acetone extract from fennel showed a significantly higher scavenging capacity than the aqueous extract, though the standards BHT and vitamin C exhibited the

greatest inhibition while *L. javanica* acetone and aqueous had low IC₅₀ which is higher than the range of concentration used. Lipid peroxidation of cellular membranes by free radicals generates malondialdehyde which reacts with DNA to cause mutations (Ynishlieva et al., 2001). Active components of *L. javanica* and *F. vulgare* significantly inhibited the generation of lipid peroxides. This implies that the combination of phenols, flavonoids and other bioactive compounds in the plant are actively involved in the antioxidant role of *L. javanica* and *F. vulgare* extracts.

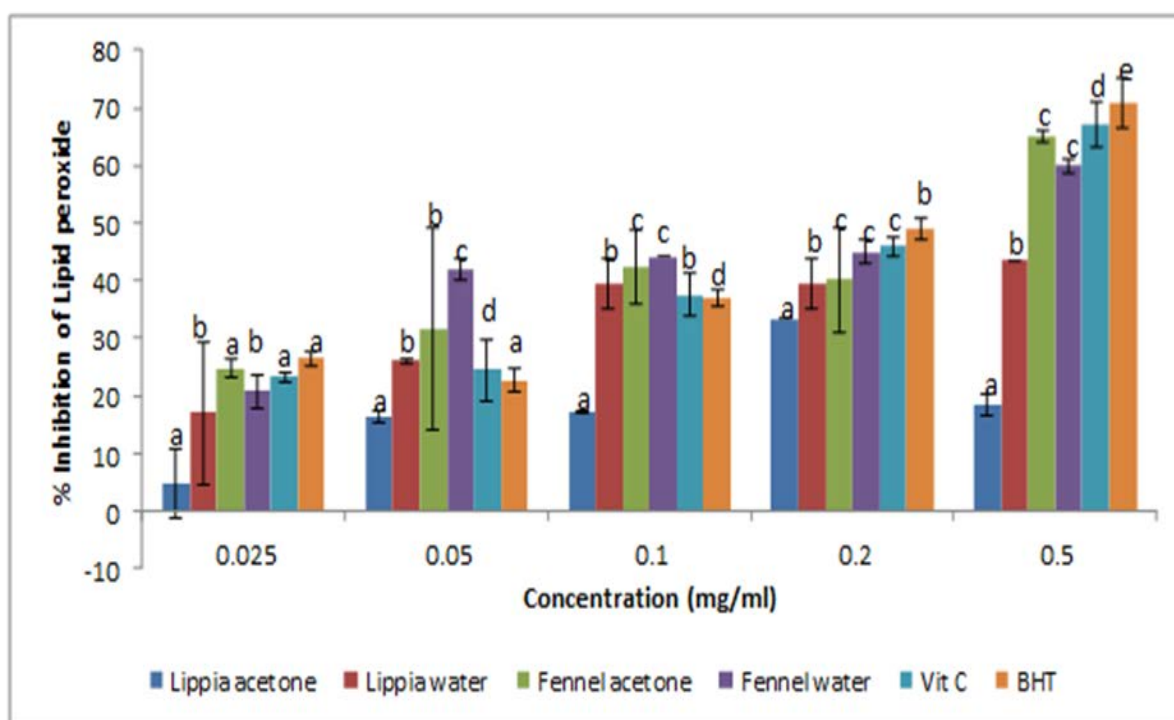


Figure 21: Inhibition of Lipid peroxidation radical (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare*

Phosphomolybdate

The extracts exhibited a concentration dependent activity against phosphomolybdate radical (Figure 22). The IC₅₀ values (Table 13) for the Lippia aqueous extract was 0.007, Fennel aqueous extract was <0.025, Fennel acetone was 0.03 mg/mL, while the Lippia acetone extract, vitamin C and gallic acid had values of 0.703, 0.113 and 0.184 mg/mL respectively. This is an indication of the potent antioxidant capacity of the aqueous extract of *L. javanica* and *F. vulgare*. Phosphomolybdate assay is used to assess the overall antioxidant capacity of plant extracts. Extracts of *L. javanica* and *F. vulgare* exhibited a high total antioxidant capacity against molybdenum radicals. This could be attributed to the presence of flavonoids in the extracts, since many flavonoids and related phenols have been reported to contribute significantly to the phosphomolybdate scavenging activity of medicinal plants.

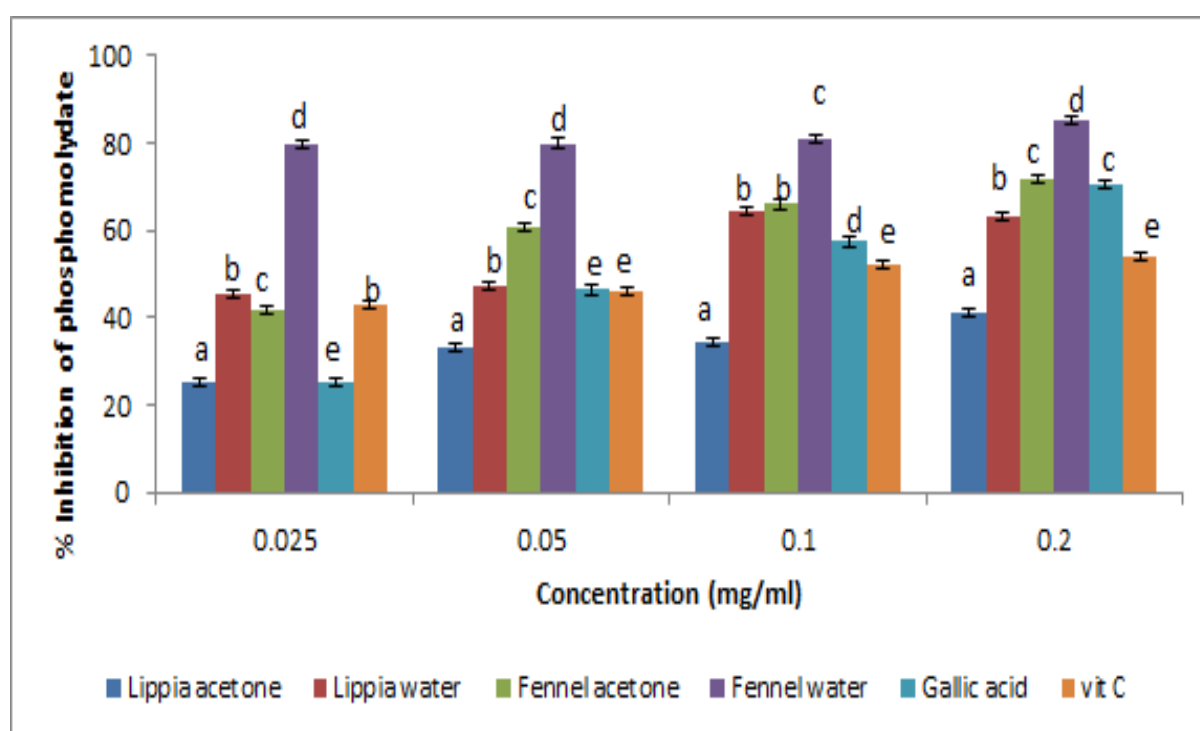


Figure 22: Inhibition of phosphomolybdate radical (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three

replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare*

Overall, *L. javanica* and *F. vulgare* exhibited high polyphenolic contents, outstanding reducing power, good radical scavenging activity against DPPH, ABTS, nitric oxide, inhibition of lipid peroxidation and phosphomolybdate. The free radical scavenging ability of *L. javanica* and *F.vulgare* is dependent on the polyphenol content. Several studies have evaluated the relationships between antioxidant activity of plant products and their phenolic content. Some authors found a correlation between the phenolic content and antioxidant activity, while others found no strong relationship. In this study, a significant total phenolic content and free radical scavenging activities was found in the acetone extract supporting the claim that a correlation exists between the total phenolic content and antioxidant activity. Also numerous monoterpenoids have been identified in the volatile extract of *L. javanica* including myrcene, caryophyllene, linalool, p-cymene and ipsdionone (Yanishlieva et al., 2001). *Lippia javanica* contains various organic acids and alcohols iridoid glycosides and toxic triterpenoids which have been detected in some Lippia species. The presence of all these compounds in *L. javanica* could probably account for the medicinal uses of the plant, as these metabolites are well known for their biological activities. This finding seems to justify the folkloric use of infusing a combination of the aerial plant parts of *L. javanica* for therapeutic purposes. Previous studies have reported that the essential oils of *Lippia javanica* had moderate antioxidant activity (Muyima et al., 2004). Another study reported that the phenylpropenes estragol and trans-anethole which are the major constituents of the oleoresin of the aerial parts of *F. vulgare* may be responsible for good radical scavenging activity (Diaaz et al., 2005).

Table 13: Inhibitory concentrations at 50% (IC₅₀) of the antioxidant activities of *Lippia javanica* and *Foeniculum vulgare* (mg/mL)

	IC ₅₀ (mg /mL)					
	ABTS	DPPH	Reducing power	Nitric oxide	Phosphomolybdate	Lipid peroxidation
<i>L. javanica</i> Acetone	0.177	0.198	0.185	< 0.025	*0.703	* > 0.5
<i>L. javanica</i> Aqueous	0.354	0.195	0.200	< 0.025	0.007	0.576
<i>F. vulgare</i> Acetone	0.129	0.228	0.364	< 0.025	0.03	0.297
<i>F. vulgare</i> Aqueous	0.07	0.166	* > 0.5	< 0.025	< 0.025	0.303
BHT	< 0.025	< 0.025	-	< 0.025	-	0.290
Rutin	< 0.025	< 0.025	0.413	< 0.025	-	-
Gallic acid	-	-	-	-	0.077	-
Vitamin C	-	-	0.220	-	0.09	0.265

*-Standard not used or * IC₅₀ values higher than range of concentration.

Anti-inflammatory activity

Results on anti-inflammatory activity showed that both plant extracts were able to inhibit protein denaturation in a concentration-dependent manner (Figure 23). *L. javanica* aqueous extract was very active followed by the *L. javanica* acetone, *F. vulgare* acetone, *F. vulgare* aqueous while the anti-inflammatory drug diclofenac had a constant excellent inhibitory effect (Figure 23). The fennel aqueous extract exhibited a higher membrane stability potential, compared with the other extracts of both plants and the anti-inflammatory drug diclofenac (Figure 24). Denaturation of proteins is a well documented cause of inflammation. The anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation (Mizushima and Kobayashi, 1968; Govindappa et al, 2011). The ability of *Lippia javanica* and *Foeniculum vulgare* extracts to inhibit protein denaturation may contribute to their anti-inflammatory properties. In the present investigation, the *in vitro* anti-

inflammatory effect of aqueous and acetone extracts were evaluated against denaturation of bovine serum albumin and hypotonic membrane stabilization of RBC (Nickavar et al., 2007; Conforti et al., 2008; Umapathy et al., 2010; Dzoyem & Eloff, 2015). Therefore, any substance that can prevent or inhibit protein denaturation will be a good anti-inflammatory agent. The precise mechanism of this membrane stabilization is yet to be elucidated; it is possible that the *Lippia javanica* and *Foeniculum vulgare* produced this effect through the surface area/volume ratio of the cells, which could be brought about by expansion of membrane proteins (Shinde et al., 1999).

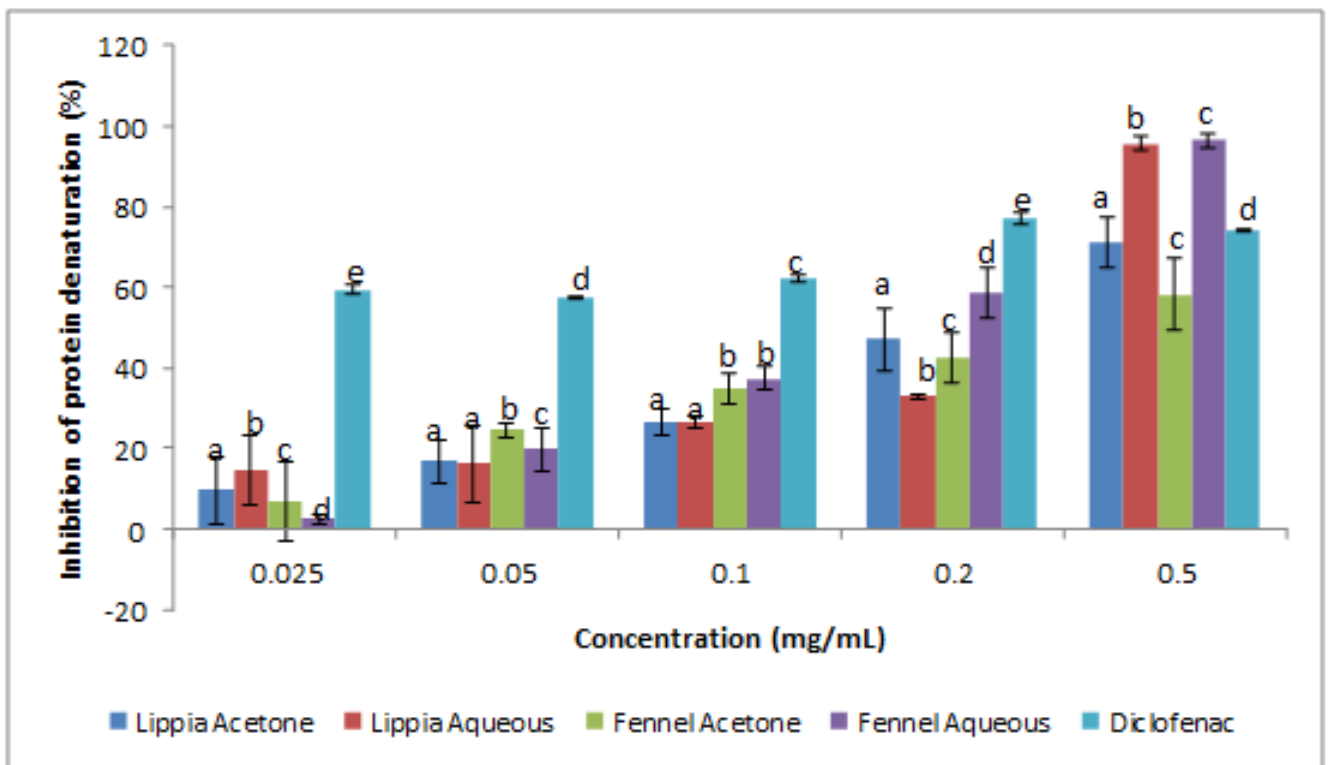


Figure 23: Inhibition of protein denaturation (%) by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar

graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * *Lippia* –*Lippia javanica*, Fennel-*Foeniculum vulgare*

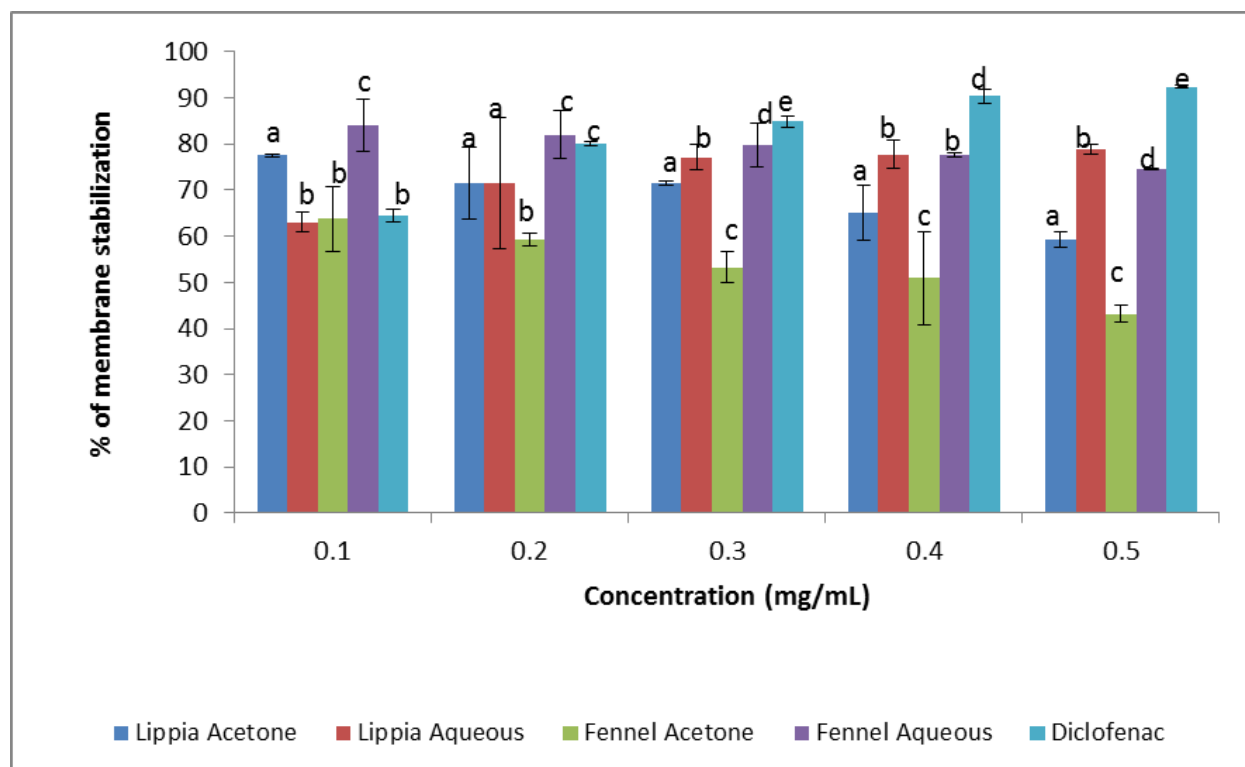


Figure 24: % Inhibition of membrane stabilization effects by acetone and aqueous extracts of *Lippia javanica* and *Foeniculum vulgare*. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * *Lippia* –*Lippia javanica*, Fennel-*Foeniculum vulgare*

Antimicrobial activities

The antibacterial result reveals that the tested extracts had similar effect on the pathogens; *Salmonella typhimurium*, *Esherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*, both acetone and aqueous extracts exhibited strong bacteriostatic effect even greater than the reference drug, amoxycillin with minimum inhibitory concentration (MIC) range from 0.625 g/mL to 1.25 μ g/mL (Table 14).

The acetone extracts from both plants were active against five fungi which are *Penicillium chrysogenum*, *Tricophyton mucoides*, *Microsporum canis*, *Tricophyton tonsurans* and *Tricophyton rubrum* while the aqueous extracts were active against *Penicillium chrysogenum*, compared to the reference drug, nystatin which was active against seven out of the nine fungi except for *Microsporum canis* and *Tricophyton rubrum*, they all exhibited zones of inhibition ranging from 5 to 35 mm (Table 15 & 16). The most susceptible fungi based on the overall mean diameter of growth inhibit was *Penicillium chrysogenum* while *Microsporum gypseum*, *Penicillium aurantiogriseum*, *Aspergillus niger* and *Tricophyton tonsurans* show strong resistance to the tested extracts even at the highest concentration of 5mg/mL, the MIC values ranges from 1.25µg/mL to 5µg/mL. Some of the phytochemical compounds e.g. glycoside, saponin, tannin, flavonoids, terpenoid and alkaloids, have variously been reported to have antimicrobial activities (Grayer & Harborne, 1994), this may imply that the phenolic compounds present in *F. vulgare* and *L. javanica* may be responsible in the inhibition of fungal and bacterial growth by interacting with the plasma or mitochondrial membrane of the microbes. The present study justifies the claimed uses of *F. vulgare* and *L. javanica* in the traditional system of medicine to treat various infectious diseases caused by the microbes.

Table 14: Zone of Inhibition (ZI) and antibacterial minimum inhibitory concentration (MIC) of *Lippia javanica* and *Foeniculum vulgare* extract

Extracts	<i>Samonella typhimurum</i>		<i>Escherichia coli</i>		<i>Listeria monocytogenes</i>		<i>Staphylococcus aureus</i>	
	ZI (mm)	MIC	ZI (mm)	MIC	ZI (mm)		ZI (mm)	MIC
<i>L. javanica</i> Acetone extract	25.0±1.5	0.625 ^a	23.0±1.7	0.625 ^a	14.3±1.5	1.25 ^b	14.3±0.6	1.25 ^b
<i>L. javanica</i> Aqueous extract	24.0±1.0	0.625 ^a	20.3±2.0	0.625 ^a	14.4±1.5	0.625 ^a	14.0±2.0	0.625 ^a
<i>F. vulgare</i> Acetone	26.0±0.0	0.625 ^a	23.5±2.1	0.625 ^a	23.5±2.1	0.625 ^a	30.0±0.0	0.625 ^a
<i>F. vulgare</i> Aqueous	25.0±1.1	0.625 ^a	30.0±0.0	0.625 ^a	21.5±2.1	0.625 ^a	23.5±2.1	0.625 ^a
Control (Amoxycillin)	18.3±1.5	<1.25 ^a	13.3±1.2	1.25 ^b	16.0±0.0	1.25 ^b	13.7±1.5	1.25 ^b

Inhibition diameters with the same superscript letters in the same column are not significantly different at P > 0.05.

*ZI denotes zone of inhibition, MIC denotes Minimum inhibitory concentration. Disc diameter; 4 mm. Values are the mean ± S.D of the mean;

Table 15: Antifungal activity of *Lippia javanica* and *Foeniculum vulgare*.

Extracts	Zone of Inhibition (mm)								
	<i>M.gypseum</i>	<i>P.aurantiogriseum</i>	<i>A.niger</i>	<i>T.tonsurans</i>	<i>A.fumigatus</i>	<i>P.chrysogenum</i>	<i>T.mucoides</i>	<i>M.canis</i>	<i>T.rubrum</i>
<i>L. javanica</i> Acetone	12.5 ± 17.7 ^a	NA	NA	12.5 ± 17.7 ^a	NA	15 ± 21.2 ^b	10 ± 14.14 ^c	NA	11 ± 15.6 ^b
<i>L. javanica</i> Aqueous	NA	NA	NA	12.5 ± 17.7 ^a	NA	25 ± 7.1 ^a	10 ± 0.7 ^c	NA	13 ± 18.4 ^b
<i>F. vulgare</i> Acetone	NA	NA	NA	NA	NA	7.5 ± 10.6 ^d	5 ± 7.1 ^d	13 ± 18.4 ^a	16 ± 22.62 ^b
<i>F. vulgare</i> Aqueous	NA	NA	NA	NA	NA	17.5 ± 10.6 ^e	NA	NA	NA
Positive Control	27.5 ± 3.5 ^b	25.5 ± 0.71 ^a	25 ± 7.1	27.5 ± 10.6 ^c	25 ± 0.71 ^b	22.5 ± 3.54 ^f	30 ± 0.7 ^e	NA	NA

NA = not active. Af = *Aspergillus fumigatus*, Mc = *Microsporum canis*, Mg = *Microsporum gypseum*, Pa = *Penicillium aurantiogriseum*, Pc = *Penicillium chrysogenum*, Tm = *Tricophyton mucoides*, Tr = *Tricophyton rubrum*, Tt = *Tricophyton tonsuran*, An = *Aspergillus niger* * Concentration of positive control drug = 0.03mg/ml.

Table 16: Minimum fungicidal concentration of *Lippia javanica* and *Foeniculum vulgare*

Extracts	Af	Mc	Mg	Pa	Pc	Tm	Tr	Tt	An
<i>L. javanica</i> Acetone	>5 ^a	-	1.25 ^b	-	1.25 ^b	>5 ^a	2.5 ^c	2.5 ^c	-
<i>L. javanica</i> Aqueous	-	-	>5 ^a	-	<0.005 ^b	>5 ^a	2.5 ^c	2.5 ^c	-
<i>F. vulgare</i> Acetone	-	2.5 ^a	-	-	-	<0.005 ^b	1.25 ^c	2.5 ^a	-
<i>F. vulgare</i> Aqueous	-	<0.005 ^a	-	-	2.5 ^b	-	-	2.5 ^b	-
Control	>5 ^a	2.5 ^b	>5 ^a	>5 ^a	>5 ^a	<0.005 ^c	>5 ^a	2.5 ^b	>5 ^a

Inhibition diameters with the different superscript letters in the same row are significantly different at $P > 0.05$. - = not tested, Af = *Aspergillus fumigatus*, Mc = *Microsporium canis*, Mg = *Microsporium gypseum*, Pa = *Penicillium aurantiogriseum*, Pc = *Penicillium chrysogenum*, Tm = *Tricophyton mucoides*, Tr = *T. rubrum*, Tt = *Tricophyton tonsuran*, An = *Aspergillus niger*

Conclusion

The polyphenolic content, free radical scavenging, anti-inflammatory and antimicrobial activities of the aqueous and acetone extracts of both plants *Lippia javanica* and *Foeniculum vulgare* were evaluated. The data presented in this study demonstrates that both plant extracts possess excellent antioxidant, free radical scavenging, anti-inflammatory and antimicrobial activities. There was correlation between the antioxidant activities and the total phenolic and flavonoids content. A concentration-dependent inhibition of protein denaturation and membrane stabilization of the aqueous and acetone extracts were also observed. Therefore, the ability of *Lippia javanica* extracts to inhibit protein denaturation and maintain stability as reported in this study could account for its folkloric use in the treatment of inflammatory diseases. The overall antimicrobial activity revealed that both plant extracts were active but the acetone extracts exhibited a higher antifungal activity compared to the reference drugs. Further studies on the characterization of various antioxidant compounds present and the mechanism of anti-inflammatory action are in progress.

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CHAPTER EIGHT

In-vitro evaluation of anti-urolithiatic potentials of *Lippia javanica* and *Foeniculum vulgare*

Introduction

Urolithiasis is the formation of calculi or stone which is a connection of material mainly salt in any part of the body, while oxalate is a metabolic end product and a major constituent of the majority of renal stones (Shoaib et al., 2014). It is one of the oldest diseases suffered by humans, can also be referred to as kidney stones, renal stones, and renal calculi. Calcium containing stones are the most common calculi comprising about 75% of all urinary calculi, which may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%). Calcium oxalate stones are found in two different varieties, calcium oxalate monohydrate (COM) or Whewellite, and calcium oxalate dihydrate (COD) or Weddellite (Menon et al., 1988; Beghalia et al., 2008). Calcium containing salts constitute one of the most important components of urinary stones that reduce urinary flow or cause obstruction, resulting in urinary stasis or reduction in urine volume and increase the risk of developing kidney stones. Low urinary flow is the most common abnormality, and most important factor to correct kidney stones (Thomas, 2010).

Urolithiasis differs according to geographical area in term of prevalence and incidence, age and sex distribution, stone composition and location as well as socio economic conditions. These factors have also generated changes in type of lithiasis in terms of both the site and the chemical-physical composition of the calculi (Alberto, 2008). Urolithiasis (Urinary calculi) is one among the three prevalent disorders in the urinary system. Approximately 80% of these calculi are composed of calcium oxalate and calcium phosphate, followed by cystine, struvite and ammonium acid urate stones (Kalpana et al., 2013). Supersaturation is the first step to

promote these undesirable conditions and further steps involved are crystallization, nucleation, aggregation and growth (Daudon et al., 2004; Kuncha et al., 2014).

Urolithiasis can occur due to adverse effect of the treatment with atazanavir in HIV-infected patients (Chan-tack et al., 2007). Also the techniques for removal of calculi such as endoscopic stone removal lithotripsy and extracorporeal shock wave lithotripsy (ESWL), cause traumatic effect of shock waves, persistent residual stone fragments, acute renal disease, possibility of infection, which leads to decrease in renal function, are all factors that can be responsible for chronic kidney stone infection (Chitra et al., 2012). Anti-urolithiatic drugs from natural sources have assumed greater importance as herbal alternatives which are cost effective with minimal side effects (Alberto, 2008; Shoaib et al., 2014). The main aim of this work is to investigate the anti-urolithiatic properties of *Lippia javanica* and *Foeniculum vulgare*.

Materials and Methods

Materials

Calcium chloride, Sodium oxalate, Tris-acid, Sodium chloride salt (NaCl), Potassium citrate, Calcium oxalate monohydrate crystal, Scanning Electron Microscope (SEM).

Nucleation assay

The stone formation begins with the occurrence of nuclei; therefore we chose the classical model for the study of oxalate crystallization as described by Hennequin et al.(1993) with some minor modifications. Solutions of calcium chloride and sodium oxalate were prepared separately at a final concentration of 3 mM/L and 0.5 mM/L respectively in a buffer containing Tris 0.5 mM/L and NaCl 0.15 mM/L of pH 6.5. Both the solutions were filtered thrice. For the assay, 950 μ L of calcium chloride and varying concentration of *Lippia javanica* and *Foeniculum vulgare* extracts (final volume of 100 μ L) were pipetted out against

a reagent blank (without extract as negative control). To this was added 950 µl of sodium oxalate and vigorously shaken. Potassium citrate was used as a positive control. The absorbance was measured at 620 nm.

Growth assay

The percentage inhibition of calcium oxalate crystal growth was evaluated in presence and absence of extracts by the procedure described by Farooq et al. (2006) and Chaudary et al. (2009). 4 mM calcium chloride and 4mM sodium oxalate of 1mL each were added to a 1.5 mL of solution containing sodium chloride (10 mM) buffered with Tris (10 mM) at pH 7.2. To this 30 µL of calcium oxalate monohydrate crystal slurry (1.5 mg/mL acetate buffer) was added. Consumption of oxalate begins immediately after calcium oxalate monohydrate crystal slurry addition and was monitored for 600 seconds for the disappearance of absorbance at 214 nm, with and without extract. Potassium citrate was used as a positive control.

The relative inhibitory activity was calculated as follows:

$$\text{Relative inhibitory activity (\%)} = [(C-S)/C] \times 100$$

where 'C' is the rate of reduction of free oxalate without any extract and 'S' is the rate of reduction of free oxalate with *Lippia javanica* and *Foeniculum vulgare* extracts.

Aggregation assay

The crystals in solution stick together to form large particles called aggregates. The inhibition in presence of extracts was determined as described by Hess et al. (1989). Calcium oxalate monohydrate crystal seeds were prepared by mixing calcium chloride and sodium oxalate at 50 mM/L. Both solutions were equilibrated to 60°C in a water bath for 1h and then cooled to 37°C overnight. The crystals were harvested by centrifugation and then evaporated

at 37°C. Calcium oxalate monohydrate crystals were used at a final concentration of 0.8 mg/mL buffered with Tris 0.05 M/L and NaCl 0.15 M/L at pH 6.5. Experiments were conducted at 37°C in the absence of the plant extract after stirring, potassium citrate was used as a positive control and absorbance was measured at 620 nm. The rate of aggregation (IR) was estimated by comparing the slope of the turbidity in the presence of the extract with that obtained from the control.

$$IR = (\text{Turbidity of sample} / \text{Turbidity of control}) \times 100$$

Microscopic study: Each calcium oxalate monohydrate crystal prepared during the different stages of the assay after reacting with the plants extracts were immediately viewed under a Scanning Electron Microscope (SEM) at a magnification of x30.

Statistical Analysis

All experiments were done in triplicates and the results were expressed as Mean \pm SD. Where applicable, the data were subjected to student t-test to compare the difference between the acetone and aqueous extracts using the Minitab program (version 12 for windows) (Minitab Inc., Pennsylvania, USA). $p < 0.05$ were considered significant.

Results and Discussion

Kidney stone is the result of supersaturation of urine with certain urinary salts such as calcium oxalate (Beghalia et al., 2008). In the present study, calcium oxalate monohydrate crystals were grown by the classical model for the study of oxalate crystallization because of its simplicity and satisfactory reproducibility. This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of any chemical species used (Kalpana et al., 2013).

Nucleation assay

Nucleation is an essential prerequisite for further formation of larger particles within the urinary tract which ultimately may form a stone (Beghalia et al., 2008). Incubation of the metastable solutions of calcium chloride and sodium oxalate resulted in the formation of calcium oxalate crystals. The extent to which the nucleation of calcium oxalate crystals were inhibited by *Foeniculum vulgare* and *Lippia javanica* were showed in Figure 25. Calcium oxalate crystals appear hexagonal in shape (control) and the disrupted calculi appear dendritic like, losing the regular hexagonal shape. The acetone extracts were able to reduce the size of the crystals formed, which is an indication of its anti-urolithiatic activity while aqueous and essential oil were found to be less effective in the disruption of calculi. The protective potency of *Foeniculum vulgare* and *Lippia javanica* were quite comparable to the effect of citrate which did not affect the rate of crystal nucleation. The results were in agreement with Pachana et al. (2010) who reported that an extract of *Tribulus terrestris* promoted the inhibitors of nucleation initiation, by decreasing their size. Pareta et al. (2011) also reported that hydroalcoholic extract of *Achyranthes indica* remarkably inhibits the crystal formation. Kalpana et al. (2013) also reported that the ethanolic extracts of banana corm could reduce the size of crystals formed. The present study revealed that Lippia acetone extract had more effect on the calcium oxalate monohydrate crystals by breaking it into smaller particles at varying concentrations (100, 200, 300, 400, 500 ug/mL) followed by the Lippia oil extract (Figure 26).

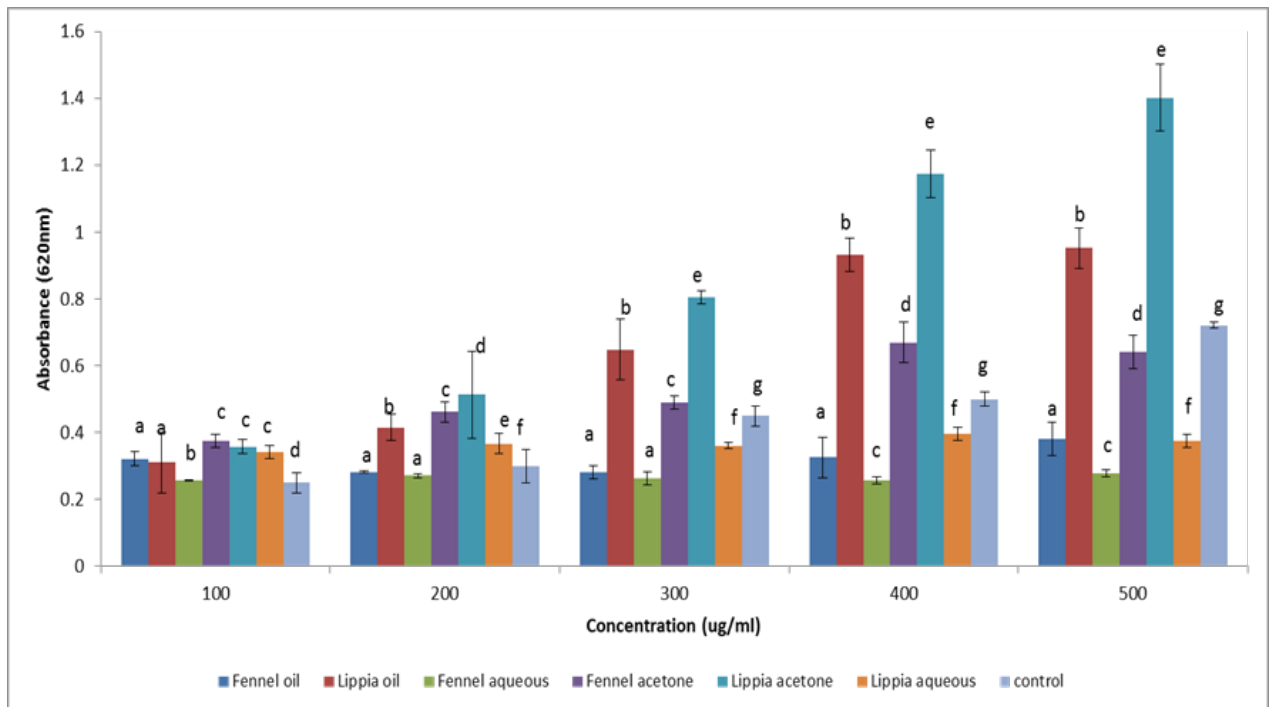


Figure 25: Effects of *Foeniculum vulgare* and *Lippia javanica* extracts on nucleation of calcium oxalate monohydrate slurry. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare*

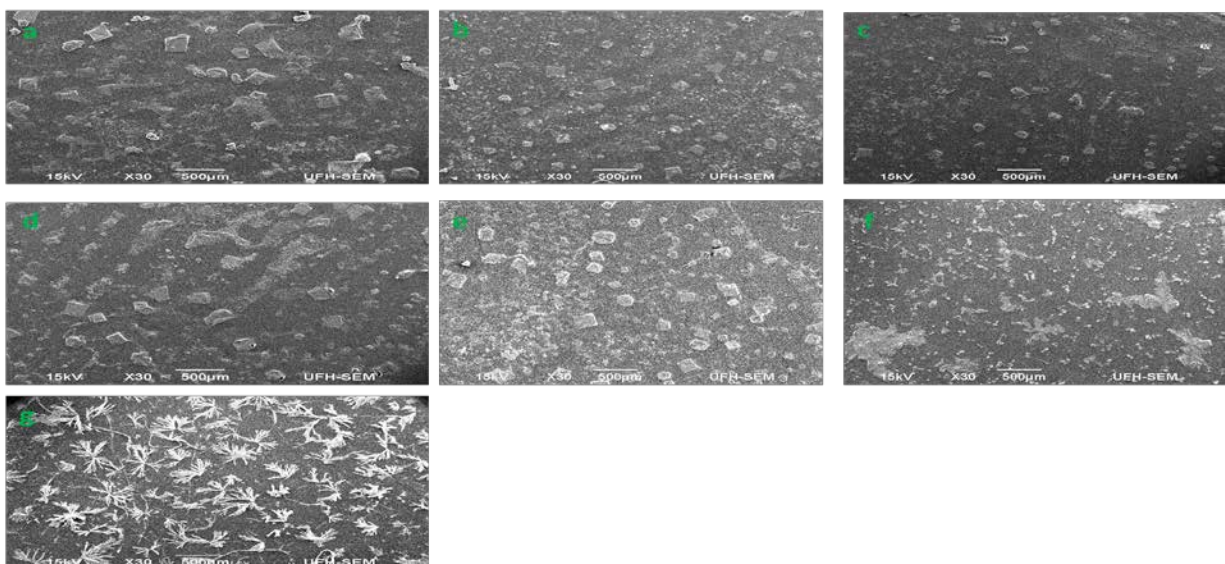


Figure 26: SEM photographs showing the effect of *Lippia javanica* and *Foeniculum vulgare* extracts on nucleation of CaOx. (plate a-without extract (negative control), plate b- *F. vulgare*

acetone extract, plate c- *L. javanica* acetone extract, plate d- *F. vulgare* water extract, plate e- *L. javanica* water extract, plate f- *F. vulgare* oil extract, plate g- *L. javanica* oil extract).

Growth assay

The aggregated clusters of calcium oxalate particles grow further. The extents to which the growths of calcium oxalate crystals were inhibited by the presence of the extracts of *Foeniculum vulgare* and *Lippia javanica* are shown in Figure 27. Among the various solvent extracts analysed, oil from *Lippia javanica* exhibited highest inhibition followed by its acetone extract. All other extracts also exhibited a considerable inhibitory action. In crystal growth assay, various mechanisms have been proposed to explain crystal retention. As a result of crystal growth, particles formed may be too large to pass freely through the renal tubules, leading to the formation of surface nuclei. The spreading of these into rows and joining of the rows to form monolayers; when placed on top of one another, they form a build-up called calculus. The rate at which this calculus is broken down by various extracts indicates their anti-lithiatic ability (Pareta et al., 2011).

Aggregation assay

Some stone crystals bind to one another through a process known as aggregation or agglomeration promoted by strong chemicals and electrical forces. Adhered crystals are held in place and cannot be easily separated and is a very important step in urolithiasis (Kalpana et al., 2013). The inhibitory potential of the different extracts used in this study compared with the controls shows that all the extracts exhibited greater antilithiatic capacity than the control. Among all the extracts, fennel oil exhibited the highest level of inhibition followed by the *Lippia* oil. The inhibition was in the order *F. vulgare* oil > *L. javanica* oil > *L. javanica* acetone > *F. vulgare* acetone > *L. javanica* water > *F. vulgare* water > control as shown by the scanning electron images (Figures 28 & 29). To the best of our knowledge there has been no previous report on inhibition of nucleation, growth and aggregation of

calcium oxalate crystallization by *Foeniculum vulgare* and *Lippia javanica*, therefore, we present this result on the *in vitro* evaluation of anti-urolithiatic potentials of the different extracts of *F.vulgare* and for the first time, that *Lippia javanica* can be used for same purpose.

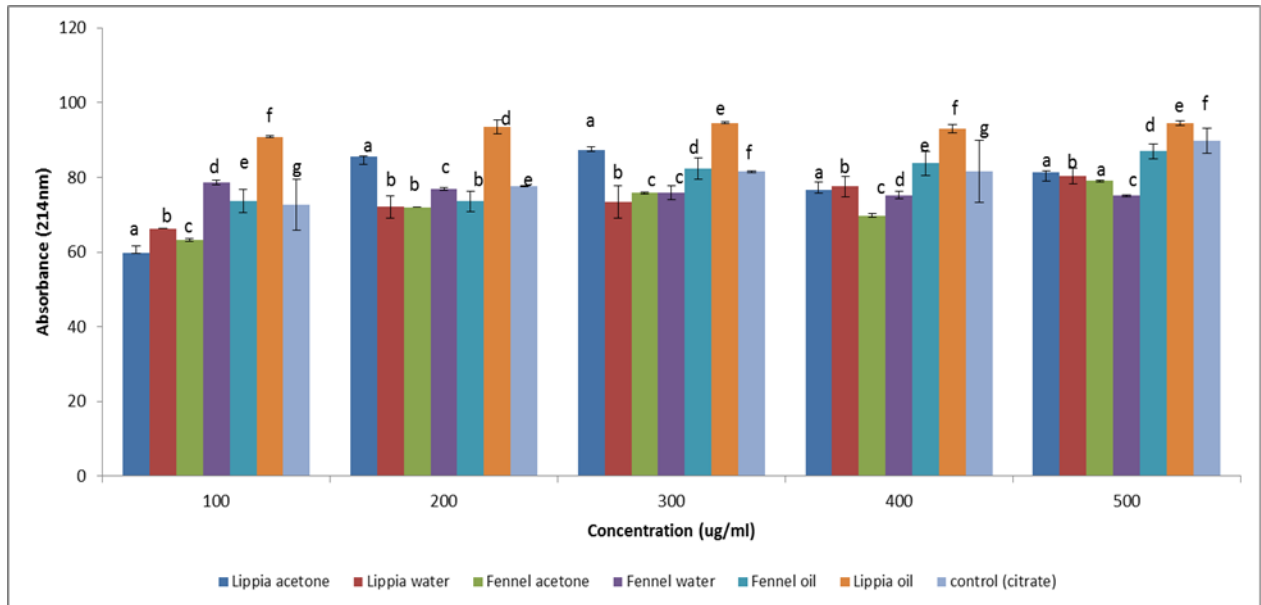


Figure 27: Effects of *Foeniculum vulgare* and *Lippia javanica* extracts on growth of calcium oxalate monohydrate slurry. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel–*Foeniculum vulgare*

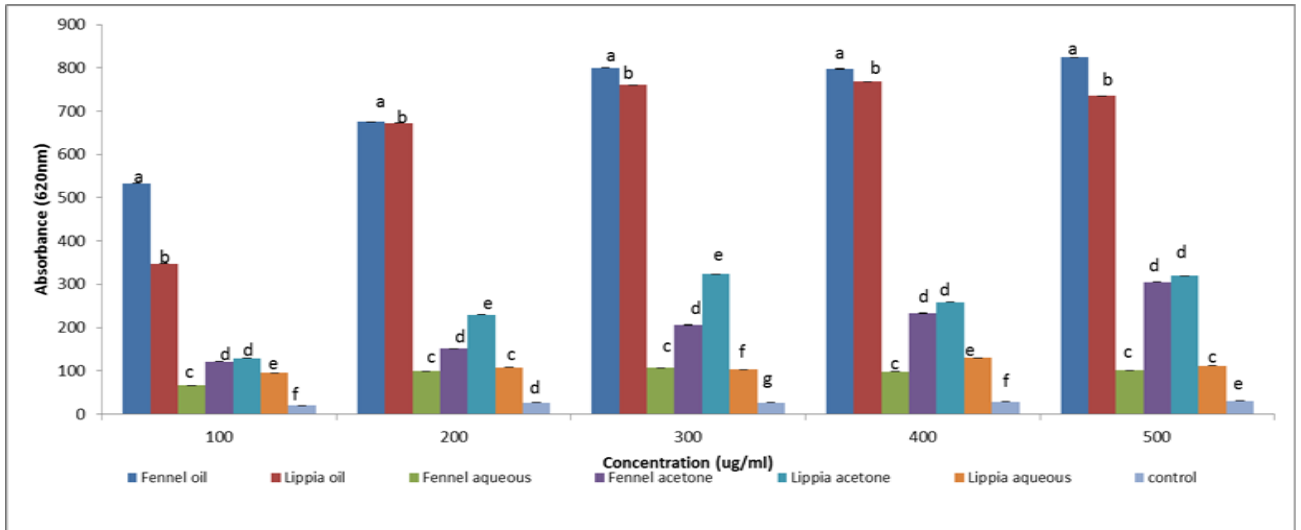


Figure 28: Effects of *Foeniculum vulgare* and *Lippia javanica* extracts on aggregation of calcium oxalate monohydrate slurry. Data are presented as means \pm SD of three replicates. Bar graphs with different letter superscript within the same concentration are significantly different ($P < 0.05$). * Lippia –*Lippia javanica*, Fennel-*Foeniculum vulgare*

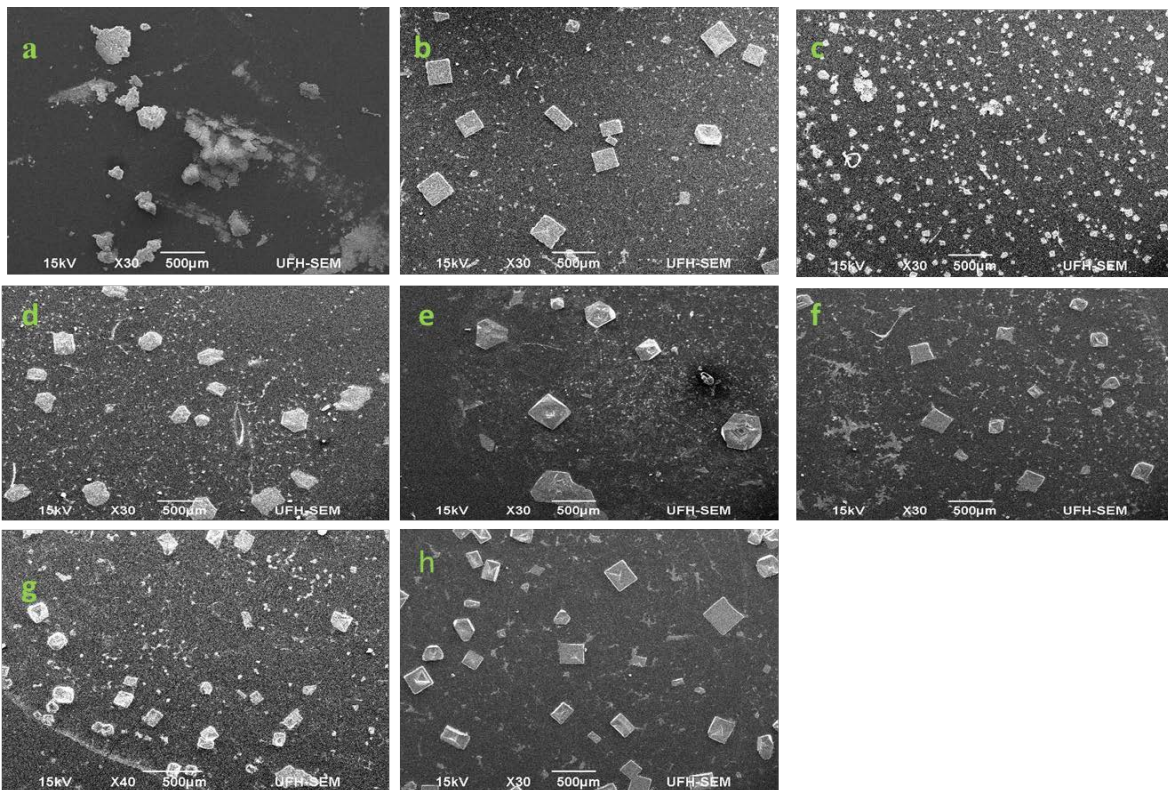


Figure 29: SEM photographs showing the effects of *Lippia javanica* and *Foeniculum vulgare* extracts on aggregation of calcium oxalate (plate a- crystal before the experiment, plate b- without extract (negative control), plate c- *F. vulgare* oil extract, plate d- *L. javanica* oil extract, plate e- *F. vulgare* acetone extract, plate f- *F. vulgare* water extract, plate g- *L. javanica* water extract, plate h- *L. javanica* acetone extract).

Conclusion

Urolithiasis is one of the most common diseases with worldwide increasing incidence and prevalence. The pathogenesis of calcium oxalate accounts for >80% of all urinary stones and incompletely understood. In the present study, calcium oxalate monohydrate crystals were grown by the classical model for the study of oxalate crystallization because of its simplicity and satisfactory reproducibility. The acetone extracts of *F. vulgare* and *L. javanica* was able to reduce the size of the crystals formed. The inhibitory potential of different extracts with the control revealed that all the extracts exhibited good inhibitory potentials compared with the control (citrate) but the essential oil from *Foeniculum vulgare* exhibited the highest inhibitory property. This study validated the traditional use of *F.vulgare* in the treatment of kidney stones in humans.

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CHAPTER NINE

General Discussion and Conclusion

Herbs and spices are traditionally defined as any part of a plant that is used in the diet for their aromatic properties with no or low nutritional values (Davidson, 1999). Spices and culinary herbs are as important today as they were in ancient times for enhancing the flavour and taste of our food, as well as serving as a source of dietary medicine. Worldwide, there are about 70 species of spices cultivated in different parts of the world but nine spices have got so much popularity and importance due to their economic importance. These spices are pepper, ginger, cloves, cinnamon, cassia, mace, nutmeg, pimento and cardamom. In Africa, few species have also been used as spices mainly as spice blends and they include chilies, cilantro, cinnamom, cloves, pepper, cumin, fenugreek, garlic, ginger and nutmeg (Carlsen et al., 2010).

In most parts of Southern Africa, there is little evidence of ancient use of spices in food. Information on plant species used as spices and condiments are inadequate or completely lacking. According to Van Wyk (2011), spices are relatively rare in South Africa but some spices such as *Heteropyxis natalensis*, *Mentha longifolia*, *Myrothamnus flabellifolia*, *Pelargonium graveolens*, *Siphonochilus aethiopicus* and *Warburgia salutaris* are of importance as potential sources of new flavours for the food industry. However, while the culinary evidence for the use of common herbs and spices have been scarce or lacking, their beneficial effects in ethnomedicinal applications abound and are generally encouraging. This gave rise to an ethnobotanical survey on indigenous spices used among the people of Nkonkobe Municipality.

The outcome of the survey revealed 58 species of plants belonging to 29 families and 50 genera that are generally used as spices. The families contributing the most taxa were Solanaceae, Apiaceae, Amaryllidaceae, Amaranthaceae and Lamiaceae. Some of the plant

species mostly used as spices and for the local ethno-medicinal system in the study area include *Lippia javanica* (Verbenaceae), *Mentha aquatica* (Lamiaceae), *Mentha longifolia* (Lamiaceae), *Mentha spicata* (Lamiaceae), *Capsicum annuum* (Solanaceae) and *Foeniculum vulgare* (Apiaceae). *Lippia javanica* has been reportedly used for flavouring drinks, treatment of fever and skin disorders (Oliveira et al., 2006) while *Foeniculum vulgare* is used in the treatment of a wide range of ailments related to the digestive, endocrine, reproductive and respiratory systems (Diaaz-Maroto et al., 2006). In the present study, the most common ailments treated using spices include colds, coughs, skin diseases, antimicrobial infections, respiratory diseases, worms, immune deficiency, diabetes, ulcers, kidney stone and cancer.

The variability in terms of age, gender and user status (laypeople, traditional healer or herbalist) of the respondents has significant implications with respects to the culinary and medicinal knowledge of spices in Nkonkobe Municipality. In terms of age, considering the fact that only 15% of the respondents were below 45 years suggests that the legacy of the indigenous knowledge of culinary spices and their medicinal uses is endangered in the study area. This finding agrees with the view of Giday et al., 2009 that indigenous knowledge of medicinal plants in Nkonkobe area is declining. Nkonkobe Municipality has a rich diversity of medicinally important plants and the tradition of using plants for medicinal purposes is still alive in the local community. However, there is a gradual decline in the knowledge of plants used as spices among the younger generation as observed in this study. This therefore calls for the need to raise awareness among the local people on the use of spices and to properly document the indigenous knowledge and sustainance of the use of spices in the area before it becomes lost to future generations. This study is therefore of utmost importance as it has fulfilled a part of this documentation of indigenous plants used as spices.

Ultra-morphological studies

The ultra-morphological study of *Lippia javanica* and *Foeniculum vulgare* revealed the presence of non-glandular and glandular trichomes on the leaf surfaces of these herbs which may serve as secretory sites where aromatic secondary metabolites found in them are produced. In this study we observed that the leaf of *Lippia javanica* possessed spine-like, non-glandular, uniseriate trichomes. These types of trichomes have been reported for *Lippia* species and verbenaceae family in general (Combrinck et al., 2007). The stomata are amphistomatic because they are characterized by more or less randomly distributed epidermis, lying almost close to each other and are fewer in number in between the veins and over finer veins. These attributes may explain the wide distribution of *L. javanica* in various climatic environments in Southern Africa.

The leaf and stem of *Foeniculum vulgare* as observed under SEM showed considerable amount of amphistomatous (equal distribution of stomata on both adaxial and abaxial surfaces) stomata densely distributed on the leaf and stem surface on the epidermal surfaces. The densely amphistomatous stomata in *F. vulgare* could account for its habitat which is in the open with adequate amount of water intake because plants growing in shady and drought prone environments have sparse distribution of stomata (Pavina et al., 2013). The stomata is diacytic while the guard cells are elliptical in shape, this is in agreement with the report of Annal et al., (2006) on taxonomic significance of leaf characters in some species of Apiaceae.

The chemical nature of the crystal deposits on *L. javanica* leaves and the leaves, stem and seed of *F. vulgare* as revealed by SEM-EDX include micro elements ((Bromine (Br), Aluminium (Al) and Silicon (Si)) and macro mineral elements (Calcium (Ca), Sodium (Na), Phosphorus (P), Sulphur (S), Chlorine (Cl), Potassium (K), Carbon (C), Magnesium (Mg) and Iron (Fe)). Calcium and silicon were observed to be higher in both plants. Calcium is

essential for muscle contraction, oocyte activation, building strong bones and teeth blood clotting, nerve impulse regulating heart beat and fluid balance within cells (Pavina et al., 2013) while silicon is an essential component of mucopolysaccharides, hyaluronic acid and chondroitin-4-sulfate, which are important constituents of connective tissue. This is a biological cross-linking agent contributing to the structure and resiliency of connective tissue and calcification of bones hence plays a role in wound healing (Price et al., 2013).

Some of these trace elements have also been observed in some other spices such as onion, pepper, cumin and vanilla and the regular consumption can make significant contribution to the daily recommended intake of minerals (Nkansah and Amoako, 2010).

Nutrient, mineral, vitamin and antinutrient composition

The presence of crude fibre, moisture, total ash, total protein, lipids and soluble carbohydrate in *Lippia javanica* and *Foeniculum vulgare* is an indication of the holistic nature of the herbal medicine. *Foeniculum vulgare* had higher ash, moisture, lipid, fibre and protein content while carbohydrate content was higher in *Lippia javanica*. Previous studies of the nutrient composition of some spices culled from an extensive search of the literature reported that spices were generally high in ash and crude fibre (Murphy et al., 1990; Dauda et al., 2014).

The elemental results revealed that *Foeniculum vulgare* showed higher Mg, K and Na content while *Lippia javanica* was higher in Ca, Zn, Cu and Fe. Spices from seeds were highest in phosphorus. A few spices such as celery seed, cumin, coriander leaf, dill weed, cloves and especially parsley flakes were very high in sodium (Murphy et al., 1990; Iheanacho and Udebuani, 2009). Similar trends were also observed for *L. javanica* and *F.vulgare* in this study. This is an indication that each of these spices can help in meeting the recommended daily allowance of these nutrients in the diet.

The vitamins A and E were also higher in *Lippia javanica* while *Foeniculum vulgare* had higher vitamin C content. No significant differences were observed in the phytate, oxalate and tannin contents of the two spices, but saponin and cyanide were significantly lower in *Lippia javanica* than in *Foeniculum vulgare*. Results of the nutritive analysis showed that *Lippia javanica* and *Foeniculum vulgare* are good sources of nutrients, minerals, vitamins and phytochemicals and they may be used to enrich the diet. The presence of phytates, tannins, saponins and other antinutrients indicates that they may have therapeutic uses as hypoglycaemic, hypocholesterolomic, antioxidant, anti-inflammatory, antihypertensive and anticancer agents compared to other plants reported (COMA, 1984; Mcneely, 1990; Nkafamiya et al., 2010).

Essential oil composition

The composition, antimicrobial activity and cytotoxicity of the essential oil of fresh and dry leaves of *Lippia javanica* and *Foeniculum vulgare* revealed that the dried samples yielded more oil compared to fresh samples. Comparatively, the yield in oil was higher in *Lippia javanica* than in *Foeniculum vulgare*. The results also revealed some bioactive compounds such as bicyclo (3.1.1) heptan-2-one and myrcene that has been identified as major constituents of *Lippia javanica* by other studies (Viljeon et al., 2005). All the components of the two oils were different except for the presence of cymene. The other components were generally monoterpenes which include anethole, α -pinene, o-cymene, D-limonene, verbenone, β -myrcene, 2-thujene, fenchone and estragole. Sesquiterpenes such as pentadecane, germacrene D, copaene and caryophyllene were also present in trace amount. Cymene (p-isopropyltoluene) is a naturally-occurring aromatic organic compound (Santana et al., 2011). It is classified as a hydrocarbon related to a monoterpene and it is a precursor of carvacrol and one of the main constituents of essential oils (Siani et al. 1999). Cymene is present in volatile oils from over 100 plants and occurs naturally in more than 200 foods

(orange juice, grape fruit, tangerine, carrots, raspberries, butter, nutmeg, oregano, and almost every spice). Cymene is an important intermediate used in pharmaceutical industries for the production of fungicides, pesticides and as a flavoring agent (Selvaraj et al., 2002).

F. vulgare is well known for its essential oil which major components have been reported to be trans-anethole, fenchone, estragol (methyl chavicol) and α -phellandrene (Diaaz-Maroto et al., 2006). Anethole has also been reported to have potent antimicrobial properties, against bacteria, yeast, and fungi (De et al., 2002). A few spices such as celery seed, cumin, coriander leaf, dill weed, cloves and especially parsley flakes have been reported to contain the monoterpenoids and sesquiterpenoids (Selvaraj et al., 2002).

Antimicrobial and brine shrimp toxicity activities

The antibacterial effect of *Lippia javanica* could be attributed to the presence of phenols and monoterpenes such as myrcene, pinene and verbenone which have been reported to have antibacterial activity against *S. aureus*, *E. coli* and *L. monocytogenes* (Ceylan and Fung, 2004; Lopez et al., 2005). This could also be responsible for the antifungal activity, as spices and herbs used today have been valued for their antimicrobial effects and medicinal powers in addition to their flavour and fragrance qualities (Ceylan and Fung, 2004 ; Davidson et al., 2005). The antimicrobial activities of *F. vulgare* are generally attributed to its essential oil. Numerous studies have shown that the essential oil of this plant and its individual constituents exhibit novel pharmacological activities (Diaaz-Maroto et al., 2006; Telci et al., 2009). For example, fenchone, anethole and P-anisaldehyde were identified as the major acaricidal agents against *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (De et al., 2002; Telci et al., 2009). The findings suggest that these plants have potential in treatment of skin infections caused by fungal species especially the oil of *F. vulgare* and *L. javanica* which exhibited great antifungal and antibacterial activity, compared to nystatin (the

reference drug), acetone and aqueous extracts of the plants. The high antifungal activity of *Foeniculum vulgare* and *Lippia javanica* oil against some pathogens especially *M.canis* and *T.rubrum* could be attributed to the presence of anethole in *F.vulgare* and other constituents present in both spices. This indicates that the plants could be further explored for their antimycotic purposes.

Polyphenolic contents and *in-vitro* antioxidant capacity

This study revealed the presence of considerable amounts of total phenols, flavonoids, and proanthocyanidins in acetone and aqueous extracts of both *Lippia javanica* and *Foeniculum vulgare*. The acetone extracts had a higher extraction over water indicating differences in the extractive capacity of the two solvents. Considerable research has been directed toward understanding the nature of polyphenols in spices. Phenolic compounds aid in the preservation of food, fresh flavour, taste, colour and in prevention of oxidative deterioration. In particular, many phenolic compounds are attracting the attention of food and medical scientists because of their antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties and their capacity to modulate some key cellular enzyme functions (Jimoh et al., 2008). Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydrogen peroxides into free radicals (Yanishlieva et al., 2001).

The antioxidant activity of plant extracts is often associated with the phenolic compounds present in them. Plant phenols constitute the major group of compounds that act as primary antioxidant (Yanishlieva et al., 2001). In this study no good correlation was found between the antioxidant activities and the total phenolic and flavonoids content because extracts with the lowest IC₅₀ values in antioxidant activities did not show a high phenolic content. This observation is similar to the report of Bajpai et al. (2005) that *Cinnamomum tamala* have low

total phenolic content but high antioxidant activity indicating the presence of other constituents other than phenols with antioxidant activity. This definitely implies that the bioactive constituent of *Lippia javanica* and *Foeniculum vulgare* include some other phytochemicals such as alkaloids, saponin, tannin and glycosides.

The data presented in this study demonstrates that almost all the reported extracts possess excellent antioxidant and free radical scavenging activity. They showed different behaviour in all the *in-vitro* assays, probably due to the different mechanisms involved in the steps of the oxidation process. A significant total phenolic content was found in acetone extracts of *L. javanica* and *Foeniculum vulgare* while a significant radical scavenging and antioxidant activities was found in aqueous extracts of the two plants.

Generation of reactive oxygen and nitrogen species (ROS) and (NOS) and other free radicals is a normal metabolic process which is compensated for, by an elaborate endogenous antioxidant defense system. However, when excessive generation of free radicals over balance the rate of their removal, oxidative stress occurs. ROS and oxidative stress have been implicated as mediators of pathological conditions such as atherosclerosis, diabetes and inflammatory diseases. The need for exogenous antioxidants to augment the endogenous antioxidant system is of current interest. Apart from green tea, several herbs and spices are the most important targets in the search for natural antioxidants from the view point of safety. Several phenolic and flavonoid compounds present in spices have been documented to possess potent antioxidant, anti-inflammatory and anti-carcinogenic properties. These bioactive compounds appear to be responsible for the chemopreventive or chemoprotective activities of spices (Srinivasan, 2014). The antioxidant potentials of *F. vulgare* and *L. javanica* as confirmed in this study has far reaching health implications of their ability to prevent oxidative stress in human and animal subjects.

Anti-inflammatory activities

Denaturation of proteins is a well-documented cause of inflammation. Therefore, the ability of *Lippia javanica* and *Foeniculum vulgare* extracts to inhibit protein denaturation may contribute to their anti-inflammatory properties. This study revealed that both plant extracts were able to inhibit protein denaturation in a concentration-dependent manner. *Lippia javanica* extracts were more effective than *Foeniculum vulgare* extracts and both extracts compared reasonably with the standard drug, diclofenac. These plant extracts have the potential to prevent cell membrane protein denaturation and membrane lysis due to the presence of membrane stabilizers and protein denaturation inhibitory agents. Many studies conducted on the anti-inflammatory activities of some spices revealed that spices may reduce inflammation in the body (Nickavar et al., 2007; Conforti et al., 2008; Umapathy et al., 2010; Dzoyem & Eloff, 2015).

Antilithiatic activity

In this study, the inhibitory potential of acetone and aqueous extracts of *Foeniculum vulgare* and *Lippia javanica* compared with the control shows that all the extracts exhibited good anti-lithiatic potentials compared with the control (citrate) but the essential oil from *Foeniculum vulgare* gave the best inhibitory action. Kidney stone formation results from a succession of several physiochemical events which include supersaturation, nucleation growth, aggregation and retention in the kidney. Worldwide, incidence of urolithiasis is quite high and more than 80% of urinary calculi are calcium oxalate stones or calcium oxalate mixed with calcium phosphate (Thomas, 2010).

Naturally occurring triterpenes of plant origin have been identified as possessing a wide range of pharmacological effects including reducing the risk of stone formation (Yadaw et al., 2011). These activities have been attributed to the presence of terpenes, saponins,

alkaloids and amides present in these plants. Similar to these reports, our study have revealed that extracts of *Foeniculum vulgare* and *Lippia javanica* have successfully inhibited stone formation by *in-vitro* model. This is a step in the validation of the antiurolithiatic capacity of the two plants. The present findings affirm the acclaimed effectiveness of *Foeniculum vulgare* against urinary infections and kidney stone formation in Nkonkobe folk medicine, as well as for the first time, the anti-urolithiatic property of *Lippia javanica*.

These results are in agreement with several authors who reported that extracts of *Tribulus terrestris*, *Achyranthes indica* Linn. and banana corm promoted antilithiasis by inhibiting nucleation, crystal formation and reduction of crystal size (Pachana et al., 2010; Pareta et al., 2011; Kalpana et al., 2013). Some spices that have been reportedly used for kidney problems are *Allium sativum*, *Apium graveolens* and *Citrus japonica* (Cheryl, 2006). The overall antilithiatic studies revealed that the essential oil of these species are active compared to extracts from acetone and aqueous.

Conclusion

This study has justified the traditional usage of *Lippia javanica* and *Foeniculum vulgare* for the management of kidney stone. The therapeutic effects of the plants may be attributed to the presence of phytochemicals which possess various bioactive constituents. The bioactive compounds present also contribute to the free radical scavenging activity and anti-inflammatory potential thus reducing or preventing the pathogenesis of diseases. The effect of these plants on microbial strains tested gives further credence to the therapeutic usage of these plants as antibacterial and antimycotic agents. The consumption of spices to derive beneficial effects has been proved to be safe even at very high dietary levels. The low toxicity, high nutrient content and various pharmacological activities observed in *Foeniculum vulgare* and *Lippia javanica* demonstrated by their antioxidant, antimicrobial, anti-

inflammatory and antiurolithiatic activities has far-reaching health implications. Taken together, these results indicate that *Foeniculum vulgare* and *Lippia javanica* both possess therapeutic and pharmacological qualities which suggest their potential applications in the pharmaceutical industry, as well as the production of health foods, beverages and nutritional supplements. Therefore the two species deserve to be considered as natural and necessary components of daily nutrition and diet.

Outcome of the study

The following are the main findings and outcome of this research work:

1. The plant families commonly used for spices in Nkonkobe Municipality were Solanaceae; this was followed by Apiaceae, Amaryllidaceae, Amaranthaceae and Lamiaceae.
2. The spices most commonly used in Nkonkobe Municipality for culinary and therapeutic purposes were *Lippia javanica* and *Foeniculum vulgare*.
3. This first report of spices in this area will serve as a reference as well as document and preserve the indigenous usage of these herbs and spices in Nkonkobe Municipality, Eastern Cape and South Africa at large.
4. The leaves of *Foeniculum vulgare* and *Lippia javanica* were amphistomatic and amphitrichomic respectively, the major constituents of crystals found in both species are Ca, O, Na and S while Al, S, P, Cl, K, C, Mg and Fe were found only in *Lippia javanica*.
5. The knowledge of ultra-morphological studies of *Foeniculum vulgare* and *Lippia javanica* has indicated that the plants contain non-glandular and glandular trichomes on their leaf surfaces which may serve as secretory sites where aromatic secondary metabolites found in them are produced.

6. There is little or no information on the morphology and ultrastructure of the leaf appendages of *Foeniculum vulgare*, as we have on *Lippia javanica*, so this is the first report on ultra-morphological studies of *Foeniculum vulgare* and *Lippia javanica* in this region. It will therefore serve as a reference on morphology of the two plants.
7. This study also provides new evidence of the nutritional values of *L. javanica* and *F. vulgare* and indicates that the two spices may provide meaningful level of nutrients, minerals and vitamins when consumed in a variety of foods.
8. A significant total phenolic content was found in acetone extracts of *L. javanica* and *F. vulgare* extracts while a significant radical scavenging and antioxidant activities were found in aqueous extracts of the two plants.
9. There was no correlation between the antioxidant activities and the total phenolic and flavonoids content because extracts with the lowest IC₅₀ values in antioxidant activities did not show a high phenolic content. This implies that other constituents of phytochemical such as alkaloids, tannins may also be responsible for antioxidant activities.
10. The most promising free radical scavenger appears to be the aqueous extract which exhibited the lowest IC₅₀ value in almost all the assays.
11. The dried samples yielded more oil compared with the fresh samples of the plants.
12. The acetone extracts from both plants were active against five fungi which are *Penicillium chrysogenum*, *Tricophyton mucoides*, *Microsporium canis*, *Tricophyton tonsurans* and *Tricophyton rubrum* while the aqueous extract was only active against *Penicillium chrysogenum*, compared to the reference drug, nysticin which was active against seven out of the nine fungi.
13. The effect of the essential oils on some pathogens such as *Salmonella typhimurium*, *Esherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* showed reduction

in the populations of these microorganisms having a strong bacteriostatic effect with minimum inhibitory concentration (MIC) range from 0.625 µg/mL to 1.25 µg/mL. The two plants showed similar activities on the tested bacteria.

14. The least concentration of 20 µg/mL used for the cytotoxicity test was toxic on the brine shrimps.
15. The low LC₅₀ values in fresh oil in this study probably means that the fresh leaves of the plant may be toxic compared to the dried oil. Therefore fresh leaves may be used more for preparation and preservation of home-made food.
16. The potential of the different extracts of *F. vulgare* and *L. javanica* on urolithiasis indicate that both plants extracts have the ability to prevent stone formation. The essential oil of *F. vulgare* exhibited the greatest potential. The *in-vitro* experiment validates the traditional use of *F.vulgare* in treatment of kidney stones in humans.
17. This is the first documented report on *in-vitro* evaluation of antiurolithiatic potential of different extracts of *Foeniculum vulgare* and *Lippia javanica*.

Contribution to knowledge

1. The ethnobotanical survey on herbs and spices used for culinary and medicinal purposes will be the first report on plants used as spices in this region. It will therefore serve as a reference as well as document and preserve the indigenous knowledge of these herbs and spices in Nkonkobe Municipality, Eastern Cape and South Africa at large.
2. Also the morphology and ultrastructure of the leaf appendages stem and seed of *Foeniculum vulgare* will be the first report on ultra-morphological studies of *Foeniculum vulgare* in this region. It will therefore serve as a reference on morphology of the *Foeniculum vulgare*.
3. This study also provides new evidence of the nutritional values of *L. javanica* and *F. vulgare* and indicates that the two spices may provide meaningful level of nutrients, minerals and vitamins when consumed in a variety of foods.
4. This study has contributed to scientific knowledge at large and may serve as reference point to the use of *Foeniculum vulgare* in folkloric medicine and revealing the possibility of using *Lippia javanica* for kidney stone treatment.

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Appendix A

Informant consensus factor

Ethnobotanical investigation of medicinal plants used as spices in the Eastern Cape Province, South Africa.

Informed consent form for informants

I have been informed that my responses, recorded in the questionnaire presented, will be used for academic purposes only as captioned above.

I acknowledge that I have been given an explanation of the objectives of the study.

I understand that the interview may take up to 15 minutes maximum

I understand that my responses will be kept anonymous

I have the right to withdraw at any time before the interview is complete

I acknowledge that my questions have been answered to my satisfaction.

UREC has approved the research and sponsors of the study, study monitors or auditors or UREC members may need to inspect research records

By my signature below, I consent to be interviewed.

Informant Signature..... Date.....

Witness / team member.....Date.....

Contact details of Investigator

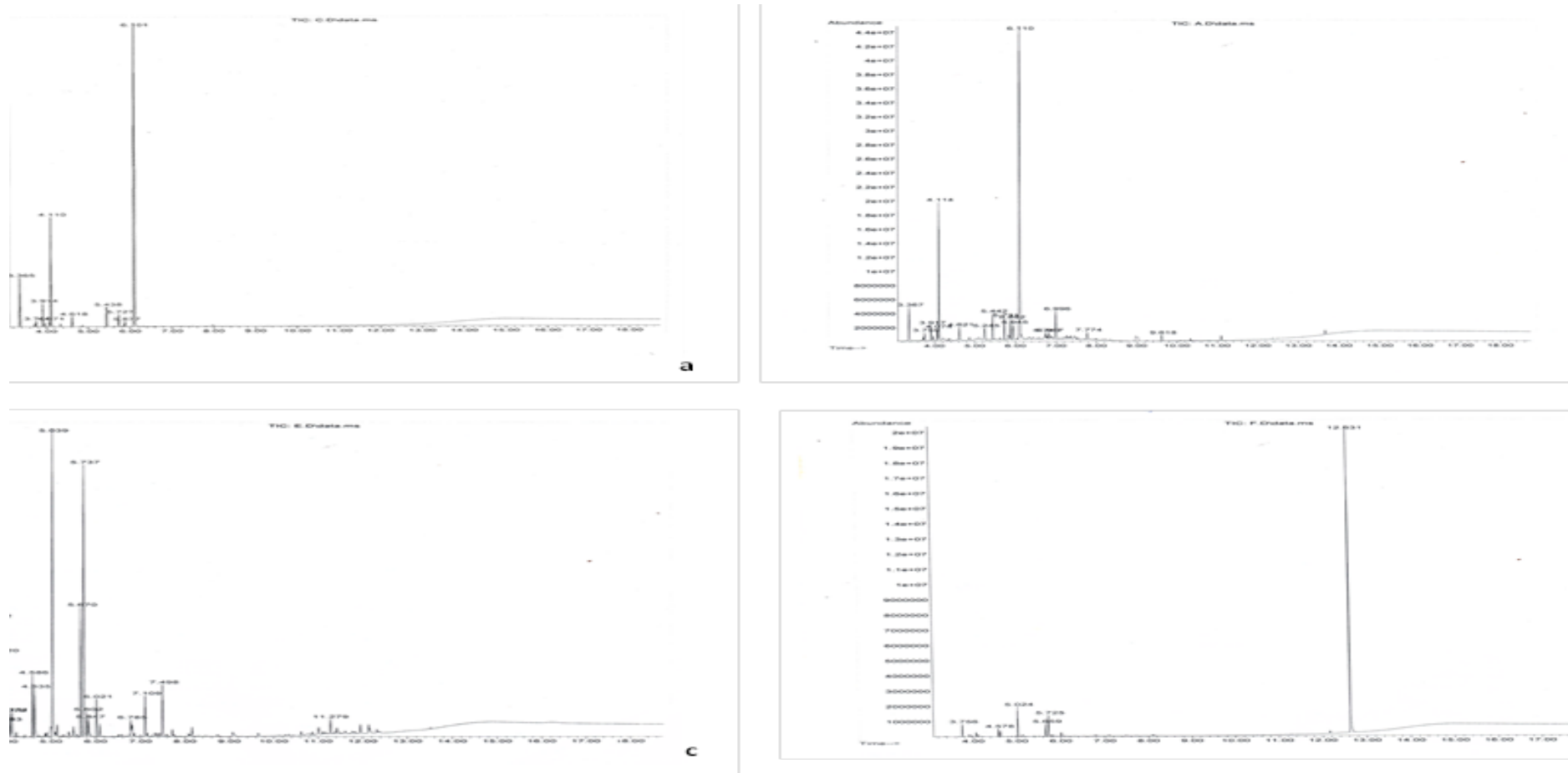
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GC/MS Chromatogram of the essential oil obtained from fresh and dried leaves of *Foeniculum vulgare* (a & b) and *Lippia javanica*.

Figure 30: GC/MS Chromatogram of the essential oil obtained from the fresh and dried leaves of *Foeniculum vulgare* (a & b) and *Lippia javanica* (c & d)



University of Fort Hare
Together in Excellence

**ETHICAL CLEARANCE CERTIFICATE
REC-270710-028-RA Level 01**

Certificate Reference Number: AFO031SASO01

Project title: **Ethnopharmacological studies of medicinal plants used as spices in Nkonkobe Municipality, Eastern Cape Province, South Africa.**

Nature of Project: PhD

Principal Researcher: Abiola Molisola Asowata-Ayodele

Supervisor: Prof AJ Afolayan

Co-supervisor:

On behalf of the University of Fort Hare's Research Ethics Committee (UREC) I hereby give ethical approval in respect of the undertakings contained in the above-mentioned project and research instrument(s). Should any other instruments be used, these require separate authorization. The Researcher may therefore commence with the research as from the date of this certificate, using the reference number indicated above.

Please note that the UREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the document
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

The Principal Researcher must report to the UREC in the prescribed format, where applicable, annually, and at the end of the project, in respect of ethical compliance.

Special conditions: Research that includes children as per the official regulations of the act must take the following into account:

Note: The UREC is aware of the provisions of s71 of the National Health Act 61 of 2003 and that matters pertaining to obtaining the Minister's consent are under discussion and remain unresolved. Nonetheless, as was decided at a meeting between the National Health Research Ethics Committee and stakeholders on 6 June 2013, university ethics committees may continue to grant ethical clearance for research involving children without the Minister's consent, provided that the prescripts of the previous rules have been met. This certificate is granted in terms of this agreement.

The UREC retains the right to

- Withdraw or amend this Ethical Clearance Certificate if
 - Any unethical principal or practices are revealed or suspected
 - Relevant information has been withheld or misrepresented
 - Regulatory changes of whatsoever nature so require
 - The conditions contained in the Certificate have not been adhered to
- Request access to any information or data at any time during the course or after completion of the project.
- In addition to the need to comply with the highest level of ethical conduct principle investigators must report back annually as an evaluation and monitoring mechanism on the progress being made by the research. Such a report must be sent to the Dean of Research's office

The Ethics Committee wished you well in your research.

Yours sincerely


Professor Gideon de Wet
Dean of Research

27 November 2015

Appendix C

