.

Effect of Rutaceae plant's essential oils and leaf extracts on dermatophytic fungal cell morphology: a hope for the development of an effective antifungal from natural origin

Submitted in fulfillment of the academic
requirements for the degree of
DOCTOR OF PHILOSOPHY

By

OLUFUNKE OMOWUMI FAJINMI

Research Centre for Plant Growth and Development
School of Life Sciences
College of Agriculture, Engineering and Science
University of KwaZulu-Natal, Pietermaritzburg

May 2016

Pictures sourced from google

A healthy, glowing, beautiful skin. . . .the pride of every woman

.

Table of Contents

.

STUDENT DECLARATION

I, Olufunke Omowumi Fajinmi, 210556317 (student number) declare that:

i. The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg.
ii. This dissertation has not been submitted for any degrees or examination at any other university.
iii. This thesis does not contain data, figures or writing, unless specifically acknowledged, copied from other researchers.
iv. Where I have reproduced a publication of which I am an author or co-author, I have indicated which part of the publication was contributed by me.

Signed at _____ on the day _____ of 2016.

_____
SIGNATURE

.

DECLARATION BY SUPERVISORS

We hereby declare that we acted as Supervisors for this PhD student:

Student's Full Name: Olufunke Omowumi Fajinmi

Student Number: 210556317

Thesis Title: Effect of Rutaceae plant's essential oils and leaf extracts on dermatophytic fungal cell morphology: a hope for the development of an effective antifungal from natural origin.

Regular consultation took place between the student and us throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science, Higher Degrees Office for Examination by the University appointed Examiners.

_____

SUPERVISOR: PROFESSOR. J. VAN STADEN

_____

CO-SUPERVISORS: PROFESSOR. J. F. FINNIE

_____

DR M.G. KULKARNI

.

COLLEGE OF AGRICULTURE ENGINEERING & SCIENCE

DECLARATION 1- PLAGIARISM

I, Olufunke Omowumi Fajinmi declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain any other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain any other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted , then:
    a. Their words have been re-written but the general information attributed to them has been referenced.
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.
6. The essential oil analysis and antioxidant activity recorded in this study was carried out by our collaborators in Czech Republic.

Signed _____

.

ACKNOWLEDGEMENTS

I give thanks to the Almighty God for giving me the opportunity to complete this study with ease. When my strength and wisdom failed me, his divine presence kept me going. He started the good work and completed it in due season. I would like to express my sincere gratitude to my supervisor Prof Van Staden, for his words of encouragement and inspiration during the course of this study, you are indeed a great mentor. I appreciate my co-supervisors; Prof Finnie for his constant support, kind efforts and words of encouragement; Dr Kulkarni for his words of encouragement.

Many thanks to all members of the Research Centre for Plant Growth and Development for the friendship we have shared over the years. Special thanks to Dr Becki Ncube and 'almost' Dr Devashan Naidoo. Mrs Warren is highly appreciated for her help as regards administrative matters. My sincere appreciation goes to Mrs Alison Young for her assistance as regards seeds and plant collection. I appreciate our Czech collaborators; Prof Dolezal and his wife (Ivana), Petre Tarkowski and others. Thanks to Tibor Beres for his help while I was in Czech. The staffs of Electron Microscopy Unit of UKZN (Subashen, Lorika Beukes and Cynthia) are highly appreciated for their help with electron microscopy work.

I appreciate Pastor and Mrs Olorunda of Dunamis faith assembly for their prayers, words of encouragement, love and support. Many thanks to my guardian Mr and Mrs Fatokun for their support since my MSc days. I appreciate Dr Siyanbola for his fatherly advice and encouragements. To Dr and Dr Mrs Ogunrombi thanks for your love. To my wonderful friend Abimbola Ajetumobi thanks for asking how far and giving me a listening ear. To my friend Renate Makekita Ntumpi, thanks for escorting me to school during late hours, you are indeed a blessing.

Special thanks to my wonderful parents and brothers for their love, financial support, encouragement and understanding, you are my greatest assets. If I have to come back to this world the second time, I would choose Mr and Mrs Fajinmi as my parents as their love and dedication is second to none. I remember all the sacrifices you made for me and my siblings to have good education. You sacrificed your time, money and efforts to make us who we are today. You are angels in human clothing!

.

To my darling husband Olaoluwa Olarewaju who I met during the course of this degree, you are the best thing that ever happened to me. The academic challenges I encountered during the first semester of my PhD brought us together as friends and we graduated to best friends and before I knew it, we got married! Your love and support kept me going during the tough moments as a student. Thanks for being there always, you are totally amazing. I cannot find the right words to accurately express how much you mean to me!

.

COLLEGE OF AGRICULTURE ENGINEERING & SCIENCE

DECLARATION 2- PUBLICATIONS

1) Growth inhibition and morphological alteration of *Trichophyton rubrum* and *T. mentagrophytes* by *Agathosma betulina* and *Coleonema album* essential oil volatiles (In preparation). Fajinmi OO, Gruz J, Tarkowski P, Kulkarni MG, Finnie JF and Van Staden J

2) Skin healing properties from the spice cupboard (In preparation). Fajinmi OO, Gruz J, Tarkowski P, Kulkarni MG, Finnie JF and Van Staden J

3) Pharmacological activities of *Coleonema album* and *C. pulchellum* against skin disease. Fajinmi OO, Gruz J, Tarkowski P, Kulkarni MG, Finnie JF and Van Staden J. Submitted to Pharmaceutical Biology (ID : NPHB-2016-1695.R1)

CONFERENCE CONTRIBUTIONS FROM THIS THESIS

1) Can the perfect skin antifungal be sourced from the Rutaceae family plants?
O.O. Fajinmi, M.G. Kulkarni, J.F. Finnie and J. Van Staden. RCPGD 14th Annual conference(2014), UKZN, PMB

2) Skin healing properties of Rutaceae plants essential oil volatiles.
O.O. Fajinmi, M.G. Kulkarni, J.F. Finnie and J. Van Staden.
RCPGD 15th Annual conference (2015), UKZN, PMB

.

LIST OF FIGURES

.

LIST OF TABLES

.

.

LIST OF ABBREVIATIONS

AD	Atopic Dermatitis
AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
B-PE	B-phycoerythrin
FGI	Fungal Growth Index
FLG	Filaggrin
GCMS	Gas Chromatography Mass Spectrometry
HIV	Human Immunodeficiency Virus
LPO	Lipid Peroxidation
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MID	Minimum Inhibitory Doses
ORAC	Oxygen Radical Absorbance Capacity
RNS	Reactive Nitrogen Species
ROS	Reactive Oxidative Species
SC	Stratum Corneum
SEM	Scanning Electron Microscope
TEWL	Trans Epidermal Water Loss

.

ABSTRACT

The skin a major organ of the body plays a crucial role in the confidence level of humans as people with skin diseases/problems are often withdrawn, shy, depressed and have a feeling of inferiority when relating with others. The increasing incidence of skin diseases of fungal origin and resistance to antifungals is a great health burden to the victims and dermatologists who struggle to restore the skin's health of their patients. Resistance to antibiotics has increased greatly as a result of the increasing population of Human Immunodeficiency Virus/Acquired Immune Deficiency Syndrome (HIV/AIDS) infected individuals, especially in the African region. The side effects of skin disease antibiotics (especially in HIV/AIDS individuals) and lengthy period of therapy are major shortcomings of both the old and newly developed range of antifungals.

Hence, there is a need to develop an effective, cheap and readily available antifungal from a natural origin in order to combat skin diseases. Members of the Rutaceae are popularly used in the production of skin care products commercially and in traditional medicine systems as a result of their active compounds such as tannins, phenols, flavonoids, terpenes, alkaloid and coumarins which have antifungal activities against several skin diseases and infections. These compounds not only possess antifungal activity but are relevant in the inhibition of processes involved in the pathogenesis of skin disease. Such processes include inflammation which arises from oxidative stress. Scientific literatures have shown a link between inflammation and oxidative stress as it plays a crucial role in the pathogenesis of chronic inflammatory diseases such as atopic dermatitis (AD) (eczema/ringworm). Restoration of the redox balance through the use of antioxidants from natural origin could be a ground-breaking approach in the treatment of inflammatory skin conditions.

In this study, the antioxidant activities of leaf extracts of seven plants belonging to the Rutaceae family (*Agathosma mucronulata, A. ovata, Calodendrum capense, Coleonema album, C. pulchellum, Murraya koenigii* and *M. paniculata*) were investigated using the oxygen radical absorbance capacity (ORAC) method. The results, expressed as µmol trolox equivalents/g shows that all the plant tested have antioxidant potentials, though at different levels. The best antioxidant activity

.

was recorded for *A. ovata* (**1202.5**) followed by *C. pulchellum* (**1126.7**), *A. mucronulata* (**1045.5**), *C. album* (**942.2**), *M. paniculata* (**930.7**), *M. koenigii* (**339.8**) and *C. capense* (**301.7**).

The antifungal activity of the leaf extracts of *C. capense*, *Coleonema album*, *C. pulchellum*, *Murraya koenigii* and *M. paniculata* against *Trichophyton rubrum* (ATCC 28188), *T. mentagrophytes* (ATCC 9533) and *Microsporum gypseum* (ATCC 24102) was investigated using the micro well dilution method. Ground leaf material of each plant used was extracted non-sequentially with petroleum ether, acetone, methanol and ethanol. Out of the 20 extracts from the five plants tested, *Coleonema pulchellum* methanol extract (**0.195 mg/ml**) and *M. koenigii* ethanol extract (**0.391 mg/ml**) gave the best antifungal activity against *T. rubrum*. *Coleonema album* and *C. pulchellum* methanol extracts gave the best antifungal activity against *T. mentagrophytes* with a MIC value of **0.390 mg/ml**. *Coleonema pulchellum* methanol (**0.049 mg/ml**) and *C. album* methanol (**0.195 mg/ml**) extracts gave the best antifungal activity against *M. gypseum*. A total of 16 extracts out of the 20 extracts from the five plants inhibited the growth of at least one fungal strain with MIC values ranging from **0.049 mg/ml** to **1.022 mg/ml**.

Apart from plant extracts, essential oils are popularly utilised in the treatment of skin diseases. The antifungal activity of essential oil volatiles of *Agathosma mucronulata*, *A. ovata*, *Calodendrum capense*, *Coleonema album*, *C. pulchellum, Murraya koenigii* and *M. paniculata* against *T. rubrum* and *T. mentagrophytes* were investigated. Essential oil derived from leaves of the plants was used. The essential oil volatiles of *A. ovata, A. mucronulata, C. album*, *C. pulchellum, C. capense* and *M. paniculata* totally inhibited the growth of *T. rubrum* with final mycelia diameters of **0.3 cm** and Fungal Growth Index (FGI) of **0%**. Out of all the essential oils tested, only the essential oil volatiles of *C. album* and *M. paniculata* totally inhibited the growth of *T. mentagrophytes.* The volatiles of these essential oils also had a fungicidal effect on both fungal species as there was no further growth when the fungi were subcultured after a 7 day exposure to the treatments. Overall, the essential oil volatiles of the plants tested had remarkable antifungal activity against *T. rubrum* compared to *T. mentagrophytes*. Essential oils of plants from the Rutaceae family have been reported to contain mainly terpenes which could be responsible for the antifungal activity recorded in this study.

.

The essential oil composition of *Agathosma mucronulata*, *A. ovata*, *Calodendrum capense*, *Coleonema album*, *C. pulchellum, M. koenigii* and *M. paniculata* leaves was investigated. *Agathosma ovata* leaves essential oil yielded forty-three components with β-myrcene (**22.03%**), 1,6-octadiene-3-ol, 3, 7-dimethyl-2-aminobenzoate (**18.75%**), trifluoroacetyl-lavandulol (**12.97%**), trans-β-ocimene (**11.81%**), β-ocimene (**5.65%**), bicyclo germacrene (**5.10%**), β-phellandrene (**3.47%**), thujone (**2.07%**), β-pinene (**1.88%**) and caryophyllene (**1.74%**) as the major components. The major components of *A. mucronulata* leaf essential oil are: β-myrcene (**34.05%**), β-pinene (**18.24%**), caryophyllene (**7.4%**), (1S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (**6.09%**), D-limonene (**6.06%**), eucalyptol (**5.46%**), bicyclo germacrene (**5.38%**), trans-β-ocimene (**4.21%**), 2,4,4-trimethyl-2-vinyl-guaia-1(10),11-diene (**3.00%**) and β- ocimene (**2.36%**) of the thirthy- three components. Compounds found in essential oil of both *A. ovata* and *A. mucronulata* are trans-β-ocimene, β-ocimene, caryophyllene, β-myrcene and bicyclogermacrene.

The essential oil of *C. capense* leaves is composed of sixty- three compounds of which cis-β-farnesene (**44.30%**), naphtalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis) (**4.88%**), β-phellandrene (**4.07%**), 1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-,[S-(E,E)] (**3.68%**), cis-β-farnesene (**3.29%**), β-bourbonene (**2.87%**), caryophyllene (**2.86%**), gamma-muurolene (**4.01%**), bicyclo[5.2.0],nonane,4-methylene (**2.03%**), isolongifolene, 9,10-dehydro (**1.64%**), trans-α-bergamotene (**1.59%**) and trans-β-ocimene (**1.53%**) are the major components.

Caryophyllene (**24.91%**), trans-β-ocimene (**9.49%**), β-myrcene (**7.96%**) and octahydro-7-methyl-3-methylene-4-(1-methylethyl)-β-copaene (**7.53%**) are the major components of *C. album* essential oil while bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene (**32.04%**), (+)-3-carene (**10.88%**), β-ocimene (**9.46%**), gamma-elemene (**8.95%**) and α-pinene (**7.43%**) are the major components of *C. pulchellum* essential oil. The major (**≥ 1.5 %**) components of the oil found in both essential oil of *C. album* and *C. pulchellum* are caryophyllene, bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene, α-pinene, gamma-elemene and carene. However, the common bioactive compounds exist in both essential oil in different quantities as shown by the results.

.

Essential oil of *M. koenigii* yielded a total of twenty-three components. The major components of the essential oil are: caryophyllene (**42.54%**), (4aR-trans)-guaia-9, 11-diene (**23.35%**), alpha-guaiene (**10.71%**) and humulene (**6.93%**). The sister species, *M. paniculata* essential oil yielded forty-two components of which 1, 6, 10-dodecatriene,7,11-dimethyl-3-methylene (**38.62%**), 1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl) (**19.0%**), caryophyllene (**10.63%**), gamma-elemene (**8.55%**) and bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl) (**5.42%**) are the major components. The major (**≥1.5%**) components of the oil found in essential oil of both species are: caryophyllene and bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl). However, the two compounds are present in different quantities in the essential oil of the two plants. The essential oil derived from all the plants (seven) used in this study contains caryophyllene as part of their main components. The compound however exists in each of the essential oil in different quantities.

It is not certain if essential oil purchased from organic product companies will consist of the same compounds and exhibit the same level of antifungal activity. The mode of action of the essential oil volatiles on the mycelia growth is also not known. It was deemed necessary to investigate the antifungal activity of essential oil (commercially produced) of two highly utilised Rutaceae plants and their mode of action on the fungal strains.

Antifungal activity and essential oil components of *Agathosma betulina* and *C. album* were investigated. Essential oil of *C. album* (EOCM10) and *A. betulina* (EOBUCB10) were purchased from Still Pure, Cilliers street, Western Cape. *Agathosma betulina* and *C. album* oil (10 µl each) was subjected to analysis using Gas Chromatography Mass Spectrometry (GCMS). There was inhibition/reduction of fungal growth in all the plates exposed to essential oil volatiles. However, the rates of inhibition varied between the fungal species, oil tested and the different volumes tested. The volatiles from *A. betulina* oil has a stronger inhibitory effect than *C. album* oil; with the highest inhibition recorded at 40 µl. Susceptibility to essential oil volatiles is more pronounced in *T. rubrum* than in *T. mentagrophytes*. The best inhibition was recorded in *T. rubrum* exposed to volatiles from 40 µl of *A. betulina* oil with a fungal growth index of **0%**, indicating its fungicidal activity on the

.

fungi. The same volume of *A. betulina* oil had a fungistatic effect on *T. mentagrophytes* with a FGI of **7 %**.

Mycelia from *T. rubrum* exposed to 40 µl essential oil volatiles did not grow when subcultured on a fresh plate of agar. This is an indication that the volatiles totally degraded the hyphae and spores. However, all the other fungal species subcultured produced mycelia after 7 days of incubation. Essential oil analysis of *A. betulina* and *C. album* using GCMS revealed the presence of terpenes. The major components of the essential oil of *A. betulina* were 1-menthone (**36.35%**), D-limonene (**29.84%**) and 6-methoxy-3(2H)-pyridazinone (**8.35%**) while that of *C. album* were β-pinene (**28.92%**), (1R)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (**27.37%**) and β-phellandrene (**22.70%**). The essential oils from both plant species is composed mainly of volatile monoterpenes.

The mode of action of the essential oil volatiles was revealed under electron microscopy. The volatiles inhibited fungal growth by inhibiting the production of spores. *Agathosma betulina* oil (40 µl) resulted in the total destruction of hyphae and spores of *T. rubrum*. This total destruction is irreversible as the subcultured mycelia did not grow after 7 days of incubation on fresh agar thus indicating the fungicidal action of the essential oil volatiles. This is noteworthy as fungicidal action is needed to avoid reinfection and resistance of fungal strains which has been attributed to the survival of fungi spores.

The outcome of this study gives credence to the use of some of the plants in skin and haircare products. The results of this study highlight the prospects of some of the plants used in this study as suitable candidates for the formulation of an effective skin antifungal/ointment of natural origin. These plants may offer a source of bioactive compounds for the treatment of symptoms involved in the pathogenesis of skin diseases.

.

CHAPTER 1. INTRODUCTION

Nature has bestowed a vast quantity of plant resources to the human race for survival on planet earth. These resources supply the primary needs of humans in the form of food, clothing, shelter, raw materials and of course medicine. Therefore, without plants the human race will cease to exist. The Rainbow Nation (South Africa) is known all over the world by plant scientists as a country with outstanding plant resources which are being extensively utilised in the horticulture industry, medicinal plant markets and for the production of art and crafts and natural products. These plant resources come from nine biomes each of which are unique and offer an amazing reflection of nature in the different parts of the country.

1.1. South Africa and its floral biodiversity

The Cape Floral Kingdom (CFK) is the smallest of the world's six Floral Kingdoms **(COWLING *et al*., 1992)**. Considering its size, it is the richest of the world's six Floral Kingdoms. The CFK is one of the 25 Global Biodiversity Hotspots **(MYERS *et al*., 2000)** with a high degree of endemism and threatened biodiversity **(HOLMES *et al*., 2012)**. The CFK is one of the most diverse regions in the world **(GOLDBLATT and MANNING, 2002)**.

The CFK comprises a land area of 90,000 km^2, less than 5% of the total area of the southern Africa **(GOLDBLATT, 1978, 1997)**. Plant species richness of the CFK is exceptional with a remarkable familial and generic composition **(BOND and GOLDBLATT, 1984; GOLDBLATT, 1997; GOLDBLATT and MANNING, 2002)**. It remains one of South Africa most valuable assets and it includes the majestic Table Mountain and Cape Peninsula (which is now a national park) with 17 nature reserves and 307 km of coastline **(HOLMES *et al*., 2012)**.

1.2. Aesthetic value and healing power of the "Cape green gold"

The CFK is home to valuable ornamental, aromatic and medicinal plants. The CFK vegetation offers a myriad of plants with unique scents and forms, which captivates the minds of nature lovers. The CFK is home to several plants known locally and internationally for their great horticultural value. These plants have been a source of income for South Africans who are involved in the trade of horticultural

.

plants. Some of these plants whilst being of great horticultural value are also of high medicinal value and are among the highly traded medicinal plants in the South African traditional medicine markets, popularly referred to as *Muthi* markets. The knowledge of the therapeutic value of South African medicinal plants is being used as a clue by researchers for the formulation and production of tinctures, ointments and essential oils from natural origin for the treatment of diseases. Pure essential oil from South Africa endemic aromatic plants is marketed locally and internationally and has in turn support the rapid establishment of natural product companies in South Africa and an increased interest in South African aromatic plants.

1.3. Aromatic plants meeting the needs of humans from time immemorial

The appeal of scented flowers and leaves and their by-products is as irresistible today as it was in the first fragrant plot, the "Garden of Eden" **(ALLARDICE, 1992)**. A garden should appeal to all senses especially the sense of smell and sight **(LA CROIX, 1984)**. Aromatic plants have been used for innumerable purposes among which are food and drink flavouring, scenting of clothes and to uplift the spirits of humans for many centuries **(ALLARDICE, 1992)**. Aromatic plants possess volatile substances in the form of essential oils in one or more parts of the plant **(SKARIA *et al*., 2007)**. The term essential oil is associated with fragrance or perfumes representing the essence or active constituents of aromatic plants **(SKARIA *et al*., 2007)**. Many essential oils are used commercially for the production of perfumes **(LA CROIX, 1984)**.

The use of fragrances for pleasure is very old; an Egyptian papyrus from around the 16th century BC gives recipes for perfumes, cosmetics and deodorants **(LA CROIX, 1984)**. Perfume was integral to ancient Egypt civilizations and the perfumes were derived from aromatic plants **(ALLARDICE, 1992)**. The Egyptians used perfumes as an offering to their Gods and the aromatic products were prepared and kept in a small room in their temples (**SKARIA *et al*., 2007**). According to **SKARIA *et al*.,** (**2007**), incense and aromatic oils were used in religious ceremonies and to ward off evil spirits.

Aromatic plants were first imported by the Ishmaelite traders who travelled from Gilead with their camels bearing spices, balm and myrrh and the Egyptians believed that the balm is the exudation of *Pistacia lentiscus* (**ALLARDICE, 1992**).

.

The oil of cedar wood collected from the dense forests of Mount Lebanon was in much demand by the Egyptians who used it for religious rites, fragrant cosmetics and ointments and they believed that cedarwood was imperishable and could preserve life (**ALLARDICE, 1992**). The Hebrews got the knowledge about aromatic perfumes from their countrymen who were held as prisoners by the Egyptians (**SKARIA *et al*., 2007**). Cedar wood was used to construct the famous temple Diana at Ephesus and it was one of the Seven Wonders of the World (**ALLARDICE, 1992**).

In Elizabethan times and earlier, floors were strewed with aromatic herbs such as *Acorus calamus*, chamomile, thyme, hyssop and lavender (**LA CROIX, 1984**). For several thousands of years, the "Incense Road" between Arabia and Egypt was travelled by the wealthy spice caravanserai and the rewards for a successful incense merchant were great and the entire economy of the kingdom of Sheba in the south of Africa depended on the sale of Frankincense and Myrrh (**ALLARDICE, 1992**). In ancient Egypt, women used cinnamon oil (from Nepal) combined with honey, myrrh and almonds to make a perfume for the feet and legs (**ALLARDICE, 1992**). The Egyptians also had knowledge about the efficacy of aromatic plants in healing diseases and this pool of knowledge was applied in medical care (**SKARIA *et al*., 2007**).

1.4. Use of aromatic plants in aromatherapy and human health since "the days of old"

When plague was a fact of life, lice and fleas were man's constant companions and waste was disposed through the nearest open window, scented flowers were used for their antiseptic qualities and also to mask the odour (**VEREY, 1981**). In an attempt to stop the spread of plague in Athens, Hippocrates fumigated streets with aromatic plants (**SKARIA *et al*., 2007**). Herbs were grown and utilized not only for their scents but also for their medicinal uses (**LA CROIX, 1984**) and some of them have remarkable application as therapeutic agents in pharmaceutical and drug industries (**SKARIA *et al*., 2007**). A rhythm exhibiting calmness is produced when essential oil with a sedative effect is inhaled as it stimulates the sense of smell and the emotional centre of the brain (**SKARIA *et al*., 2007**). "The breeze of essential oils of the world has blown from yesterday's narrow definition as a symbol of luxury to indispensable necessities for both the rich and poor from the cradle of infancy to the silence of the grave" (**SKARIA *et al*., 2007**).

.

LA CROIX (**1984**) highlighted that scented plants have always played a vital role in the traditional cottage garden and today, there seems to be a renaissance of interest in aromatic and fragrant plants. An old man said when he was young; he did put bergamot (*Moranda*) into his hair-grease as “it pleased the girls” (**LA CROIX, 1984**). Essential oil from several aromatic plant species have been incorporated into skin and hair care products. The family Rutaceae which includes citrus is known for the presence of punctuate oil glands and almost all the members have aromatic leaves (**LA CROIX, 1984**).

1.5. The Rutaceae, a family of green aromatics in the treasure-box of the Rainbow Nation

Rutaceae species are among the most traded plants in the international herbal medicine market. The family Rutaceae has several members of economic importance (**CHASE *et al.,* 1999**). The Rutaceae family also known as the citrus family is a large group of about 161 genera and 1815 species (worldwide), with 21 genera and 289 species native to southern Africa. The most notable globally are the species of *Citrus* that produce both the citrus fruits of commercial importance (lemons, oranges, mandarins, tangerines, limes, kumquats, etc.) and the essential oils used in perfumery, species of *Pilocarpus* are the source of pilocarpine, a drug used to treat glaucoma, and species of *Boronia*, *Choisya*, *Poncirus*, and *Skimmia* that are used as ornamentals (**CHASE *et al.,* 1999**). Several species of the family Rutaceae are popularly used as medicinal plants due to the presence of highly therapeutic components found in them. The majority of the native Rutaceae species in southern Africa are endemic to the Cape region of South Africa. Some Rutaceae species are extensively exported to other countries of the world due to their essential oils which have unique scent and therapeutic components. Examples are the *Agathosma* species, *Coleonema album* and others. The Rutaceae species have been a green gold plant family in South Africa.

The economic and medicinal value of Rutaceae species of South African origin cannot be over-estimated. The export value and commercial importance of Rutaceae species popularly known as *Buchu* have been recorded since the early 18th and 19th centuries. In the late eighteen century, *Buchu* leaves were dried, baled and exported to Europe and America and for over 30 years, buchu oil has been exported to the world’s major flavour houses(. http://africanaromatics.com/buchu-

.

agathosma/). *Agathosma species* is exported in large volumes for use as a fixative in the food industry (**COETZEE *et al*., 1999**). **PAPPE** (**1857**) recorded that *Buchu* leaves is an article of export and listed their medicinal uses.

The medicinal value of *Buchu* is attributed to the essential oil of the leaves (**PAPPE, 1857**). **MACOWAN** (**1893**) advocated that the oil from *Buchu* should be distilled in the Cape instead of exporting the raw materials for distillation. **PILLANS** (**1910**) recorded that up to five shillings per pound of *Buchu* leaves were being realised in the London market. The standard buchus of commercial importance were *Barosma betulina*, *B. crenulata* and *B. serratifolia* and among the commercial buchus, *B. betulina* is the most valued in all pharmacopoeias of other countries (**PHILLIPS, 1917**). A plantation of *Buchu* was established in Kirstenbosch and the local production of Buchu oil was encouraged (**PHILLIPS, 1917**).

Most of the species of Rutaceae have gland-dotted leaves and a large proportion of the family is relatively rich in essential oils (**SWAIN, 1963**). In the field, Rutaceae species can be easily recognized by the conspicuously dotted oil cells on the leaves (**WIART, 2006**). Several Rutaceae species have been used to treat diseases of the respiratory tract, to reduce fever, and to promote digestion on account of their essential oils and a growing body of evidence suggests that coumarins of the Rutaceae have anti-tumor activities (**WIART, 2006**). Oil produced by several genera of the family is used in perfumes and as medicine while other species are used as ornamentals (**MABBERLEY, 2008**). *Ruta chalepensis* is used in traditional medicine in many countries to treat a variety of diseases like rheumatism, neuralgia, menstrual bleeding, fever, arthritis, hepatic diseases, antifertility, and gastrointestinal disorders (**GUNAYDIN and SAVCI, 2005; GONZALEZ-TRUJANO *et al.,* 2006**). In the Italian countryside, *R. graveolens* leaves were set under the bed to repel bugs and mice (**GUARRERA, 1999**). A decoction of *Ruta* species also has been used topically against scabies, lice, and fleas, to repel insects and to treat intestinal worms in livestock (**FEO *et al*., 2002**).

The antimicrobial activity of the chloroform, methanol and water extracts of leaves, fruits and barks of *Aegle marmelos* was tested against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Proteus mirabilis*, *Escherichia coli*, *Salmonella paratyphi* A and *Salmonella paratyphi* B. The methanol extract showed

.

significantly higher activity against above mentioned bacteria than that of the other extracts (**POONKOTHAI and SARAVANAN, 2008**).

Extracts of *Aegle marmelos* leaves gave remarkable antibacterial activity against *Escherichia coli*, *K. pneumoniae*, *Proteus vulgaris*, *Micrococcus luteus*, *Enterococcus faecalis* and *Streptococcus faecalis* (**SARADHA and RAO, 2010**). Hesperidin rich species of the Rutaceae have been reported to have anti-allergic effects (**KUBO and MATSUDA, 1992**) and significant anti-inflammatory and analgesic effects (**GALATI *et al.,* 1994**). *Clausena excavata* extracts have been reported to show anti-cancer, antimicrobial, antioxidant, antifungal, antiviral, antidiabetic, immunomodulatory and antiplasmodial activities (**ARBAB *et al.,* 2012**).

Since *Pilocarpus* species have been confirmed to be a potent stimulant of the secretory system, showing sialagoue, diuretic and diaphoretic activities(**CORRÊA, 1969**), infusions of their leaves have been used to treat fevers, stomatitis, bronchitis, gout, psoriasis, kidney diseases and several other illnesses (**HOLMSTED *et al.,* 1975**). It is noteworthy that these Rutaceae species (*Pilocarpus* species) contain the Imidazole alkaloid, pilocarpine which has been utilised in ophthalmology (**WEBER, 1876**) for the treatment of glaucoma (**KOROLKOVAS, 1996**) and hence has made the genus one of the most important plants in the Brazilian flora (**PINHEIRO, 1997**). The effectiveness of pilocarpine in the treatment of xerostomy (dry mouth) caused by the chemotherapy of throat cancer or those of the head area (**MILLER, 1993; VALDEZ *et al.,* 1993**) has been reported.

Zanthoxylum americanum Mill., leaf, fruit, stem, bark and root extracts exhibited a broad spectrum of antifungal activity and inhibited at least eight out of eleven fungal strains (including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*) representing diverse opportunistic and systemic pathogens and thus justify the popular use of this plant in North American traditional medicine for fungal infections (**BAFI *et al.,* 2005**). The essential oil extracted from the epicarp of *Citrus sinensis* (L.) Osbeck exhibited absolute fungitoxicity against ten post-harvest pathogens (**SHARMA and TRIPATHI, 2006**). In an analysis of the chemical composition and antifungal activity of the essential oil of *Haplophyllum tuberculatum* (**AL BURTAMANI *et al.,* 2005**), the oil affected the mycelial growth of *Curvularia lunata* and *Fusarium oxysporum* in a dose-dependent manner, but had no effect on the germination of their spores. The authors further stated that the most abundant

.

components of the oil were α- and β-phellandrene, limonene, β-ocimene, β-caryophyllene and myrcene.

The quality and quantity of essential oil derived from five species of *Coleonema* have been characterised (**BAŞER *et al*., 2006**). Forty-three compounds were characterized in the oil of *C. album* representing 99.1% of the total composition. Main compounds were β-phellandrene (30.4%) and myrcene (20.5%). Forty-three compounds were characterized in the oil of *C. aspalathoides* representing 94.1% of the oil with sabinene (24.0%), terpinen-4-ol (12.2%), p-cymene (12.1%), linalool (11.3%) and α-pinene (9.1%) as main constituents. In the oil of *C. calycinum*, 35 compounds were characterized representing 99.4% of the total volume. β-phellandrene (42.0%) and linalool (11.0%) were the main constituents. Monoterpene hydrocarbons were the main constituents in three oil samples of *C. pulchellum*. Myrcene (63.0%) was the main constituent in the oil of *C. virgatum* in which 23 compounds were identified representing 97.3% of the total. The presence of coumarins, terpene and terpenoids is responsible for the various medicinal use of Rutaceae species among which is the use for skin diseases of fungal origin.

1.6. Significance of the study

There is a strong relationship between the skin and emotions in humans as embarrassment causes blushing, anxiety causes cold and a sweaty palm and anger reddens the face (**WELLER *et al*., 2008**). The relationship between the skin and mind is quite complex and the incidence of psychiatric abnormalities is higher among patients with skin disorders than the general population (**WELLER *et al*., 2008**). Skin diseases can have a negative effect on the physical and psychological wellbeing of an individual (**APPGS, 2013**). Psychological problems associated with skin disease patients include feelings of anxiety, anger, shame, stress, social isolation and a low self-esteem (**APPGS, 2013**). The presence of disfiguring skin lesions can distort the emotional development of a child as they can become withdrawn or aggressive (**WELLER *et al*., 2008**) and can be a reason to be absent at school (**APPGS, 2013**).

Chronic skin disease is capable of ruining the quality of life as it interferes with work, social activities (**WELLER *et al*., 2008; APPGS, 2013**), sexual relationships (**APPGS, 2013**), causes pain and itching and thus makes a patient feel like an outcast (**WELLER *et al*., 2008**). The unforgiving itching resulting from skin diseases

.

can cause misery (**COOPOOSAMY and NAIDOO, 2011**) and their physical appearances may be a source of stigmatization (**COOPOOSAMY and NAIDOO, 2011; APPGS, 2013**). Feelings of stigmatisation and rejection are common in patients with chronic skin diseases (**WELLER *et al*., 2008**). The increased incidence of resistance of skin fungal diseases to antibiotics is of great concern. The situation is more prominent among HIV/AIDS individuals thus adding to the burden of skin diseases in Africa as a region. Owing to the high incidence of HIV/AIDS in South Africa (**GLOBAL INFORMATION AND ADVICE ON HIV AND AIDS, 2016**), there is an urgent need for the development of new medicines to cure various HIV/AIDS opportunistic infections most especially skin diseases.

Skin diseases are often more associated with HIV/AIDS infected individuals as a result of a decrease in their immunity. South Africa has the highest population of HIV/AIDS positive individuals in Africa and the KwaZulu-Natal region has the highest incidence in South Africa. The African continent has a high prevalence of HIV/AIDS and hence is in dire need of appropriate and adequate management of dermatologic diseases (**HU *et al.,* 2011**). In many developing countries, health care centers are often run by clinical officers or nurses (rather than physicians) who act as the primary care workers but have inadequate training on the diagnosis of dermatologic conditions (**SATIMIA *et al*., 1998; HAY and MARKS, 2004**).

Skin diseases apart from being a form of stigmatization among HIV/AIDS positive individuals, can also have an adverse effect on the economic growth of a society/country as it reduces the productivity of those infected. Dermatitis of the hand can destroy a manual worker's earning capacity such as with hairdressers, nurses, cooks, and mechanics (**WELLER *et al*., 2008**). In times of unemployment, people with skin diseases find it difficult to get a job (**WELLER *et al*., 2008**).

Hence, the need to develop a plant based remedy as a cure for skin diseases is crucial as it will relieve the burden of skin diseases among HIV/AIDS individuals and the general populace. The Rutaceae family plants are generally rich in terpenes and coumarins (and its derivatives) which have been reported to have anti-dermatophytic properties. Natural products offer a cheap, affordable and accessible option for the treatment of several diseases, including skin diseases of fungal origin such as eczema and ringworm which are often referred to as 'Atopic Dermatitis' (AD). Several skincare product formulations include plant extracts and tinctures as

.

they are good source of natural pigments such as carotenoids; and other skin health enhancing compounds which include vitamins, A, B and E.

In an attempt to achieve an increased therapeutic rate of treatments of skin diseases, a combination of topical and oral anti-inflammatory drugs has been used (**SOARES *et al.,* 2013**). Natural products offer a variety of anti-inflammatory (**YASSER and NABIL, 2012**) and anti-oxidant agents (**KOSTOVA, 2006**) which can counter the oxidative stress, dryness, scaling and inflammations that comes into play during the pathogenesis of skin diseases. Rutaceae family plants are known to be a source of coumarins, terpenes, flavonoids and other therapeutic agents that have been reported to be effective in the treatment of skin diseases and alleviation of oxidative stress and inflammation.

Extracts and tinctures from several Rutaceae species have been incorporated into skin care products. Thus, if the extracts can enhance skin beauty, they may also contain vital ingredients that can heal skin diseases. Various Rutaceae species have been used to treat skin diseases in the traditional medicine system of different African countries.

1.7. Aims and objectives

The aims and the objectives of this study are to;

- Investigate the antioxidant activity of the selected Rutaceae species as oxidative stress is responsible for skin inflammation and crucial in the pathogenesis of atopic dermatitis/ringworm;
- Investigate the antifungal activities of leaf extracts of selected Rutaceae plant species against *Trichophyton rubrum* (ATCC 28188), *T. mentagrophytes* (ATCC 9533) and *Microsporum gypseum* (ATCC 24102);
- Investigate the antifungal activities of the commercially produced essential oils of two popular South African endemic Rutaceae species, *Coleonema album* and *Agathosma betulina* against *Trichophyton rubrum* and *T. mentagrophytes*;
- Investigate the effects of essential oil on the fungal cell morphology and growth such as mycelia diameter, hyphae and spore production; and
- Analyse the bioactive compounds present in the essential oils of the selected plants in order to be able to determine the compounds that could be responsible for the antifungal activity of the essential oil *in vitro*.

.

CHAPTER 2. LITERATURE REVIEW

2.1. HIV/AIDS: a trauma to the human race

The human race has been ravaged by diseases such as malaria, polio, smallpox, cholera, tuberculosis, hepatitis, yellow fever, influenza, whooping cough, bird flu, meningitis, syphilis and cancer, which have led to loss of lives but have been recently controlled through the production of vaccines and drugs. “HIV/AIDS” emerged during the 20th century and has been a great trauma to the human race ever since. The disease finds its way into the human body mostly during moments of pleasure (sexual intercourse) and through the use of unsterilized sharp objects. The syndrome inflicts pains not just to the body but also to the soul and psychologically leaves the victims depressed. This depression coupled with the stigmatization by the public and in worst cases by loved ones makes the patient to lose hope and drains them of the strength to continue the journey of life. Irrespective of colour, race, financial and societal status, the syndrome drowns humans in a pool of shame, anxiety, pain and unavoidable diseases which are often referred to as “Opportunist Infections” (OI). One of the major symptoms of a full blown HIV/AIDS condition is the manifestation of diseases and infections on the skin.

2.2. The human skin, a vital irreplaceable organ

The skin is a vital organ in humans as it is closely related to the identity of an individual. The skin reveals an individual’s race and in some cases the tribe to which they belong. More importantly, the skin serves as a key indicator of an individual’s state of health. The human skin is a unique organ that permits terrestrial life by regulating heat and water loss from the body whilst preventing the entry of harmful chemicals or microorganisms (**WILLIAMS, 2003**). The skin is the largest organ of the human body which amounts to 10% of the body weight of an average individual and can be classified into four main layers according to its structure and function (**WILLIAMS, 2003**);

- The innermost subcutaneous fat layer;
- The overlying dermis;
- The viable epidermis; and

.

- The outermost layer of the tissue

The subcutaneous fat layer, or hypodermis, bridges the overlying dermis and the underlying body constituents. This layer of adipose tissue principally serves to insulate the body and to provide mechanical protection against physical shock. The subcutaneous fatty layer can also provide a readily available supply of high-energy molecules, whilst the principal blood vessels and nerves are carried to the skin in this layer.

The dermis is typically 3-5mm thick and is the major component of human skin. It is composed of a network of connective tissue predominantly collagen fibrils which provides support while elastic tissue provides flexibility, embedded in a mucopolysaccharide gel (**WILKES *et al*., 1973**). Numerous structures are embedded within the dermis. They include: blood and lymphatic vessels, nerve endings, pilosebaceous units (hair follicles and sebaceous glands), and sweat glands (eccrine and apocrine). There are 3 main appendages found on the surface of human skin that originate in the dermis. These have been described in detail by **KATZ and POULSEN** (**1971**). Hair follicles are found over the entire surface of the skin with the exception of the sole of the feet, palms of the hands and the lips. The sebaceous gland associated with hair follicles secretes sebum which is composed of free fatty acids; waxes and triglycerides which lubricates the skin surface and helps to maintain the pH of the skin surface at around 5. Eccrine (or sweat) glands and apocrine glands also originate in the dermal tissue. The eccrine glands secretes sweat (a dilute salt solution at a pH of around 5) in response to heat and emotional stress. The apocrine glands are located near the dermo-epidermal layer but are limited to specific areas of the skin including the axillae, nipples and ano-genital regions.

The epidermis is a complex multiple layered membrane. It contains no blood vessels and hence nutrients and waste products must diffuse across the dermo-epidermal layer in order to maintain tissue integrity. The epidermis contains four histologically distinct layers which from the inside to the outside are: the stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum.

Studies have shown that the stratum corneum barrier is highly compromised for patients with AD with transepidermal water loss from the body increasing by up to 10-fold (**OGAWA and YOSHIIKE, 1992; AALTO-KORTE and TURPEINEN, 1993**).

.

An intact, healthy skin has a highly effective barrier against the ingress of micro-organisms but hosts microbial flora which include bacteria and yeasts, and if breached, it can result in skin disease/infection (**WILLIAMS, 2003**).

2.3. Skin diseases: a health burden across the globe

Skin diseases and their complications are a significant burden on the health system of several nations (**ATRAIDE *et al.,* 2011**). In assigning health priorities, skin diseases are sometimes regarded as small-time players in the global league of illness compared with diseases that cause significant mortality, such as HIV/AIDS, community-acquired pneumonias, and tuberculosis **(HAY *et al.,* 2006)**. However, skin problems are generally among the most common diseases seen in primary care settings in tropical areas, and in some regions where transmissible diseases such as *Tinea imbricata* or onchocerciasis are endemic; they become the dominant presentation **(HAY *et al.,* 2006)**.

Skin diseases are not usually recognized as a major public health problem in developing countries, despite the fact that recent reports by the World Health Organization (WHO) estimates that 21–87% of the general population in developing countries has a skin disease. Problems with the skin are among the main reasons for seeking healthcare, amounting up to 24% of primary care visits, and are one of the most common causes of morbidity (**SCHMELLER, 1998; NNORUKA, 2005**). In many Sub-Saharan African countries, infections and infestations make up the majority of skin diseases which accounts for 85% of skin diseases in Tanzania, 78% in Malawi, 71.5% in Ethiopia, and 40.1% in Uganda (**GIBBS, 1996**). In addition to the immense burden of skin disease in Africa, diseases such as dermatitis and prurigo are commonly either untreated or over-treated with strong topical steroids and antibiotics which have been found to cause considerable side effects (**JOBANPUTRA and BACHMANN, 2000**). The health consequences of dermatologic disease extend beyond the individual (**FIGUEROA *et al*., 1998**). Hence, the public health is at risk since common skin conditions include parasitic infestations and infections (**FIGUEROA *et al*., 1998**).

Several factors which include occupation, nutrition, genetic, environment, race, age, and habits can influence the pattern of skin diseases (**PARTHASARADHI and AL GUFAI, 2004**). **HAY *et al.,* (2006)** stated that although mortality rates

.

resulting from skin infections and diseases are generally lower than that of other health conditions, it is important to meet people's needs for effective remedies for skin conditions for a number of reasons:

- Skin diseases are so common to the extent that patients represent large numbers in primary health care settings. Children particularly tend to be affected, adding more burden of disease to a group which is already vulnerable;
- Generally, families must meet the costs of skin disease treatment from an overstretched household budget, and such expenses in turn reduce the capacity to purchase essential items such as food (**HAY *et al*., 1994**);
- Screening the skin for signs of disease is an important strategy which gives a clue to the presence of a wide range of illnesses; and
- Morbidity is significant through disfigurement, disability, or symptoms such as intractable itch, which can contribute to social isolation and in worse scenarios the reduction in quality of life.

Skin diseases can also result in psychological or mental disorders (**MURRAY *et al*., 2013**). In health clinics, skin diseases constitute up to 15% of all attendances. The rate of treatment failure, above 80%, are common as shown from results of studies assessing success in the management of skin diseases in primary care settings in the developing world (**FIGUEROA *et al*., 1998**).

2.4. Skin diseases in Africa, the culprits and way forward

The World Health Organization's report on the global burden of disease indicate that skin diseases were associated with mortality of 20,000 in Sub-Saharan Africa in 2001(**WORLD HEALTH ORGANIZATION, 2005**). The distribution of dermatomycoses, their aetiological agents and the predominating anatomical infection patterns varies with geographical location and a wide range of environmental and cultural factors **(MALE, 1990; MACURA, 1993**).

Naturally, dermatophytes thrive at surface temperatures between 25 to 28ºC and human skin infections are thus supported by warm and humid conditions (**HAVLICKOVA *et al*., 2008**). Superficial fungal infections are relatively common in tropical countries and are aggravated by wearing of occlusive clothing; crowded living conditions; skin to skin contact; close proximity to animals; and suboptimal

.

hygienic practices (**HAVLICKOVA *et al*., 2008**). Fungal, bacterial and parasitic skin diseases flourish in a hot and humid climate (**NAAFS *et al.,* 2013**). The regional incidence of dermatophyte infections is not static due to booming mass tourism; international sports activities and increasing migration (**HAVLICKOVA *et al*., 2008**).

Skin lightening creams are a significant cause of disease in urban women (**DOE *et al*., 2001; DEL GUIUDICE and YVES, 2002**). People who wear closed shoes may more often develop athlete's foot and mycosis pedis. Fungal infections form part of the most commonly diagnosed skin diseases in the Africa region (**HAVLICKOVA *et al*., 2008**). Fungal species of *Trichophyton* are the most common dermatophytes associated with fungal skin diseases in Africa (**HAVLICKOVA *et al*., 2008**). The overall incidence of *Tinea* in Sub-Saharan Africa for the year 2005 was estimated to be 78 million (**HAY *et al.,* 2006**).

2.4.1. The increased burden of skin diseases resulting from HIV/AIDS

In Sub-Saharan Africa, skin diseases are dominated by bacterial and fungal infections and their clinical expressions are often modified by HIV-induced immune-suppression (**HAY *et al*., 2006).** The HIV/AIDS epidemic in Africa further adds to the burden of skin disease (**HU *et al.,* 2011**). Worldwide, dermatophytoses frequently associated with people living with HIV/AIDS is 20-40% more than in the general population (**D'ANTUONO *et al*., 2001**). Skin manifestations are often the first sign of HIV infection and conversion to AIDS (**NNORUKA, 2005; TSE, 2007**) and 90% of individuals with HIV/AIDS are diagnosed with skin disease at some point during the course of the disease (**PENNYS, 1995; FIGUEROA *et al*., 1998; MOSAM *et al*., 2004; NATIONAL AIDS AND STI CONTROL PROGRAM, 2008**).

Fungal infections of the skin and nails represent the majority of all mycoses (**HAVLICKOVA *et al*., 2008)** and are among the most common skin disease manifestation in HIV/AIDS patients. They can occur at any stage of the disease and some of the dermatoses are unique to HIV infection while others are common conditions, which also occur in HIV-negative individuals (**NATIONAL AIDS AND STI CONTROL PROGRAM, 2008**). Patients with HIV infection exhibit a wide range of skin pathology which includes bacterial, fungal, and viral infections, skin tumors, inflammatory and eczematous eruptions, and drug rashes **(RUDIKOFF, 2002).** Anyone caring for HIV-infected individuals could be burdened by the unusual prevalence of skin disorders in this patient population **(RUDIKOFF, 2002).** The skin

.

problems may have a prolonged course and may cause diagnostic and treatment challenges in HIV/AIDS-positive individuals (**NATIONAL AIDS AND STI CONTROL PROGRAM, 2008**).

In addition to ordinary and esoteric skin infections, tumors, and drug reactions, HIV-infected patients typically exhibit unusually dry skin, and may thus display inflammatory or eczematous eruptions **(RUDIKOFF, 2002)**. These include seborrheic dermatitis, psoriasis, pruritic popular eruption of HIV, eosinophilic folliculitis, papular urticaria, prurigo nodularis, and notably, a dermatosis that strongly resembles AD **(RUDIKOFF, 2002).**

2.4.2. Prevalence of skin diseases in HIV/AIDS individuals and the microorganisms responsible for the infections/diseases

In a study involving 186 HIV-positive patients, 175 (94%) suffered from one or more cutaneous disorders (**HO and WONG, 2001**). It is impossible from the current literature to estimate the prevalence of AD in HIV-infected children and adults **(RUDIKOFF, 2002).** According to the HIV/AIDS Epidemiological Surveillance Report for the WHO African Region 2005 Update, recent trends in HIV prevalence show that nearly 30% of women aged 15 to 49 attending antenatal clinics in southern Africa are infected (**BALDRIDGE *et al*., 1988**). More than 60% of all the people living with HIV/AIDS across the world live in Sub-Saharan Africa (**BALDRIDGE *et al*., 1988**). The population of hospital admissions for dermatologic diseases in HIV/AIDS individuals as a proportion of the admissions for dermatologic disease in all individuals has drastically increased from nearly 40% to 60% in various regions of South Africa (**MOSAM *et al*., 2004**).

Dermatophytoses manifests as an opportunistic infection which is four times more prevalent in acquired immunodeficiency syndrome (AIDS) patients (**GOODMAN *et al.,* 1987**). The most common clinical manifestation of dermatophytosis is *Tinea pedis* (athlete's foot) and nail infections, which can come into play at any time during the course of illness in HIV-positive patients (**BHAGRA *et al.,* 2013**). The incidence of dermatophyte infection of the glabrous skin has been reported to be approximately 40% in these patients (**ELMETS, 1994**). *Tinea* infections and onychomycosis are common in HIV disease (**TSE, 2007**). The features are similar to the HIV negative individuals (**TSE, 2007**). *Tinea unguium* (ringworm of the nails) frequently associates with *Tinea pedis* (athlete's foot) and

.

produces sub-ungual hyperkeratosis, onycholysis and nail discoloration (**TSE, 2007**). Generally, the increased number and severity of skin disorders are associated with declining immunity (**RAJU *et al.*, 2005**). The spectrum of skin disorders depends on; the immunologic stage as reflected by CD_4 count, concurrent use of HAART and the pattern of endemic infections (**TSE, 2007**).

The frequently isolated species are *Trichophyton rubrum, T.mentagrophytes,* and *Epidermophyton floccosum* (**FERNANDES *et al*., 1998**). *Microsporum gypseum* is a geophillic dermatophyte, which is rarely isolated from skin lesions of immunocompromised patients (**BHAGRA *et al.,* 2013**). *Microsporum gypseum* may cause ringworm of the scalp and hair and *Tinea corporis* (ringworm of the glabrous skin) in immunocompetent hosts (**PANDEY and PANDEY, 2013**). The most common skin disorder identified in HIV/AIDS individuals was fungal infections (**TSE, 2007**).

2.4.3. Fungi as major causative agents of skin diseases in humans

Fungi have emerged as major causes of human diseases in recent times, especially in immunocompromised individuals (**ELDER *et al.,* 2005)**, causing considerable morbidity and mortality (**MURRAY *et al*., 2013**). Fungi are eukaryotic organisms that are different from other eukaryotes as a result of the presence of a rigid cell wall composed of chitin, glucan and a cell membrane in which ergosterol is substituted for cholesterol as a major sterol component (**ELDER *et al.,* 2005; MURRAY *et al*., 2013**). Fungi may be unicellular or multicellular; yeasts are usually unicellular while molds are multicellular organisms consisting of threadlike tubular structures known as hyphae (**ELDER *et al.,* 2005; MURRAY *et al*., 2013**).

The hyphae are closely associated to form a mat-like structure called a mycelium (**MURRAY *et al*., 2013**). The hyphae may produce a specialised structure, the conidia, an asexual reproductive element which is easily airborne and serves as a means of fungus dissemination (**MURRAY *et al*., 2013**). Fungal spores can survive dry and harsh conditions and can tolerate a range of pH conditions (**ELDER *et al.,* 2005).** The size, shape and certain developmental features of the conidia are critical tools used in the identification of fungi into genus and species (**MURRAY *et al*., 2013**).

.

2.4.3.1. Classification of fungi

There are two main groups of fungi: the molds and yeasts (**ELDER *et al.,* 2005)**. In the molds, fungal cells are joined together to form filaments called hyphae (**ROBERTS *et al*., 1990**). The hyphae branches to form an interwoven mass called the mycelium (**ELDER *et al.,* 2005)** on which the fungus produces its spores (**ROBERTS *et al*., 1990**). The type and number of spores produced varies among species and is used as an identification tool in mould fungi (**ROBERTS *et al*., 1990**). Yeasts in contrast to molds are predominantly unicellular, usually with round oval cells (**ROBERTS *et al*., 1990**). These cells reproduce by budding, a process involving the development of a protuberance at the poles of the cell; which develops into a daughter cell which eventually separates from the parents (**ROBERTS *et al*., 1990**).

In some yeasts, the budding cell becomes elongated in the form of a chain known as a pseudomycellium. Some yeasts produce a true mycelium which is similar to, and cannot be distinguished from, that produced by molds (**ROBERTS *et al*., 1990**). A small number of fungi are dimorphic, meaning that they are capable of growth in either the yeast or mould form depending on the environmental conditions they are exposed to during growth (**ROBERTS *et al*., 1990**). A number of the human fungal pathogens are dimorphic in nature (**ROBERTS *et al*., 1990**).

2.4.3.2. Types of fungal diseases in humans and their mode of survival

There are approximately 100,000 species of fungi distributed globally. A greater number of fungal infections in both temperate and tropical countries are superficial infections of the skin (**HAVLICKOVA *et al*., 2008**). The most common pathogens in practice are dermatophytes, yeasts and molds (**HAVLICKOVA *et al*., 2008**). Fungal diseases of the human keratinized tissue may be caused by dermatophytes, non-dermatophytic fungi or a combination of both (**PATEL *et al*., 2006**).

The dermatophytes are a phylogenetically related group of filamentous ascomycetes (**PATEL *et al*., 2006**), parasitic fungus (**HARUNA *et al,* 2011**) which can be classified into the genera *Epidermophyton*, *Microsporum* and *Trichophyton* (**PATEL *et al*., 2006; HARUNA *et al,* 2011**) as characterized according to the formation and morphology of their conidia (**WEITZMAN and SUMMERBELL, 1995; WHITE *et al.*, 2008; PERES *et al.*, 2010**). These molds have keratinases which

.

allow them to grow in keratinized tissues on the host (**PATEL *et al*., 2006**). Fungi that are able to use the protein keratin for growth are referred to as keratinophilic fungi (**PATEL *et al*., 2006**). All dermatophytes are keratinophilic but not all keratinophilic fungi are dermatophytes (**PATEL *et al*., 2006**). The distinct characteristic between the two is based on the fact that dermatophytes grow on a living host while the other fungus does not require a living host (**PATEL *et al*., 2006**).

Dermatophytes produce proteolytic enzymes known as keratinases (**SOARES *et al*., 2013**). These enzymes hydrolyze keratin which is the main protein constituent of hair, nails and skin (**SIMPANYA, 2000; HARUNA *et al,* 2011; SOARES *et al.,* 2013**) thus causing injuries (**SIMPANYA, 2000)** and an inflammation by the host immune response to the metabolic by-products (**HARUNA *et al,* 2011**). The extent of the infection can be mild to severe, depending on the immune response of the host (**AKCAGLAR *et al.*, 2011**). The severity of the infection is partly dependent on the reaction of the host to the invading organism, and other factors, such as the species or virulence of the infecting strain, the reaction of the host to the metabolic products produced by the fungus, anatomic site of infection, and environmental factors (**SOARES *et al.,* 2013**).

In mammals, keratin, collagen and elastin constitute 25% of the body mass (**SOARES *et al.,* 2013).** The enzymes needed to hydrolyse these macromolecules can be found in infected tissues and is thus regarded as being essential to the virulence of dermatophytes (**SIMPANYA, 2000**). The ability of a dermatophyte to cause an infection in the host is dependent on several factors, among which are the "escape" mechanisms of the host resistance, which includes dry skin, a slightly acidic pH, the fungicidal effect of fatty acids, the continuous regeneration of skin, the state of the keratinized layer and other factors, such as competition with the normal skin microbiota (**ERBAGCI, 2004**). The infection is initiated through the inoculation of arthrospores deposited on the skin and favoured by a pre-existing skin lesion or abrasion (**SIDRIM and ROCHA, 2004**) and established by the “remarkable ability of the microorganisms to degrade keratin” (**SIMPANYA, 2000; ABDEL-RAHMAN, 2001; MACÊDO *et al.*, 2005**).

Animals are reservoirs of zoophilic dermatophytes and their zoonotic infections are of significant importance (**CABAÑES, 2000; BARANOVA *et al.*, 2003**). Anthropophilic species are not opportunistic microorganisms and are not part

.

of the normal microbiota of humans but pathogens that infect keratinized tissues, nails or hair of healthy individuals (**BAEZA *et al.*, 2007; MONOD, 2008**). Transmission can occur by direct contact or from exposure to desquamated cells (**SANTOS *et al*, 2006**). Direct inoculation through breaks in the skin often occurs in individuals with depressed cell-mediated immunity (**SANTOS *et al*, 2006**). The choice of appropriate treatment is determined by the site and extent of the infection and the species involved as well as by the efficacy, safety profile, and kinetics of the available drugs (**FERNANDEZ-TORRES *et al*., 2002**).

Superficial disease caused by non-dermatophytes is limited to the stratum corneum, hair or both (**PATEL *et al*., 2006**). The dermatophytes usually do not invade living tissues but colonize the outer layer of the skin (**HARUNA *et al,* 2011**). The growth of fungi on the host leads to manifestation of skin diseases as there is little or no host immune response (**PATEL *et al*., 2006**). The fungi *Trichophyton tonsurans, T. mentagrophytes* and *Microsporum gypseum* causes *Tinea* (popularly referred to as ringworm) such as *Tinea capitis* (ringworm of the scalp and hair), *Tinea corporis* (ringworm of the glabrous skin), *Tinea unguium* (nail infection) and *Tinea manuum* (ringworm of the hand) (**HARUNA *et al,* 2011**).

2.4.4. Classification of Human mycoses

Human mycoses are classified into; superficial, cutaneous and subcutaneous mycoses, the endemic mycoses and opportunistic mycoses (**MURRAY *et al*., 2013**). Superficial mycoses are limited to the superficial surfaces of the skin and hair (**MURRAY *et al*., 2013**). The fungal species responsible for this type of mycoses include *Piedraia hortae*, *Hortae werneckii*, *Malassezia furfur* and *Trichosporon spp* (**MURRAY *et al*., 2013**).

Cutaneous mycoses infects the keratinized layer of skin, hair and nails and can be symptomatic with signs like itching, scaling, broken hairs, ring-like patches of the skin, and thickened discoloured nails (**MURRAY *et al*., 2013**). Fungi of the genera *Trichophyton*, *Microsporum* and *Epidermophyton* are responsible for cutaneous mycoses and the infections involving these organisms are referred to as dermatophytoses (**MURRAY *et al*., 2013**). Cutaneous disease involves the epidermis and dermis and results to an inflammatory reaction similar to that of contact dermatitis (**PATEL *et al*., 2006**). The most frequent type of onychomycosis (a fungal infection of the nails) is caused by dermatophytes which represents

.

between 18% and 40% of all onychopathies (**ZIMMERMAM-FRANCO *et al*., 2013**). Dermatophytes, most especially *Trichophyton rubrum* and *T. mentagrophytes* are the primary cause of 90% of onychomycosis (**KAUR *et al*., 2008**).

Subcutaneous mycoses involve the deeper layers of the skin such as the cornea, muscle, and connective tissues (**MURRAY *et al*., 2013**). The fungus finds its way to the tissues by traumatic inoculation and remains localised causing abscess formation, non-healing ulcers and draining sinus tracts (**MURRAY *et al*., 2013**). The infections may be caused by hyaline molds such as *Acremonium* species, *Fusarium* sp and pigmented or dermatiaceous fungi such as *Alternaria* spp, *Cladosporium* sp and *Exophiala* sp.

Endemic mycoses, also often referred to as systemic mycoses are caused by dimorphic fungal pathogens such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidiodes immitis*, *C. posadasii*, *Paracoccidiodes brasiliensis*, and *Penicillium marneffei* (**MURRAY *et al*., 2013**). The fungi exist in two morphological forms; yeast or spherules at 37ºC and molds at 25ºC (**MURRAY *et al*., 2013**).

Opportunist mycoses are infections caused by fungi that are human commensals. With the exception of *Cryptococcus gattii* and *C. neoformans* these organisms exhibit a low level of virulence and are responsible for infection in individuals that are debilitated and immunosuppressed (**MURRAY *et al*., 2013**). The most common species in this group is the yeasts *Candida* sp, the molds *Aspergillus* sp and *Pneumocystis jirovecii* (**MURRAY *et al*., 2013**). Infections caused by pathogenic fungi (mycoses) can be classified into 3 groups namely superficial, subcutaneous and systemic (**ROBERTS *et al*., 1990**).

Superficial infections are the most common and are responsible for a great deal of morbidity in the general population (**ROBERTS *et al*., 1990**). The major superficial mycoses which may affect the hair, skin, nail and mucous membranes are: ringworm caused by dermatophytes, candidiosis caused by species of *Candida* and *Pityriasis versicolor* caused by *Malassezia furfur* (**ROBERTS *et al*., 1990**). Superficial fungi can be grouped according to their habitats into anthropophilic, zoophilic and geophilic organisms (**HAVLICKOVA *et al*., 2008**).

Anthropophilic fungi generally cause superficial dermatomycoses which are characterised by relatively low inflammatory activity because of an immunological arrangement between the fungus and its human host (**MALE, 1990; MACURA,**

.

1993). Household dust may serve as a reservoir of antropophilic dermatophytes which can preserve dermatophyte spores for years (**HAVLICKOVA *et al*., 2008**).

Zoophilic germs are found in animals, but are also sporadically transmitted to humans by cats, dogs (*Microsporum canis*), guinea pigs and rabbits (*Trichophyton mentagrophytes* var. *granulosum*) (**HAVLICKOVA *et al*., 2008**). They are associated with highly inflammatory and infectious skin infections (**HAVLICKOVA *et al*., 2008**). Geophilic fungi grow in the soil and only intermittently infect humans (**MACURA, 1993**). The result of which varies from high to low inflammation (**HAVLICKOVA *et al*., 2008**). *Microsporum gypseum* strains which are common geophilic pathogens are more virulent when isolated from humans than those isolated from the soil and occasionally causes an epidemic spread under favourable growth conditions (**FARAH *et al.*, 2000**).

A high number of superficial fungal infections of the skin are caused by five or six species of dermatophytes, of which *Trichophyton rubrum* is the most common (**ALY, 1994**). As described in the clinical manifestations and global patterns of superficial fungal infections, the predominant species of dermatophytes vary according to their clinical localisation (**FREEDBERG *et al*., 2003**). Most of the basic fungal skin infections are caused by dermatophytes and the major clinical manifestations are detailed below (**HAVLICKOVA *et al*., 2008**).

Tinea corporis (ringworm of the glabrous skin)

This affects the trunk, often in exposed areas like the abdomen or limbs, causing red patches (**HAVLICKOVA *et al*., 2008**). It is more often seen in children than in adults and occurs most frequently in hot climates (**MACURA, 1993**). Dermatophytes of the genera *Trichophyton* and *Microsporum* are the most common causative agents (**HAVLICKOVA *et al*., 2008**).

Tinea capitis (ringworm of the scalp)

This is a dermatophyte infection of the scalp and hair and seems to affect young children globally (**MACURA, 1993**). Presentation depends on the aetiology and can be non-inflammatory, inflammatory or black dot type. The non-inflammatory form is most commonly caused by *M. audouinii* or *M. ferrugineum* and usually begins as a small erythematous papule surrounding a single hair shaft, which spreads to other hairs (**HAVLICKOVA *et al*., 2008**). Scaling occurs and the hair turns grey

.

(**HAVLICKOVA *et al*., 2008**). The inflammatory type is usually associated with zoophilic or geophilic germs such as *M. canis* and *M. gypseum* respectively (**HAVLICKOVA *et al*., 2008**). Black dot *Tinea capitis* (ringworm of the scalp and hair) is caused by *T. tonsurans* or *T. violaceum.* The fungus *Trichophyton verrucosum* is highly contagious and virulent and is the only dermatophyte with the ability to grow well at 37ºC (**HAVLICKOVA *et al*., 2008**). Ringworm of the scalp and hair caused by *T. verrucosum* can result in irreversible scarring and alopecia (**HAVLICKOVA *et al*., 2008**). It is widespread and a well-known occupational disease of cattle keepers (**MACURA, 1993**).

Tinea cruris (ringworm of the groin)

This presents as an itchy red rash in the groin and surrounding area and is prevalent in young men living in a warm climate (**MACURA, 1993**). It can coexist with *Tinea pedis* (athlete's foot) if the infection is spread by scratching the feet and then the groin (**HAVLICKOVA *et al*., 2008**). The most common agents are *Epidermophyton floccosum*, *T. mentagrophytes* and *T. rubrum* (**HAVLICKOVA *et al*., 2008**).

Tinea unguium (onychomycosis, nail infections)

The causative agents of onychomycosis include dermatophytes, *Candida* spp and non-dermatophytic molds (**HAVLICKOVA *et al*., 2008**). Dermatophytes are the most common culprits of onychomycosis in temperate Western countries, while *Candida* and non-dermatophytic molds are more frequently involved in countries with a hot and humid climate (**CHI *et al*., 2005**). *Trichophyton rubrum* is the most common dermatophyte associated with onychomycosis. Others include *T. interdigitale, Epidermophyton floccosum, T. violaceum, M. gypseum, T. tonsurans*, and the cattle ringworm fungus, *T. verrucosum* (**MACURA, 1993**). The fungus *T. soudanense* is considered by some to be an African variant of *T. rubrum* rather than a full-fledged separate species. It should be noted that *T. interdigitale* is still sometimes referred to as *T. mentagrophytes* var. *interdigitale*. The latter should be used to describe the zoophilic form of the dermatophyte, and *T. interdigitale* used to describe its anthropophilic form (**HAVLICKOVA *et al*., 2008**). Recent genetic research has shown that *T. mentagrophytes* var. *granulosum* is the same as *T. interdigitale* (**NENOFF *et al.*, 2007**). Other causative agents include *Candida spp*

.

(**MACURA, 1993**) and non-dermatophytic molds, in particular members of the genera *Scytalidium* (now *Neoscytalidium*), *Scopulariopsis* and *Aspergillus*.

Tinea pedis (athlete's foot)

This is a very common infection which occurs in one out of five adults and the incidence increases with age from adolescence (**MALE, 1990**). It occurs more frequently in people who wear occlusive shoes (**MACURA, 1993**). It may be associated with several different fungi which includes yeasts, the most common being *T. rubrum* and *T. interdigitale* (formerly *T. mentagrophytes* var. *interdigitale*). The incidence of *Tinea pedis* (athlete's foot) has increased in the developed world during the past 30 years and its prevalence is around 10% (**NELSON *et al*., 2003**). The "One hand two feet syndrome" is characterised by dermatophyte infection of both feet and one hand is common in patients with lower immunocompetence (**HAVLICKOVA *et al*., 2008**). The condition is frequently associated with *T. rubrum*.

2.4.5. Nail infections caused by fungi

Dermatophytes are the most common nail pathogen and only 3 species are often responsible for nail infections (**ROBERTS *et al*., 1990**). Dermatophyte infection of the nails is associated with athlete's foot (**ROBERTS *et al*., 1990**). There is a spread of ringworm infection from the skin of the feet, usually toe webs to toe nails and other parts of the body like groin, hands and fingernails (**ROBERTS *et al*., 1990**). Hence, dermatophytoses of the nails is often associated with current or past ringworm infection of the feet and it spreads with the use of communal bathing places and its prevalence is highest in adult males (**ROBERTS *et al*., 1990**). The widespread occurrence of *T. rubrum* nail infections could be a reflection of the persistent nature of skin infections resulting from this species, its relative resistance to therapy and its ability to invade nail keratin better than other species (**ROBERTS *et al*., 1990**).

All species of fungus responsible for nail infections are endemic on the floors of communal places. Thus, the incidence of infection is high in regular users of public/communal facilities; these include regular swimmers, sportsmen, coal miners, members of the armed forces and any other regular user of communal showers and bathrooms (**ROBERTS *et al*., 1990**). Nail infections caused by yeasts are

.

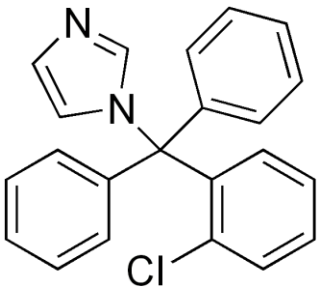
predominantly due to *Candida species*, particularly *Candida albicans* (**ROBERTS *et al*., 1990**). These organisms are commensals of the mouth, gastrointestinal tract, the vagina and to a lesser extent the skin. There is no concrete evidence that yeasts are keratinolytic (**ROBERTS *et al*., 1990**). However, it is likely that they have some proteolytic activity which destroys the integrity of the keratin (**ROBERTS *et al*., 1990**). In chronic paronycia, a chronic inflammation beneath the nail fold and secondary disruption of the nail plate, *C. albicans* is the predominant organism (**ROBERTS *et al*., 1990**). Dermatophytes slowly destroy the nail keratin, causing the nail to crumble and *T. rubrum* is the most common nail pathogen (**ROBERTS *et al*., 1990**). Fungal skin infections are treated with topical preparations containing azoles which may be in the form of creams, lotions, sprays but are not specifically formulated for nail infection as the vehicle does not allow adequate penetration of the antifungal agent into the nail and hence are not recommended (**ROBERTS *et al*., 1990**).

2.4.6. Treatment of Mycosis

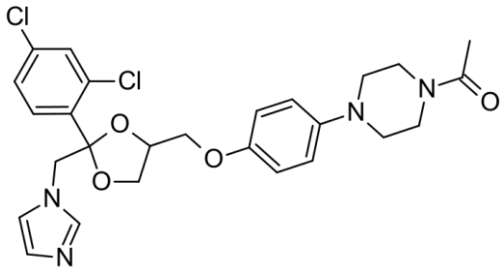
Azoles are the most widely used antifungals in clinical practice, and thus are also the most studied by the scientific community regarding their mode of action, pharmacological properties, and the resistance mechanisms which have been developed by microorganisms (**VANDEPUTTE *et al*., 2012**). Azole antifungals are also highly researched by pharmaceutical companies, who seek to improve their efficacy and to develop the perfect antifungal (**VANDEPUTTE *et al*., 2012**). Azoles are cyclic organic molecules which can be divided into two groups on the basis of the number of nitrogen atoms in the azole ring (Fig 2.1): the imidazoles contain two nitrogen atoms, and the triazoles contain three nitrogen atoms (**MAERTENS, 2004**).

Azole drugs target the ergosterol biosynthetic pathway of the fungus by inhibition of a key enzyme, the lanosterol 14-alpha demethylase, encoded by the ERG11 gene (**VANDEPUTTE *et al*., 2012**). This inhibition occurs through the binding of the free nitrogen atom of the azole ring to the iron atom of the heme group of the enzyme (**VANDEPUTTE *et al*., 2012**). This results in the accumulation and metabolism of 14-alpha methylated sterol species and gives impetus to the synthesis of toxic compounds, which are unable to successfully replace ergosterol (**CARRILLO-MUÑOZ *et al*., 2006**).

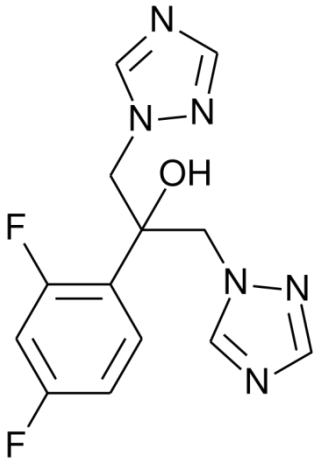
.



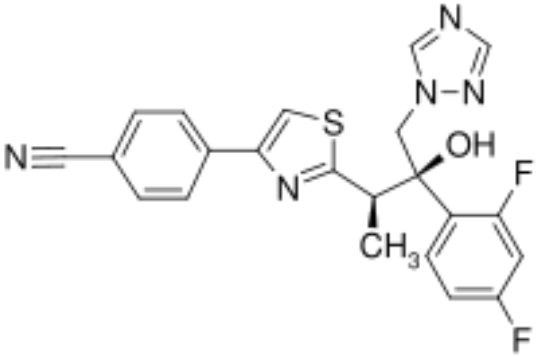
Clotrimazole



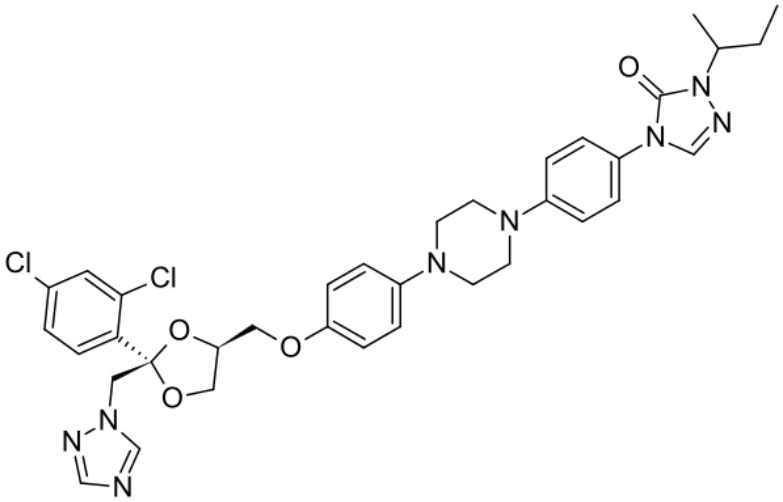
Ketoconazole



Fluconazole



Ravuconazole



Itraconazole

Figure 2.1: Azoles used for dermatophytic infections in clinical practice

.

In 1944, the first azole was synthesized by Woolley (**WOOLLEY, 1944**), but it was not considered as a potential antifungal agent by the scientific community until 1958 (**VANDEPUTTE *et al*., 2012**). Clotrimazole, econazole, and miconazole were made available for treatment in the late 1960s (**FROMTLING, 1988).** Their use was however restricted to external application as a result of their high toxicity when administered orally (**BURGESS and BODEY, 1972; TETTENBORN, 1974**). In 1968, miconazole became the first antifungal made available for parenteral injection, but due to its toxicity and relatively limited range among fungal species (**HEEL *et al*., 1980**), its use decreased to the point that it was no longer commercialized (**VANDEPUTTE *et al*., 2012**).

In the 1970s, a new class of antimycotic drugs was developed, the azoles. The first azole made available for systemic use was clotrimazole. However, its use was limited largely because of its irregular concentrations in the blood. Miconazole was the first effective azole for systemic infections, but its shortcoming is that it could only be given intravenously and it offered few advantages over amphotericin, aside from lessened toxicity (**STEVENS, 1977**). The first azole to be orally available was ketoconazole and it showed consistent blood levels (**BORELLI *et al*., 1979**).

In 1981, the Food and Drug Administration (FDA) approved a new antifungal known as ketoconazole which was developed by **HEERES** and his co-workers (**HEERES *et al*., 1979**). Susceptibility to ketoconazole was documented for various *Candida* species. Ketoconazole also shows activity against dermatophytes. This drug was the only antifungal made available for the treatment of systemic fungal infections caused by yeasts for the next ten years (**VANDEPUTTE *et al*., 2012**). However, there are several drawbacks to this drug as it is poorly absorbed when administered orally and no ketoconazole form has ever been developed for intravenous injection (**VANDEPUTTE *et al*., 2012**).

Another shortcoming of the drug is that it cannot pass through the cerebrospinal barrier and is less active in immunosuppressed patients (**VAN DER MEER *et al*., 1980; BRASS *et al*., 1982; PERFECT *et al*., 1982; FROMTLING, 1988**). It causes some severe side effects such as a decrease in the level of testosterone or glucocorticoids production and liver and gastrointestinal complications (**PONT *et al*., 1982; LEWIS *et al*., 1984; DISMUKES *et al*., 1985**). For these cogent reasons, the triazoles were developed. Fluconazole was made

.

available for use by clinicians in 1990 and it provided several advantages over the use of imidazole (**VANDEPUTTE *et al*., 2012**). Fluconazole has a high hydro-solubility and can therefore be easily injected intravenously (**VANDEPUTTE *et al*., 2012**). It is almost completely absorbed through the gastrointestinal tract, and it diffuses throughout the whole body including the cerebrospinal fluid (**ARNDT *et al*., 1988, BRAMMER *et al*., 1990**). Fluconazole was the gold-standard treatment of fungal infections during the 1990s as a result of its good pharmacokinetic properties as well as its wide range of activity (**VANDEPUTTE *et al*., 2012**). Unfortunately, fluconazole was over-prescribed by physicians for prophylaxis or treatment, this led to an increase in resistance to azole drugs (**VANDEPUTTE *et al*., 2012**). Moreover, fluconazole is almost ineffective against most molds (**VANDEPUTTE *et al*., 2012**).

Itraconazole was approved and made available by the FDA in 1992 and it possesses a broad spectrum of activity across fungal species comparable to ketoconazole and wider than fluconazole, but less toxic than ketoconazole (**VANDEPUTTE *et al*., 2012**). However, itraconazole is hydrophobic and is thus more toxic than fluconazole (**VANDEPUTTE *et al*., 2012**). Thus, a new itraconazole formulation with an enhanced absorption and a decreased toxicity was approved by the FDA in 1997 (**BARONE *et al*., 1998**) and an injectable formulation of itraconazole was made available in 2001 (**BOOGAERTS *et al*., 2001**). Fluconazole and itraconazole are still not the perfect antifungals as they have some non-negligible drug interactions with such drugs that are prescribed in chemotherapy or with AIDS treatment (**VANDEPUTTE *et al*., 2012**). These interactions can lead to a decrease in azole concentration or in the worse scenario an increase in toxicity (**ALBENGRES *et al*., 1998**).

New generation triazoles have also been developed, voriconazole and posaconazole were approved by FDA in 2002 and 2006, respectively. Ravuconazole is presently in the clinical trial phase of drug development (**VANDEPUTTE *et al*., 2012**). They possess a wide range of activity as they show good activity against several fungus among which are *Candida* and other dermatophytes (**SABO and ABDEL-RAHMAN, 2000; CHIOU *et al*., 2000**). While the new generation triazoles have been more effective against *Candida* (**CHIOU *et al*., 2000**) as compared to classical triazoles, their side effects and drug interactions are similar to those observed with fluconazole and itraconazole (**POTOSKI and BROWN, 2002**).

.

Likewise, fungal isolates resistant to the classical triazoles are also cross-resistant to the new generation triazoles (**VANDEPUTTE *et al*., 2012**).

In the treatment of fungal infections on the skin, topical medications are suitable only for early or mild infections, especially those caused by *T. rubrum*, the main fungus responsible for athlete's foot (**SOARES *et al.,* 2013**). Systemic therapy is usually used in nail infections (onychomycosis) and infections caused by zoophilic dermatophytes which are the microorganisms involved in the development of ringworm of the scalp and hair, and glabrous skin (**TANI *et al.*, 2007; WHITE *et al.*, 2008**). The growth of dermatophytes of all genera is inhibited by terbinafine and is the main drug of choice for the treatment of dermatophytoses (**SOARES *et al.,* 2013**).

Naftifine and terbinafine are a group of synthetic drugs, the allylamines, which were identified with the clinical treatment of superficial mycoses (**BALFOUR and FAULDS, 1992**). Allylamine has been reported to show fungicidal activity against a variety of dermatophytes, molds and certain dimorphic fungi, and fungistatic activity against *Candida albicans* (**ABDEL-RAHMAN and NAHATA, 1997**; **MCCLELLAN *et al.*, 1999**). Its mechanism of action involves blocking the biosynthesis of ergosterol, an essential component of the fungal cell membranes through the inhibition of fungal squalene epoxidase (**ABDEL-RAHMAN and NAHATA, 1997**).

In comparative studies, the incidence of adverse effects related to the use of terbinafine orally was lower than that detected in the treatment with griseofulvin and similar to that of itraconazole (**ABDEL-RAHMAN and NAHATA, 1997**). Griseofulvin, a weakly fungistatic agent, toxic to fungal cell nuclei has been the mainstay of systemic treatment for dermatophyte nail infections for the past 30 years (**ROBERTS *et al*., 1990**). It is prescribed in a dose of 1g daily to be taken with meals and need to be used for a period of 12-18 months in the case of toenail infection and 6-12 months in the case of fingernail infections (**ROBERTS *et al*., 1990**). Although Griseofulvin is devoid of serious side effects in the treatment of nail infection, its cure rate is approximately 30% making it an imperfect antifungal as it needs to be taken for a long period of time (**ROBERTS *et al*., 1990**). Ketoconazole is active against dermatophytes but in the case of nail infections its use is limited as a result of its side effects (**ROBERTS *et al*., 1990**). Griseofulvin controls the development of keratinized tissue infection by presenting only fungistatic and not fungicidal action

.

(**SOARES *et al.,* 2013**). Griseofulvin is orally administered and the treatment varies depending on the clinical form of the mycosis (**GUPTA *et al.*, 2001**) and must be prolonged in cases where the infection is severe (**DEACON, 1998**). The drug has been reported to interfere with the polymerization of microtubules in its mechanism of action and thus causing abnormalities in cell division due to achromatic spindle formation and abnormal growth, probably resulting from the disruption of intracellular transport associated with microtubules (**ODDS, 2003**). The imidazole derivatives currently represent a major advancement in the oral and topical treatment of superficial mycoses (**KATZUNG *et al.*, 1998**).

In the treatment of dermatophytoses, clotrimazole (topical), ketoconazole, itraconazole and fluconazole are commonly used as they are easily absorbed through the gastrointestinal tract and maintain an effective activity (**KATZUNG *et al.*, 1998**). In therapy, antifungal azoles used in the clinic fall into two groups: the imidazoles (ketoconazole, clotrimazole, miconazole, and econazole) and triazoles (fluconazole, voriconazole, itraconazole and posaconazole) (**SOARES *et al.,* 2013**). The use of imidazoles is limited to the treatment of superficial mycoses while the triazoles have a wider application (**SOARES *et al.,* 2013**).

Chronic infections of dermatophytoses are treated with intense drugs such as terbinafine, itraconazole and fluconazole, which are the most used (**ABDEL-RAHMAN *et al.*, 1998; FERNÁNDEZ-TORRES *et al.*, 2001; FERNÁNDEZ-TORRES *et al.*, 2003**). In an attempt to achieve an increased therapeutic rate of treatments, a combination of topical and oral anti-inflammatory drugs has been used (**SOARES *et al.,* 2013**). In recent times, there have been cases of treating onychomycosis using topical amorolfine and ciclopirox (**GUPTA *et al.*, 2001**).

Itraconazole shows a broad *in vitro* activity against the dermatiaceous fungi. Fluconazole is sensitive and showed resistance against the key dermatophytes (**OLSEN *et al*., 2000)** but has limited activity against molds and other filamentous fungi. Voriconazole which is structurally related to fluconazole shows a spectrum similar to that of itraconazole.

2.4.6.1. Mode of action of antifungals

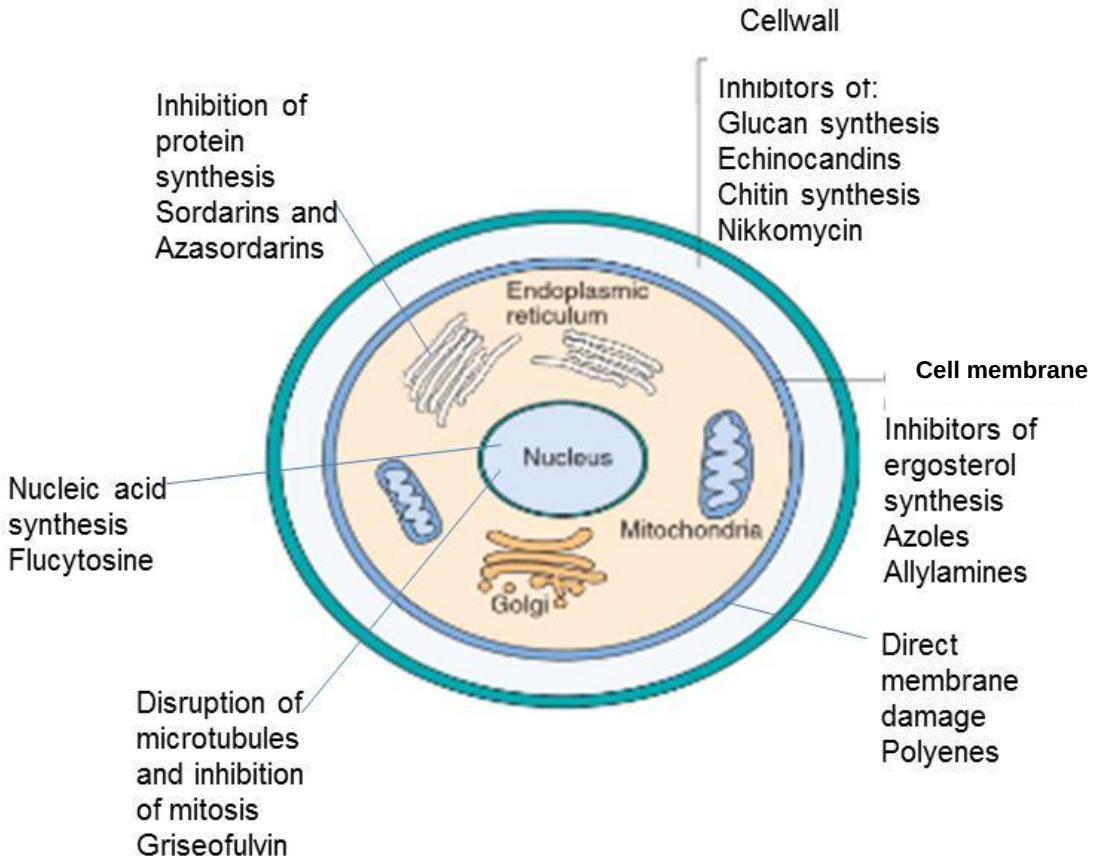
There is a large variety of antifungal drugs; their cellular targets are however limited due to the similarity that exists between fungi and host cells (**SOARES *et al.,* 2013**). Drugs that are commonly used target the biosynthetic pathway of the fungus

.

(**MARTINEZ-ROSSI *et al.*, 2008**). Mode of action involves: direct membrane damage, disruption of microtubules and inhibition of mitosis, inhibition of other processes within the fungal cell such as protein, glucan and ergosterol synthesis (Fig 2.2) (**MURRAY *et al*., 2013**). Ergosterol is the most common sterol found in the fungal plasma membrane (**MURRAY *et al*., 2013**). Azoles inhibit ergosterol synthesis by binding to lanosterol demethylase which is a specific enzyme in ergosterol biosynthesis (**MURRAY *et al*., 2013**). The active binding site of lanosterol demethylase contains a heme domain (**HITCHCOCK, 1991; VAN DEN BOSSCHE, 1991**). Azoles bind to the iron atom of the heme domain using a specific nitrogen atom in the azole ring nucleus and thus preventing the demethylation of lanosterol (**JOSEPH-HORNE and HOLLOMON, 1997**). Azoles may also target lipids of the fungal plasma membranes (**JOSEPH-HORNE and HOLLOMON, 1997**) and may interact with the 3-ketosteroid reductase, an enzyme in methylsterol biosynthesis. The principal effect of azoles is to inhibit a cytochrome, P-450, which is dependent on the fungal enzyme system and is involved in the final stages of ergosterol biosynthesis (**ROBERTS *et al*., 1990**).

The current treatment against the dermatophytoses is based on antifungal drugs, mainly the imidazolic derivatives and allylamines (**ZIMMERMAM-FRANCO *et al*., 2013**). However, various shortcomings have been associated with synthetic antifungal drugs which range from its high price, adverse reactions and its slow action (**ZIMMERMAM-FRANCO *et al*., 2013)**. Fluconazole and itraconazole are still not the perfect antifungals, since they have some non-negligible drug interactions with such drugs that are used in chemotherapy or with AIDS treatment. These interactions can result in a decrease in azole concentration or even an increase in toxicity (**ALBENGRES *et al.,* 1998**). In addition to ketoconazole's rare hepatotoxicity, resistance most especially in patients with AIDS was reported (**ROSENBLATT *et al*., 1980; HORSBURGH and KIRKPATRICK, 1983; WARNOCK *et al*., 1983; RODRIGUEZ-TUDELA *et al*., 1995**). The treatment can also increase the chances of recurrence and resistance of strains when the drugs are not properly administered (**VALDÉS, 2005; DIOGO, 2010**).

.



Picture source- MURRAY PR, ROSENTHAL KS, PFALLER MA., 2013. Medical Microbiology.

Figure 2.2: Mode of action of various antifungals on the fungal cells

.

2.4.6.2. Skin disease resistance and other shortcomings of antifungals

The incidence of resistance to antifungal agents may be increasing (**DENNING, 1995; JOHNSON *et al*., 1995; WALSH *et al*., 2000; DODGSON *et al*., 2004; PUJOL *et al*., 2004; BLIGNAUT *et al*., 2005**) with drug resistant fungal strains as a particular common causative pathogen of infection in high-risk patient groups, such as HIV/AIDS patients (**JOHNSON *et al*., 1995; PANKHURST, 2001**). The reports of recurrences of infections are usually attributed to the discontinuation of therapy (**SOARES *et al.,* 2013**). The frequent use of azoles (especially fluconazole) in the treatment and prevention of fungal infections has led to reports of emerging resistance to antifungal agents (**MURRAY *et al*., 2013**). The resistance to azoles has been reported in 32% of symptomatic and in 14% of asymptomatic patients (**REVANKAR *et al*., 1996; MAENZA *et al*., 1997; MARTINS *et al*., 1997**). The prevalence correlates with the amount of cluster of differentiation 4 (CD_4) cells, the fungal load, and the duration and doses of therapy (**MAENZA *et al*., 1996**). **VUFFRAY *et al*.,** (**1994**) showed that resistance can also occur in patients with high single doses of fluconazole. There is no evidence showing that fungi have the ability to destroy or modify antifungal agents as a means of achieving resistance to antibiotics (**MURRAY *et al*., 2013**). Unlike in bacteria cells, antifungal resistance genes are not transmissible from cell to cell (**MURRAY *et al*., 2013**).

The biochemical mechanisms that may be responsible for drug resistance in a fungus involves a decrease in drug uptake, structural alterations in the target site and an increase in drug efflux or in intracellular target levels, gene amplification, gene transfer, gene deletion, point mutations, loss of cis- and trans-acting regulatory elements and transcriptional activation (**KAMAI *et al.*, 2004**). Fungal resistance to antifungals develops slowly and involves the emergence of intrinsically resistant species or a gradual and stepwise alteration of the functions and structures of the fungal cell that leads to resistance to an agent which the species have been exposed to (**MURRAY *et al*., 2013**).

It is noteworthy that the use of immune-modulating drugs, concomitant infections such as HIV and the nutritional status of the host are more crucial factors that determine the outcome of the infection than the ability of an antifungal to inhibit or totally destroy the infecting organism (**MURRAY *et al.,* 2013**). The *in vitro* resistance of an isolate can be classified as either intrinsic or acquired (**SOARES *et***

.

***al.,* 2013**). Intrinsic resistance allows all normal members of a species to tolerate a particular drug while acquired resistance means that a resistant strain emerges from a population that was previously drug-sensitive (**KAMAI *et al.*, 2004**). The clinical resistance to griseofulvin, or relapses after treatment, is common in dermatophytoses (**SOARES *et al.,* 2013**).

At present, antibiotics and antifungals play a vital role in the control of fungal diseases (**SOARES *et al.,* 2013**). However, some of these antifungals have shortcomings such as toxicity, fungistatic activity and a limited spectrum of action or resistance (**GARCÍA-SOSA *et al.*, 2011**). As a result of these shortcomings, there is a need to develop innovative antifungal products of natural origin (**ZIMMERMAM-FRANCO *et al*., 2013)**.

2.5. Plants, a source of therapeutic compounds

The search for plants with therapeutic activities dates back centuries (**SOARES *et al.,* 2013**). Thousands of plants have been used by people of all races, in the form of poultices, infusions, decoctions and others (**SOARES *et al.,* 2013**). The increased interest in plants with medicinal properties have resulted into an alternative therapeutic approach because of the prospect of isolating substances with significant efficacy and a lower side effects (**QUEIROZ *et al.*, 2009**). Plants have been used in medicine for several centuries, having been extensively used in folk medicine because they represent an economical alternative and are applicable to various pathologies (**ROJAS *et al.*, 2006**). Plants are an important source of biologically active compounds which serve as models for the synthesis of a large number of drugs (**YUNES and CALIXTO, 2001; SIMÕES *et al.*, 2003**).

Natural products have been a great resource for investigation into drug research and thus giving impetus to chemical research and discovery of new drugs (**CHIN *et al.*, 2006**). The success of natural products results from several factors which include their great chemical biodiversity and the effects of evolutionary pressure in creating biologically active molecules (**HARVEY, 2007**). These agents are synthesized by manipulating the natural product, thus demonstrating that the synthesis of molecules can be used to discover and develop novel agents of therapeutic value (**WILSON and DANISHEFSKY, 2006**). Special attention has been directed towards natural derivatives, based on the knowledge of the production of

.

compounds with antifungal activities in nature (**WOJTASZEK, 1997; GURGEL *et al.*, 2005**).

2.5.1. Plants as a potential source of antifungals

A high percentage of antifungal drugs have some connection with natural products (**SOARES *et al.,* 2013**). These natural products can be used as therapeutic agents, a source of raw materials for synthesis and can serve as prototypes for new pharmacologically active models (**BRAZIL, 2006**). The polyenes and griseofulvin are natural products (**BUTLER, 2005**). Griseofulvin is produced by *Penicillium griseofulvum* (**KAR, 2003**) while polyenes (amphotericin B and nystatin) were isolated from *Streptomyces species* (**DI SANTO, 2000**). Pharmacological research into medicinal plants and their derivatives serve as a crucial tool for drug development (**SOARES *et al.,* 2013**). Among the antifungal compounds sourced from nature are the coumarins. The potential of coumarins as dermatophyte-specific antifungal agents has been reported (**MERCER *et al*., 2013**).

The coumarins are a very large class of compounds found throughout the plant kingdom (**EGAN *et al.,* 1990; EGAN and O'KENNEDY, 1992; FINN *et al.,* 2002**) but abundant in certain plant families including the Rutaceae. Coumarins are plant-derived polyphenolic compounds which are best known for their anti-inflammatory, anti-thrombotic (e. g. warfarin) and vasodilatory properties (**RIVEIRO *et al*., 2010**). Coumarins have been reported to possess antimicrobial properties (**DUNCAN *et al*., 2004; WU *et al*, 2009; DEKIC *et al*., 2010; KUMAR *et al*., 2012**). Generally, coumarins are poorly water-soluble, but the coumarin glycosides are significantly more water-soluble but with reduced/no antimicrobial activity and are readily available or can be easily synthesized (**LIM *et al*., 2004**).

Although, the coumarins are distributed throughout all parts of the plant, the highest levels of coumarins occur in the fruits, followed by the roots, stems and leaves (**LACY and O'KENNEDY, 2004**). Environmental conditions and seasonal changes can influence the natural occurrence of coumarins in different parts of the plant (**JAIN and JOSHI, 2012**). Approximately 1,300 coumarins have been isolated from natural sources (**MELLO *et al*., 2003**). **MERCER *et al*., (2013)** described for the first time the potential of coumarins as topical antifungals for superficial mycoses

.

using a prodrug approach. A variety of pharmacologically active coumarins and their derivatives have been isolated from Rutaceae species.

2.5.2. Isolation of coumarins and other bioactive compounds from Rutaceae species

Clausena excavata is rich in coumarins and several coumarins have been isolated from the plant. **HE *et al*. (2000)** reported a O-terpenoidal coumarin named excavacoumarin A isolated from the plant. Chemical investigation on the fruits and stems of *C. excavata* led to the isolation and identification of a coumarin, namely clausenaexcavin (**LAPHOOKHIEO *et al*., 2009**). Lenisin A was isolated from the aerial part of *C. excavata* (**HE *et al*., 2006**). The isolation and identification of chemical constituents of the leaves of *C. excavata* cultivated in a greenhouse yielded 10 new furanonecoumarins named clauslactones A, B, C, D, E, F, G, H, I, and J , together with a known carbazole, clauszoline M, and a coumarin, umbelliferone (**WU and FURUKAWA, 1982**). **TAKEMURA *et al*., (2004**) reported the presence of a dimeric coumarin, diseselin B, and 3 phenylpropanoids, lenisin A-C, together with eight O-terpenoidal coumarins, isolated from the aerial part of *C. excavata*. Clausenidin, O-methylmukonal, 3- formyl-2,7-dimethoxycarbazole, and clauszoline-J, were isolated from the rhizomes and roots of *C. excavata* (**KONGKATHIP *et al.,* 2010**).

HONG *et al*., (**2000**) isolated two O-terpenoidal coumarins named excavacoumarin A and B from the leaves of *C. excavata*. Phytochemical investigation on the aerial part of *C. excavata* by **HONGPING *et al*.,** (**2000**) led to the isolation of six O-terpenoidal coumarins, named excavacoumarins B, C, D, E, F and G. **XIN *et al***. (**2008**) reported that three coumarins containing a C10 terpenoid side chain, clauslactones R, S and T, together with 14 known coumarins, were isolated from the leaves and stems of *C. excavata*. The coumarins, excavatins A-M have been isolated from *C. excavata* (**THUY *et al.,* 1999**). Moreover, four furanone-coumarins, clauslactones- N, -O, -P and -Q were isolated from the leaves and twigs of *C. excavata* (**TAKEMURA *et al*., 2000**).

A carbazole alkaloid, clausine-L and 11 known compounds were isolated from the leaves of *C. excavata* (**WU and FURUKAWA, 1982**). A dimeric coumarin, diseselin B and three phenylpropanoids, lenisin A–C, together with eight known *O*-

.

terpenoidal coumarins, were isolated from the aerial parts of *Clausena lenis* (**ARBAB *et al.,* 2011**). Two geranyl coumarins have been isolated from the leaves of *Clausena anisata* and identified as anisocoumarins I and J (**NGADJUI *et al*., 1991**). The coumarin, 7-[(2′E,6′E)-7′-carboxy-5′(ζ)-hydroxy-3′-methylocta-2′,6′-dienyloxy]-coumarin, was isolated from the leaves of *C. suffruticosa* (**BEGUM *et al*., 2010**). *Clausena dentata* contains sabine, biofloractriene, borneol and β-bisabolol (**RAJKUMAR and JEBANESAN, 2010**).

The leaves of *Murraya paniculata* yielded four coumarins; auraptene**,** gleinadiene, 5,7-dimethoxy-8-(3-methyl-2-oxo-butyl)coumarins and toddalenone (**AZIZ *et al.,* 2010**). The coumarin 7-methoxy-8-(2′-isovaleryloxy-3′-hydroxy-3′-methylbutyl) coumarin, also known as murrayatin was isolated from the leaves of *Murraya exotica (***BARIK *et al*., 1983**). Trioxygenated C-8 prenylated coumarin, 5,6,7-trimethoxy-8-(2′,3′-dihydroxyisopentenyl)-coumarin was isolated from the leaves of *Murraya gleinei* together with the coumarins: meranzin-hydrate, meranzin, murralongin, murrangatin and scopoletin (**WICKRAMARATNE *et al*., 1984**). The ethanolic extract of the fruit of *Murraya omphalocarpa* yielded coumurrayin, mexoticin, 3,5,6,7,3′,4′,5′-heptamethoxyflavone and 5,7-dimethoxy-8-(3′-methyl-2′-oxobutyl) coumarin (**WU *et al*., 1980**).

Essential oils possess antiviral (**HAYASHI *et al.*, 1995**), antioxidant (**DEANS *et al.*, 1992**) and anticancer activities (**HAILAT *et al.*, 1995; ARUNA and SIVARAMAKRISHNAN, 1996**) as well as bactericidal effects (**DEANS and RITCHIE, 1987; DEANS *et al.*, 1992; TASSOU *et al.*, 1995; BARATTA *et al.*, 1998; FYFE *et al*., 1998; MANGENA and MUYIMA, 1999**) and are commonly utilized to enhance skin beauty and treat skin diseases. The use of essential oil is determined by various factors which include chemical, physical, and sensory properties, which differ greatly from one essential oil to the other (**GEDIYA *et al*., 2011**). Essential oils are effective when used topically as they contain very small molecules capable of penetrating through the skin, and subsequently act by using their lipophilic fraction to react with the lipid parts of the skin cell membranes (**ABURJAI and NATSHEH, 2003**).

Aromatic plants and oils have been used for thousands of years, as incense, perfumes, cosmetics, and for their medicinal and culinary applications (**ABURJAI and NATSHEH, 2003**). Essential oils contain volatile and liquid aroma compounds

.

derived from natural sources such as in plants (**GEDIYA *et al*., 2011**). Limonene has been reported to inhibit lipopolysaccharide induced inflammation and inflammatory cell migration (**SOUZA *et al*., 2003**). Limonene has been reported to display strong 5-lipoxygenase inhibitory activity (**BAYLAC and RACINE, 2003**).

MOOLLA (**2006**) investigated the chemical composition of *Agathosma ovata* essential oil and identified 145 compounds in 10 different samples. They all contain a large number of common monoterpenes with very similar compositions and minor quantitative variations (**MOOLLA, 2006**). The similar compounds found in all the samples include: sabinene, *p*-cymene, β-pinene, α-pinene, α-thujene, myrcene, limonene, linalool and terpinen-4-ol (**MOOLLA, 2006**). In all the samples, sabinene was the most dominant component ranging between 25.6% and 44.4% (**MOOLLA, 2006**). Most of the variations in the chemical compositions were associated with flowering and the results obtained revealed that the chemical composition of the essential oil of *A. ovata* is subject to seasonal variation (**MOOLLA, 2006**). The presence of coumarins may be one of the factors responsible for the antimicrobial properties of *A. ovata* (**MOOLLA, 2006**). *Agathosma ovata* varies its secondary metabolite production in relation to the prevailing climatic conditions (**MOOLLA, 2006**).

The essential oils from the leaves of *M. koenigii* yielded 39 compounds with 3-carene (54.22%) as the major compound followed by caryophyllene (9.49%) (**CHOWDHURY *et al*., 2008**). Other notable compounds in the essential oil of *M. koenigii* include; caryophyllene oxide (1.02%), 3-phenylbutyrophe-none(1.15%), cis-sabinenehydrate (1.46%), α-thujene (1.47%), β-elemene (1.92%), γ-elemene (1.96%), α-terpinene (2.39%), allyl(methoxy)dimethylsilane (2.58%), γ-terpinene (2.7%), 4-terpineol (2.8%), α-caryophyllene (2.81%) and β-myrcene (3.2%) (**CHOWDHURY *et al*., 2008**).

The phyto-constituents isolated from the leaves of *M. koenigii* include mahanine (**NARASIMHAN *et al.,* 1970**), koenine, koenigine, koenidine (**NARASIMHAN *et al.,* 1975**), girinimbiol, girinimibine (**ADEBAJO *et al.,* 2006**), koenimbine, O-methyl murrayamine A, O-methyl mahanine, isomahanine, bismahanine, bispyrayafoline (**TACHIBANA *et al.,* 2003**) and coumarin glycosides (scopotin, murrayanine) (**ADEBAJO and REISCH, 2000**). The essential oil from the leaves yielded di-alpha phellandrene, D-pinene, D-sabinene, D-terpinol, dipentene,

.

and caryophyllene (**GOPALAN *et al.,* 1984**). The essential oil of *M. paniculata* leaves from the mountains of the Central Region of Cuba yielded eighteen compounds, accounting for 95.1% of the total constituent with beta-caryophyllene (30%) as the major component (**RODRÍGUEZ *et al*., 2012**).

Essential oil of *M. paniculata* contains 58 compounds with caryophyllene oxide (16.63%), β-caryophyllene (11.81%), spathulenol (10.21%), β-elemene (8.94%), germacrene D(6.95%) and cyclooctene, 4-methylene-6-(1-propenylidene) (6.37%) as the major components (**CHOWDHURY *et al*., 2008**). The other major compounds in the *M. paniculata* essential oil are cyclohexene, 5,6-dietheyl-3-methyl (3.30%), δ-elemene (3.57%), α-cubebene (2.96%), retinal (1%), α-caryophyllene (3.13%), copaene (2.33%), cubenol (2.36%), nerolidyl acetate (1.20%), 12-oxabicyclo(9.1.0)dodeca-3,7-diene,1,5,5,8–tetramethyl (2.07%), eremophilene (1.54%), ledol (2.2%) and aromadendrene oxide (1.47%) (**CHOWDHURY *et al*., 2008**).

Thirty compounds, constituting about 99.7% of the *Haplophyllum tuberculatum* oil were identified and the most abundant compounds were α- and β-phellandrene, limonene, β-ocimene, β-caryophyllene and myrcene (**AL BURTAMANI *et al*., 2005**). Five simple coumarins namely; umbelliferone, scopoletin, 7-isoprenyloxycoumarin, umbelliprenin, and osthenol, and three furanocoumarins; columbianetin, angelicin and psoralen were isolated from *Haplophyllum patavinum* (**FILIPPINI *et al*., 1998**).

The root bark of *Zanthoxylum dipetalum* contained the pyranocoumarins avicennol and xanthoxyletin (**FISH *et al*., 1975**). Five coumarins, 50-methoxyauraptene, 6,50-dimethoxyauraptene, 50-methoxycollinin,7-((20E,50E)-70-methoxy-30,70-dimethylocta-20,50-dienyloxy)coumarin, and 6-methoxy-7-((20E,50E)-70-methoxy-30,70-dimethylocta-20,50-dienyloxy)coumarin have been isolated from the leaves of *Z. avicennae* (**CHO *et al*., 2012**).

Two coumarins, hystrixarin and (+)-hopeyhopin were isolated from the roots of *Citrus hystrix* (**PANTHONG *et al*., 2013**). Two coumarin glycosides 8-(1-β-D-glucopyranosyloxy-1-methylethyl)-8,9-dihydro-2H-furo-[2,3-h]-1-benzopyran-2-one and 8-(3-β-D-glucopyranosyloxy-2-hydroxy-3-methylbutyl)-7-methoxy-2H-1-benzopyran-2-one have been isolated from aqueous extracts of bitter orange flavedo (**MCHALE *et al*., 1987**).

.

2.5.3. Rutaceae species used in traditional medicine for skin related diseases and infections

Carotenoids from citrus fruits help against sun burns and lipid peroxidation in human skin cells induced by UV radiation, among others (**AUST *et al*., 2001**). In a survey of ethnomedicinal plants used against skin diseases in Darjeeling Himalayas, the Rutaceae species were among the plants utilised for skin diseases (**SHARMA, 2013)**. *Citrus aurantifolia*, *C. limonia*, *C. medica* fruits; *Evodia fraxinifolia* fruit and bark; *Zanthoxylum nitidum* leaf, fruit and seed are used for skin diseases by topical application (**SHARMA, 2013)**. Mode of application was topical but in many cases also orally and fresh plants were prevalently used and found to be effective compared to the dried or stored plant materials (**SHARMA, 2013)**. A mixture of a Rutaceae species, *Toddalia asiatica* (leaves), coconut oil and three other plants is applied externally to cure skin diseases among the Kani tribals in southern India (**AYYANAR and IGNACIMUTHU, 2005**).

Clausena dentata leaves made into paste and mixed with turmeric is applied for skin infection (**RAJKUMAR and JEBANESAN, 2010**). Leaves of *Acalypha fruticosa*, *Zanthoxylum chalybeum* and *Suregada zanzibariensis* are pounded together and juice extracted from the mixture is applied to the skin to treat skin diseases (**HEDBERG *et al*., 1983; CHHABRA *et al*., 1991**). *Clausena anisata* decoction is used in the treatment of skin diseases, 50% aqueous ethanol extract exhibited antifungal activity against *Aspergillus niger*, *Microsporum canis* and *Trichophyton mentagrophytes* (**ASWAL *et al*., 1984).**

Clausenol, a carbazole alkaloid isolated from alcoholic extracts exhibited antifungal activity against *Candida albicans* (**CHAKRABORTY *et al*., 1995**). *Vepris louisii* is used as a cure for skin diseases of bacterial origin (**FOCHO *et al*., 2009**). *Calodendrum capense* is popularly used for “African hair and skin” care (**LALL and KISHORE, 2014**). The bark of *C. capense* is utilised traditionally as an ingredient for skin ointment (**VAN WYK and GERICKE, 2000**). The leaves and bark of *C. capense* is used as a facial mask, in soap preparations and for skin-hyperpigmentation problems (**MAPUNYA *et al*., 2012**).

Antifungal activity of ethanol extracts of grapefruit, *Citrus paradisis* seed and pulp was examined against ten yeast strains (**CVETNIC and VLADIMIR, 2004**). The yeasts were sensitive to extract concentrations ranging from 4.13 to 16.50%.

.

Leaves, fruits, stems, bark and roots of a Rutaceae species, *Zanthoxylum americanum* Mill., were investigated for antifungal activity with eleven strains of fungi representing diverse opportunistic and systemic pathogens, including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (**BAFI *et al*., 2005**). All extracts demonstrated a broad spectrum antifungal activity and inhibited at least eight fungal species in a disk diffusion assay (**BAFI *et al*., 2005**). The results provide a pharmacological basis for the very widespread use of this plant in the indigenous North American ethnomedical tradition for conditions related to fungal infections (**BAFI *et al*., 2005**).

The essential oil extracted from the epicarp of *Citrus sinensis* (L.) Osbeck exhibited absolute fungitoxicity against ten post-harvest pathogens (**SHARMA and TRIPATHI, 2006**). Three preparations from the essential oil of *Citrus bergamia* (bergamot natural essence, furocoumarin-free extract and the distilled extract) showed antifungal activity against dermatophytes and yeast pathogens (**VERZERA *et al*., 2003; ROMANO *et al*., 2005; SANGUINETTI *et al*., 2007**).The chemical composition and antifungal activity of the essential oil of *Haplophyllum tuberculatum* was analysed (**AL BURTAMANI *et al*., 2005**). The oil affected the mycelial growth of *Curvularia lunata* and *Fusarium oxysporum* in a dose-dependent manner, but had no effect on the germination of their spores (**AL BURTAMANI *et al*., 2005**). Bioassay-guided fractionation of the hexane/ethyl acetate/water crude extract of the aerial parts of *Haplophyllum sieversii* was performed after preliminary screening data indicated the presence of growth-inhibitory compounds against *Colletotrichum fragariae*, *C. gloeosporioides* and *C. acutatum* (**CANTRELL *et al*., 2005**).

The seeds of *Calodendrum capense* are crushed and boiled to obtain oil that is suitable for soap production (**LALL and KISHORE, 2014**). Oil extracted from Cape Chestnut (*C. capense*) seeds also known as *Yangu* oil (**RAMOROKA and MAPUNYA, 2006**) has natural UV protection, a high content of fatty acids (especially linoleic) and antioxidants (**LALL and KISHORE, 2014**). The main fatty acids present in the oil of *C. capense* are palmitic, oleic, linoleic and stearic acid (**LALL and KISHORE, 2014**). Apart from the crude plant extracts, essential oil derived from various Rutaceae family plants have also been reported to be effective in the treatment of skin diseases.

.

2.5.4. Sources and components of essential oil

Essential oils are produced by blossoms, leaves and fruits of different plants and stored in special tissues such as glandular hairs, oil cells and oil ducts (**WALTERS and ROBERTS, 2008)**. Essential oil is a complex aromatic volatile mixture of several compounds with diverse chemical structures (**WALTERS and ROBERTS, 2008)**. It is generally believed that the biological activity of an essential oil is the result of both its active and inactive substances (**WALTERS and ROBERTS, 2008)**. The inactive components may have an influence on resorption, skin penetration, rate of reaction, or bioavailability of the active compounds while several active components may have a synergistic effect (**WALTERS and ROBERTS, 2008)**.

Many of the substances that make up essential oil are able to penetrate into different layers of the skin and even into the blood stream (**WALTERS and ROBERTS, 2008)**. The extent of this penetration into the skin explains why essential oils often have systemic side effects after topical application on the skin (**WALTERS and ROBERTS, 2008)**. The application of undiluted essential oil on the skin may cause irritation (**WALTERS and ROBERTS, 2008)**.

The components of essential oils are important as their qualitative and quantitative composition determine the characteristics of the oils, which in turn could be responsible for its antimicrobial potentials (**DUGO *et al*., 2000**). Citrus essential oils contains 85-99% volatile and 1-15% non-volatile components (**FISHER and PHILLIPS 2008**). The volatile constituents are composed of a mixture of monoterpene (limonene) and sesquiterpene hydrocarbons and their oxygenated derivatives including: aldehydes (citral), ketones, acids, alcohols (linalool) and esters (**SMITH *et al*., 2001; BORGMANN *et al*., 2004; FLAMINI *et al*., 2007**). Component(s) of essential oils can only be regarded as volatile if there is a loss of weight over a period of time or temperature (**FISHER and PHILLIPS, 2008**).

The activation energies for lemon oil, limonene and linalool are 33.2, 37.87 and 65.64 kJ mol^{-1} respectively (**HAZRA *et al*., 2002**) implying that they are highly volatile especially at temperatures above 25ºC (room temperature) (**FISHER and PHILLIPS, 2008**). Evaporation of the oils results from external factors such as temperature, humidity, concentration and pressure (**AUMO *et al*., 2006**) i.e. the diffusion across a homogenous membrane increases with an increase in

.

temperature (**CLARYS *et al*., 1998**), similar to the evaporation of aldehydes such as citral (**MAKI-ARVELA *et al*., 2006**).

In 1970, various chemical compounds (amounting to 200 in total) had been described from orange oil of which 100 had been identified (**WOLFORD *et al*., 1971**). Monoterpenes constitute 97% of citrus oil composition with alcohols, aldehydes and esters being the lowest percentage components, ranging from 1.8 to 2.2% (**MOUFIDA and MARZOUK, 2003**). The major chemical component of citrus oils is limonene, ranging from 32 to 98%, with sweet orange containing 68 to 98%, lemon 45 to 76% and bergamot 32 to 45% (**SVOBODA and GREENAWAY, 2003**).

2.5.4.1. Scientific methods used for the analysis of antimicrobial activities of essential oils

Various methods have been used to analyse the antimicrobial properties of essential oils. The antimicrobial effect of essential oils is assessed by *in vitro* screening method, usually that of the disc diffusion method which involves the placing of an impregnated (with antimicrobial agent) filter disc on the surface of inoculated agar plate and inhibition of growth is observed (**BAUER *et al*., 1966; DEANS and RITCHIE, 1987; SMITH-PALMER *et al*., 1998; SKANDAMIS and NYCHAS, 2001; WANNISSORN *et al*., 2005; FISHER and PHILLIPS, 2006**). In the late 1950s, this method was adopted for the screening of essential oil vapours by placing the filter disc on the lid of the petri dish (**MARUZZELLA and SICURELLA, 1960**). Another method of assessing antimicrobial activity is to make a well in the inoculated agar and then add the test substance (**DEANS and RITCHIE, 1987; DORMAN and DEANS, 2000**). None of these screening methods is quantitative as they only give an indication of the essential oils having antimicrobial properties (**FISHER and PHILLIPS, 2008**).

The antimicrobial effect of essential oils vapour has been assessed using the direct disc diffusion method and the outcome of the assessment indicated that only the water-soluble components diffused across the agar while the re-deposition of the vapourised components on the surface of the agar accounted for the remainder of the inhibition (**FISHER and PHILLIPS 2008**). The Minimum Inhibitory Concentrations (MICs) of essential oils can be assessed by two protocols (**FISHER and PHILLIPS, 2008**). Visible growth can be observed using the agar dilution method; while optical density, absorbance or viable counts are measured in addition to visible growth

.

observed using the broth dilution method (**BURT, 2004**). The MIC is determined as the lowest concentration at which growth is inhibited. The main problem with determination of the level of antimicrobial activities of essential oils in this way is their hydrophobic nature which makes them insoluble in water-based media (**FISHER and PHILLIPS, 2008**). This problem can be solved by the use of emulsifiers such as Tween 20 or 80 alone or in combination with solvents such as acetone, polyethylene glycol or ethanol (**TASSOU *et al*., 2000; BURT, 2004; FISHER and PHILLIPS, 2006**).

Another factor to consider when determining the MIC of an essential oil is that the absolute concentration of inhibition can be between the lowest MIC and the next concentration in which growth is observed (**FISHER and PHILLIPS, 2008**). Minimum inhibitory doses (MID), which is the minimum dose of the essential oil volatiles to inhibit growth is determined by a parallel method to the MIC (**FISHER and PHILLIPS, 2008**). This is carried out in a sealed container by either placing the essential oil on the surface of the container to give a slow evaporation or an impregnated disc to give a faster evaporation (**INOUYE *et al*., 2003; FISHER and PHILLIPS, 2006**). The incubation temperature can have an effect on the microbial inhibition when using this method as the MID value is dependent on the evaporation rate of the volatile components (**FISHER and PHILLIPS, 2008**). The differences between MIC/MID in studies against the same organism might be as a result of the differences in oil composition, which could have ensued from the regional differences in temperature, season of oil extraction and the different methods of extraction used (**BURT, 2004**).

The evaporation rate of essential oil volatiles can be increased by heating. However, some of the components of the essential oil can be altered which can in turn affect its resulting antimicrobial activities (**FISHER and PHILLIPS, 2008**). The use of water to disperse oils has also been investigated using an air washer and the phenolic components of the oil showed a decreased antimicrobial activity. This is assumed to be as a result of their poor solubility in water (**FISHER and PHILLIPS, 2008**). The water could also reduce their volatility as the essential oil components with hydroxyl groups may be more solvated and thus remain in the water phase (**SATO *et al*., 2006**).

.

Terpenoids are some of the substances in essential oils (**MELLO *et al*., 2003**) and terpene compounds from natural vegetable oils are frequently monoterpenes (approximately 90% of the volatile oil) and sesquiterpenes (**NEGRI *et al*., 2014)**. Other terpenoids, such as diterpenes, are found only in volatile oils (**MELLO *et al*., 2003**). Studies have shown that terpenoids may have antioxidant and antimicrobial properties against pathogenic fungi, including *Candida* and dermatophytes (**HAMMER *et al*., 2000; PAULI, 2006; BARCHIESI *et al*., 2008; PALMEIRA-DE-OLIVEIRA, *et al*., 2009; MARCOS-ARIAS *et al*., 2011**). Studies of three preparations from the essential oil of *Citrus bergamia* (bergamot natural essence, furocoumarin-free extract and the distilled extract) showed antifungal activity against dermatophytes and yeast pathogens (**VERZERA *et al*., 2003; ROMANO *et al*., 2005; SANGUINETTI *et al*., 2007**).

Isolated terpenes should be more thoroughly researched in order to establish the mechanism of their antifungal activity (**NEGRI *et al*., 2014**). The pharmacological, biochemical and therapeutic applications of coumarins are solely dependent on the particular substitution patterns (**MELLO *et al*., 2003**). Bergamot oil is directly obtained from the fruit and consists of a volatile fraction (93%–96%), whose main components are, with approximate percentages, limonene (40%), linalool (8%) and linalyl acetate (28%), and a non-volatile fraction (4%–7%) consisting primarily of coumarins and psoralens (*i.e.*, bergamottin, citroptene, bergaptene *etc*.) (**VERZERA *et al*., 2003; ROMANO *et al*., 2005**). The furocoumarin-free extract (bergaptene-free) and distilled extract (absolutely devoid of non-volatile residues) showed more activity than the natural essence against all of the species tested (**SANGUINETTI *et al*., 2007**).

It is recommended that essential oil should only be used in diluted forms for external application (**CLARKE, 2008; WALTERS and ROBERTS, 2008)**. Essential oils differ in their biological activities against various microbial species (**WALTERS and ROBERTS, 2008)**. The variation in the susceptibility of bacteria to different essential oils may be due to the variable chemical composition, variation in the cell wall structure, lipid and protein composition of the cytoplasmic membrane (**WALTERS and ROBERTS, 2008).** The most common application of essential oils is topical application to the skin or mucous membranes with direct skin contact in the form of perfumes, cosmetic fragrances and pharmaceuticals (**WALTERS and**

.

ROBERTS, 2008). Topical application of essential oils results in clinical side effects such as allergic contact dermatitis, skin and mucous membrane irritation, hyperpigmentation and cytotoxic effects (**DE GROOT and FROSCH, 1997; AHMED, 2001).**

In order to benefit from the therapeutic activity of essential oil and avoid its side effects, it must be used with caution and in diluted form. Several essential oil have antifungal activities and can be used to treat skin diseases of fungal origin because of their notable antifungal activities and pleasant flavours (**RAI and MARES, 2003)**. The Rutaceae plant species are known for their scents and their use in African traditional medicine systems for the treatment of skin diseases. A variety of extracts, tinctures and oil from Rutaceae plant species have been incorporated into hair and skin care products. Essential oil and plant extracts have been incorporated into some emollients and ointments used in the treatment of skin inflammation and oxidative stress which arise from the manifestation and pathogenesis of skin diseases.

2.6. Pathogenesis of skin diseases

The skin offers an efficient permeability barrier system and protects the body from microbial invasion and oxidative stress (**SCHAFER *et al.,* 2012)**. The dysfunction in the epidermal barrier system is a major contributory factor in the physio-pathogenesis of skin diseases, most especially in AD (**RAMOS-E-SILVA, and JACQUES, 2012**). A prolonged topical steroid therapy on the skin increases the Transepidermal Water Loss (TEWL) (**RAMOS-E-SILVA, and JACQUES, 2012**). Damage to the skin epidermal barrier function is caused by changes in skin permeability as a result of a reduction in the corneal thickness, depletion of the intercorneocyte lipid matrix, and a decrease in the number of lamellar bodies in the granular layer (**SHEU *et al*., 1997; SHEU *et al*., 1998**). A deeper knowledge of the mechanisms that disrupts the skin epidermal barrier system can give impetus to the development of therapies that will improve the quality of life of individuals with skin diseases by providing a healthier skin (**RAMOS-E-SILVA, and JACQUES, 2012**).

The skin is a continuously self-renewing organ, serving as a cover for the body surface and separating it from the outside world, providing protection against external agents such as mechanical and chemical insults, heat, infections, water,

.

and electromagnetic radiation (**BARONI *et al.,* 2012**). The skin is divided into two major structural compartments: the epidermis and the in-depth dermis or connective component of nutrition (**BARONI *et al.,* 2012**). The epidermis prevents the loss of water and other components of the body to the environment (inside–outside barrier) and protects the body from a various environmental insults (outside–inside barrier) (**BARONI *et al.,* 2012**). The stratum corneum which is the outermost layer of the epidermis provides a permeability barrier that prevents skin desiccation (**WERTZ, 2000; MADISON, 2003**).

The movement of water from the stratum corneum into the outer environs is referred to as transepidermal water loss (TEWL). Measurements of TEWL may be a useful parameter for the identification of skin damage caused by certain chemicals, and pathologic conditions such as eczema, because the rates of TEWL increase in proportion to the level of skin damage (**BARONI *et al.,* 2012**). The intercellular space of the stratum corneum is composed of a unique mixture of lipids (**BARONI *et al*., 2012**) whose function is to counteract the loss of water and salts from the skin and the penetration of water-soluble substances and is thus important in the protection of the skin (**WERTZ and VAN DEN BERGH, 1988; BOUWSTRA *et al.,* 2006**). The function of cholesterol in the epidermal barrier system could be the provision of a degree of fluidity and flexibility to what could otherwise be a rigid and fragile membrane system (**BARONI *et al.,* 2012**).

TEWL is influenced by the eccrine sweat gland activity and the stratum corneum thickness, and the highest TEWL values are observed on the palms, soles, and forehead (**PINNAGODA *et al.,* 1990**). HIV negative individuals have some degree of dysfunction in the epidermal barrier system, with an increase in TEWL regardless of antiretroviral therapy and even in the absence of xerosis (**GUNATHILAKE *et al.,* 2010**). As a result of HIV infection, there is an increase in the production of Th2 (T helper type 2) cytokines which can lead to an increased cutaneous permeability by a decrease in the production of ceramides and changes in epidermal differentiation (**GUNATHILAKE *et al.,* 2010**).

The main components of the epidermal layer of the skin are keratinocytes, a few Langerhans cells which are specialized dendritic cells and some melanocytes (**FELDMEYER *et al.,* 2010**). The keratinocytes are responsible for the production of different keratins that generate the toughness of the epidermis (**FUCHS and**

.

RAGHAVAN, 2002). Different types of immune cells such as macrophages, T-cells and mast cells are implanted in the connective tissue of the underlying dermis (**NESTLE *et al.,* 2009**). The connective tissue is made up of an extracellular matrix which is produced and secreted by fibroblasts, the major cell type of the dermis (**SORRELL and CAPLAN, 2004**). Under homeostatic conditions, the surface of the skin is inhabited by a variety of microorganisms (**WAGENER *et al.,* 2013**).

However, a dynamic, healthy balance between the epidermis and the micro organismal population is controlled by the production of antibiotic and antifungal compounds by dermal sebocytes and the microorganisms themselves (**WAGENER *et al.,* 2013**). In addition, keratinocytes produce antibacterial substances after infection or injury (**GLASER *et al.,* 2005**). Some of the keratinocyte-produced antimicrobics and cytokines influence the immunological properties of dendritic cells and T-cells (**NESTLE *et al.,* 2009**; **FELDMEYER *et al.,* 2010**). Hence, the skin creates a balance between ensuring a competent pathogen defense and immunosurveillance and to reduce excessive immune responses that can result to a disease (**CONTASSOT *et al.,* 2012**).

Excess reactive oxygen species produce pathological changes in cells and tissues such as in inflammatory skin conditions (**WAGENER *et al.,* 2013**). Inflammatory skin diseases range from severe rashes which involve itching and redness to chronic conditions such as dermatitis often referred to as eczema (**WAGENER *et al.,* 2013**). Chronic skin inflammation results from a continuous, inflammatory response which negatively affects skin health (**WAGENER *et al.,* 2013**). Ordinarily, inflammation of the skin is a beneficial and protective process which occurs after injury or infection (**NESTLE *et al.,* 2009**; **FELDMEYER *et al.,* 2010**). The skin can however be subjected to excessive inflammatory responses which lead to chronic inflammation, auto-inflammation and auto-immunity (**NESTLE *et al.,* 2009**).

A continuous release of excess ROS in the skin can intensify inflammatory injury and promote chronic inflammation (**WAGENER *et al.,* 2013**). The cellular redox balance is controlled by several (enzymatic) antioxidants and pro-oxidants; and in the case of chronic inflammation, the antioxidant system may be depleted, and this subsequently leads to prolonged oxidative stress (**WAGENER *et al.,* 2013**). Oxidative stress is an imbalance in the production/inactivation of ROS and causes

.

damage of biological molecules (such as DNA, carbohydrates, lipids, and proteins) and release of inflammatory cytokines (**BRIGANTI and PICARDO, 2003; BICKERS and ATHAR, 2006**). Lipid peroxidation (LPO) is deleterious as it leads to a diffuse spread of free radical reactions (**CATALÁ, 2009**). Membrane lipids, especially phospholipids containing polyunsaturated fatty acids (PUFAs) (**DEIGNER and HERMETTER, 2008**), are particularly prone to peroxidation as a result of their association within the cell membrane with enzymatic and non-enzymatic systems generating pro-oxidative free radical species (**BRACONI *et al.,* 2010**).

Under various pathological conditions, an oxidative cascade may be generated which can induce cytotoxicity and apoptosis and may have a significant role in inflammation, enhancing the release of cytokines and modifying lipoproteins to pro-inflammatory forms (**GUTTERIDGE, 1988; DEIGNER and HERMETTER, 2008**). LPO products react with sugars, proteins, and DNA (**NIKI, 2009**). The end products of LPO, such as malondialdehyde, 4-hydroxy-2-nonenal (4-HNE), and 4-hydroxy-2-hexanal (4-HEE) can damage proteins by reacting with various amino acids both *in vivo* and *in vitro* (**CATALÁ, 2009**). Oxidative stress causing the production of lipid peroxides may thus be a major factor in the development of skin inflammatory diseases (**BRACONI *et al.,* 2010; SIVARANJANI *et al*., 2013**).

Generally, it is recognized that a combination of environmental factors such as allergens, microbial infection and genetic factors induce the multiple immunologic and inflammatory responses seen in AD patients (**LÜ *et al.,* 2009**). Atopic dermatitis is associated with an impaired oxidative status, and systemic alterations in antioxidant patterns of the skin have been found in involved skin of AD patients as well as in uninvolved non-lesioned skin as an adaptive response to chronic inflammation of the epidermis (**BRIGANTI and PICARDO, 2003**). Filament aggregating protein (filaggrin, FLG) is a key protein that plays a major role in the formation of the cornified cell envelope, which is crucial for an effective skin barrier (**CANDI *et al.,* 2005**). Studies have shown that loss-of-function (null) mutations in the gene encoding FLG leads to skin barrier impairment in AD (**MARENHOLZ *et al.,* 2006; PALMER *et al.,* 2006; WEIDINGER *et al.,* 2006**). Many AD patients acquire a deficiency of filaggrin with subsequent barrier disruption as a result of the local inflammatory immune response (**HOWELL *et al.,* 2009**).

.

Free radicals mediate lipid peroxidation, which is considered to be a major mechanism involved in cell membrane destruction and cell damage (**SEN, 1995; MITCHELL and COTRAN, 2003**). The skin barrier function of the skin occurs almost entirely in the epidermis, most especially in its superficial layer, the stratum corneum (**KALIA *et al.,* 1996**). The stratum corneum (SC) consists of a protein component (the corneocyte) which provides mechanical resistance to the SC and a medium for the extracellular matrix of the SC which is the second component (**DARLENSKI and FLUHR, 2012**). The extracellular matrix consists of multiple layers of lipids (mortar) which functions to mediate the permeability barrier against excess water and electrolyte loss (**DARLENSKI and FLUHR, 2012**).

Hence, a combination of events such as transepidermal water loss, disruption of the skin epidermal barrier system, oxidative stress, inflammation and ultimately, invasion by microbial pathogens are responsible for the manifestation of skin diseases. In view of this, an effective antifungal should encompass all the activities needed to restore skin health to normalcy. As a result of the principal role of ROS in inflammatory pathologies, the restoration of redox balance forms a ground-breaking therapeutic target in the development of new approaches for the treatment of inflammatory skin conditions (**WAGENER *et al.,* 2013**). Nrf2 links antioxidant defense mechanisms in the epidermis with the control of skin permeability barrier and antimicrobial defense (**SCHAFER *et al.,* 2012**). The production of ROS and ROS-induced oxidation results in the disruption of the skin barrier (**THIELE, 2001**).

This implicates that restoration of redox balance through the use of effective antioxidant formulations in combination with an effective antifungal could ameliorate skin inflammation, reduce transepidermal water loss and thus restore the skin barrier function and prevent further invasion by microbial pathogens. Eight Rutaceae species were chosen for this study based on the reports of their use in African traditional medicinal systems and the scientific reports of their *in vitro* activities against skin diseases of fungal origin. They are: *Agathosma betulina* (essential oil), *A. mucronulata*, *A. ovata*, *Coleonema album*, *C. pulchellum*, *Calodendrum capense*, *Murraya koenigii* and *M. paniculata*. Herbarium specimens have been deposited in the University of KwaZulu-Natal (Pietermaritzburg) herbarium. Three fungal strains: *Trichophyton rubrum* (ATCC 28188), *T. mentagrophytes* (ATCC 9533) and

.

Microsporum gypseum (ATCC 24102) were chosen for this study based on the reports of the fungus linked to skin diseases in Africa.

.

CHAPTER 3. ANTIOXIDANT ACTIVITY OF SEVEN RUTACEAE PLANT LEAF EXTRACTS

3.1. Introduction

Oxidative stress is the underlying mechanism of various diseases, and convincing evidence has been produced to demonstrate a link between oxidative stress (especially lipid peroxidation) and a variety of inflammatory dermatologic conditions which include atopic dermatitis (AD) (**BANG *et al*., 2001; BRIGANTI and PICARDO, 2003; BICKERS and ATHAR, 2006; BRACONI *et al.,* 2010; SIVARANJANI *et al*., 2013**). Oxidative stress causes the production of lipid peroxides which is a major factor in the development of skin inflammatory diseases (**BRACONI *et al.,* 2010; SIVARANJANI *et al*., 2013**).

There is increasing interest in the use and quantification of antioxidant capacity in the pharmaceutical and cosmetic industries (**OU *et al.,* 2001**). This interest results from the enormous evidence of the importance of reactive oxygen/nitrogen species (ROS/RON) in aging and pathogenesis of diseases (**HALLIWELL and ARUOMA, 1991; WINK *et al*., 1991; NGUYEN *et al*., 1992; STADTMAN, 1994**). The skin is a major target of oxidative stress from reactive oxygen species (ROS) which originate from the environment and the skin itself (**TROUBA *et al*., 2002**). An increase in the amount of the oxidants in the body, exceeding the antioxidant defence system capacity is referred to as oxidative stress which leads to chronic inflammation, and can subsequently cause collagen fragmentation and the disorganization of skin cell functions, and thus contribute to the manifestation of skin diseases (**KRUK and DUCHNIK, 2014**).

Therapeutic approaches with the potential ability to restore antioxidant levels in the skin could be achieved by: reducing the ROS production, increasing the endogenous antioxidant enzymatic defences, and enhancing the non-enzymatic antioxidant defence system through dietary or pharmacological approaches (**WAGENER *et al*., 2013**). Preparations composed of natural compounds have been used for centuries for the treatment of skin conditions **(ABURJAI and NATSHEH, 2003)** and has been evaluated for several pharmacological activities, including antioxidant activity (**LI *et al*., 1988; ROUT *et al*., 2007; SHARKER and SHAHID, 2009**).

.

CAO *et al.,* (**1993**) developed a method called oxygen radical absorbance capacity (ORAC). This method is used to measure antioxidant scavenging activity against the peroxyl radicals induced by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) at 37°C (**CAO *et al.,* 1993; CAO *et al.,* 1995**). In this method of antioxidant capacity evaluation, B-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum* was the chosen fluorescent probe and the assay offers exceptional assessment in which the time and degree of inhibition are measured as the reaction goes to completion (**OU *et al.,* 2001**).

However, the $ORAC^{PE}$ has some limitations which include the use of B-PE as the probe which gives inconsistency from lot to lot and leads to variations in reactivity to peroxyl radicals (**CAO and PRIOR, 1999**). Furthermore, B-PE is not photo-stable and it can be photo-bleached due to its photo-instability; it interacts with polyphenols; lack of accessibility due to the rare availability of the FARA COBAS II analyser and more importantly, 75% of the cost of $ORAC^{PE}$ is for B-PE (**OU *et al.,* 2001**). Hence, in terms of the criteria for method validation and cost-effectiveness, B-PE is ineffective as a fluorescent probe and these shortcomings prompted the validation of a stable fluorescent probe to substitute B-PE by **OU *et al.,*** (**2001**). Researchers used fluorescein (FL) (3′,6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) as a fluorescent probe and this improved $ORAC^{FL}$ method offers a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radicals. Contrary to PE, FL and its derivatives are frequently utilised fluorescent probes in the labelling and sensing of biomolecules (**SRUTKOWSKA *et al.,* 1999**).

Several species of the Rutaceae have been analysed for antioxidant activities and the remarkable activities displayed by several species of the family has been attributed to the presence of secondary metabolites such as coumarins and terpenes which exist in abundance in the family. Members of the family are also known to have oil glands on the reverse side of their leaves and thus offer a source of essential oil which has been incorporated into formulations/creams/lotions. These contain active ingredients with therapeutic effects against skin complaints which include skin inflammations **(YANG *et al.,* 2009; WAGH *et al.,* 2015)** which result from oxidative stress during the pathogenesis of skin diseases and itching **(KAPOOR, 2005; KAMAL *et al.,* 2011)**.

.

Natural antioxidants, such as essential oils seem to have the potential to offer considerable protection against oxidative skin damage (**RHIND, 2012)**. Several essential oils have anti-oxidant and anti-inflammatory activities, and some appear to be significant specifically in relation to the skin as there's a possibility that anti-oxidant activity is linked with keratinocyte differentiation, and hence barrier function and texture (**RHIND, 2012)**. Essential oils have been reported to have anti-inflammatory activities and their constituents such as terpene hydrocarbons, sesquiterpene hydrocarbons and sesquiterpene alcohols have been reported to display 5-lipoxygenase inhibitory activity (**ALEXANDER, 2001; BAYLAC and RACINE, 2003**). Anti-oxidant activity is one of the major and most important biological activities attributed to essential oils and is at the root of many other properties which include anti-inflammatory activity (**RHIND, 2012)**. However, evaluation of the antioxidant activity of essential oil could be problematic as a result of the hydrophobic nature of the essential oil which could lead to inaccurate and inconsistent results. The aim of this study was to investigate the antioxidant activity of leaf extracts of *Agathosma mucronulata*, *A. ovata*, *Calodendrum capense*, *Coleonema album*, *C. pulchellum*, *Murraya koenigii* and *M. paniculata*.

3.2. Materials and methods

Fresh plant materials (leaves) were sourced from the South African National Biodiversity Institute, Kirstenbosch. The sample was freeze-dried, ground into powders and stored in air-tight containers at 23±1ºC. 100 mg of the sample was dissolved in 1ml of 80% methanol and sonicated for 30 mins. It was subsequently filtered using a syringe filter. The antioxidant activity of the leaf extracts was determined using the ORAC method according to **OU *et al*.** (**2001**). Briefly, 100 µL of 500 nM fluorescein and 25 µL of diluted extracts were pipetted into each working well of a microplate preincubated at 37°C. Then 25 µL of 250mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was added and the microplate was shaken for 5 s on a shaker. The fluorescence (Ex. 485 nm, Em. 510 nm) was read every 3 min for 90 min. Net area under the curve was used to calculate antioxidant capacity which was expressed as trolox equivalents.

.

3.3. Results

The antioxidant activity expressed as trolox equivalents (µmol trolox equivalents/g) of *C. album* and *C. pulchellum* leaf extracts are recorded in Table 3.1 The leaf extracts of *C. pulchellum* had a higher trolox equivalent value (**1126.7 ± 68.1 µmol Trolox Equivalents/g**) compared to that of its sister species *C. album* (**942.2 ± 34.9 µmol Trolox Equivalents/g**). This indicates that *C. pulchellum* has a higher antioxidant activity than *C. album*. The leaf extract of *M. koenigii* displayed moderate antioxidant activity (**339.8±27.7 µmol Trolox Equivalents/g**). However, *M. paniculata* displayed a much higher antioxidant activity (**930.7±67.7 µmol Trolox Equivalents/g**) more than double of what was detected for *M. koenigii* leaf extract. The two *Agathosma species* have remarkable antioxidant activity. The trolox equivalents of *A. ovata* recorded is particularly noteworthy. The values of the ORAC are **1045.5±48.8** and **1202.5±92.0 µmol Trolox Equivalents/g** for *A. mucronulata* and *A. ovata* respectively. *Calodendrum capense* leaf extract displayed moderate (**301.7±19.1 µmol Trolox Equivalents/g**) antioxidant activity.

.

Table 3.1: ORAC^{FL} values of plant leaf extracts expressed as Trolox Equivalents

	µmol Trolox Equivalents/g
Agathosma mucronulata	1045.5±48.8
Agathosma ovata	1202.5±92.0
Calodendrum capense	301.7±19.1
Coleonema album	942.2 ± 34.9
Coleonema pulchellum	1126.7 ± 68.1
Murraya koenigii	339.8±27.7
Murraya paniculata	930.7±67.7

.

3.4. Discussion

There is a growing interest toward antioxidants of natural origin (**LARSON, 1988; GAZZANI *et al*., 1998; VELIOGLU *et al*., 1998**) and increasing evidence highlights a connection between oxidative stress with several human chronic diseases including inflammation and skin diseases (**HALLIWELL *et al*., 1992**). The involvement of ROS in the pathogenesis of atopic dermatitis (eczema) has been suggested (**CHEESEMAN and SLATER, 1993**). Oxidative damage of any cellular constituent can be a contributory factor to the development of diseases (**SIES, 1991; DARR and FRIDOVICH, 1994; DREHER and JUNOD, 1996; WISEMAN and HALLIWELL, 1996; HALLIWELL and GUTTERIDGE, 1999; DURACKOWA, 2010**). Hence, the use of natural antioxidant could help to achieve an effective treatment against several diseases, including skin diseases.

According to **BASKARAN *et al*.,** (**2014**), *C. pulchellum* contains biologically potent therapeutic phytochemicals with high antibacterial and antioxidant activities. The result of this study support the report of **BASKARAN *et al*.,** (**2014**) as the leaf extracts of *C. pulchellum* displayed a remarkable antioxidant activity. The fact that *C. pulchellum* leaf extract displayed a higher antioxidant activity compared to *C. album* extract is noteworthy. Caryophyllene and limonene have been reported to display strong 5-lipoxygenase inhibitory activity (**BAYLAC and RACINE, 2003**). *Coleonema album* extracts possessed significant *in vitro* antioxidant activity (**ESTERHUIZEN *et al*., 2006b**).

In this study, *C. pulchellum* leaf extracts gave higher antioxidant activity compared to the *C. album* extract. The antioxidant activity in an array of different natural sources is often attributed to coumarins (**MOURE *et al*., 2001; HEIM *et al*., 2002**). The presence of coumarins and various structural derivatives in the genus *Coleonema* have been reported in previous investigations (**DREYER *et al*., 1972; GRAY, 1981**). Coumarins and flavonoids are phenolic compounds and have been demonstrated to have potent antioxidant activities as a result of their phenolic hydroxyl groups (**DEMMIG-ADAMS and ADAMS, 2002**) and a suitable structural chemistry for free radical scavenging activity (**RICE-EVANS and BURDON, 1994**). The phenolics have received increased attention as a result of the various scientific reports indicating a correlation between consumption of food and beverages rich in phenolics and reduced incidence of diseases linked to oxidative stress (**RICE-**

.

EVANS and BURDON, 1994; MARTINEZ-CAYUELA, 1995; HEINECKE, 1998; CHISOLM and STEINBERG, 2000).

KRUK and DUCHNIK (**2014**) recommended that the redox balance should be considered in the development of new antioxidant strategies connected to the prevention and treatment of skin diseases. A relevant contribution (>1 mmol) to the total intake of plant antioxidants can be achieved by consuming a normal diet of culinary herbs containing a very high concentration of antioxidants and thus be a good source of dietary antioxidants (**DRAGLAND *et al.,* 2003**). The plants *M. koenigii* and *M. paniculata* could offer a natural source of antioxidant as a result of the good antioxidant activities they both displayed in this study. Dietary and naturally occurring antioxidants such as flavonoids, carotenoids, and several vitamins have been shown to support skin health and rejuvenation (**SCHAGEN *et al*., 2012**). These flavonoids, carotenes, vitamins and other curative bio-compounds beneficial to skin health can be derived from spices, skin ointments, antifungal formulations from natural origin and cosmetics.

The epidemiological and *in vitro* studies of medicinal plants strongly support the fact that plant constituents with antioxidant activity have the ability to exert protective effects against oxidative stress in biological systems (**BLOCK and PATTERSON, 1992; CAO *et al.,* 1996; NESS and POWLES, 1997; EASTWOOD, 1999**). Many herbs are composed of antioxidant compounds which shields the cells against the harmful effects of reactive oxygen species (**NARAYANASWAMY and BALAKRISHNAN, 2011**). A lot of aromatic, medicinal, spice and other plants are composed of chemical compounds that exhibit antioxidant properties (**VEERU *et al.,* 2009)**. All the plants used in this study have the capacity to shield the skin from oxidative stress as they displayed good antioxidant activity.

Although the body is safeguarded by natural antioxidant defence system, there is a continuous demand for antioxidants from natural sources (**RIMBACH *et al.,* 2005**). Free radicals formed within the body react with various biological molecules such as lipids, proteins and deoxyribonucleic acids resulting in the imbalance between oxidants and antioxidants (**NARAYANASWAMY and BALAKRISHNAN, 2011**). Oxidative stress in atopic dermatitis is usually caused by an increase in lipid peroxidation and decreased levels of antioxidants (**SIVARANJANI *et al*., 2013**). Antioxidants are known to inhibit lipid peroxidation

.

through the inactivation of lipoxygenase in order to scavenge free radicals and active oxygen species (**SUDARAJAN *et al*., 2006**). Among the several naturally occurring antioxidants; ascorbic acid, carotenoids and phenolic compounds are more effective than other compounds (**DUH *et al*., 1999**). They are commonly referred to as radical scavengers, reducing agents, metal chelators, singlet oxygen quenchers, and hydrogen donors (**PROESTOS *et al.,* 2006**).

According to **MOOLLA *et al.,*** (**2007**), *A. ovata* (round-leaf) displayed a weak anti-oxidant activity, with IC_{50} values > 100 µg/ml using the DPPH assay. The report of **MOOLLA *et al.,*** (**2007**) contradicts the result of the antioxidant activity of *A. ovata* in this study. This could be as a result of the inconsistencies of the DPPH method which has been reported in scientific literatures to have various shortcomings (**BRAND-WILLIAMS *et al.,* 1995; ANCEREWICZ *et al.,* 1998; MIN, 1998; SANCHEZ-MORENO *et al.,* 1998; ARNAO, 2000; PRIOR *et al.,* 2005**). In this study, the ORAC method which is presently one of the best methods for the analysis of antioxidant activity was adopted. *Agathosma* species popularly referred to as '*Buchu*' in the South African traditional medicine; are rich in flavonoids (**MOOLLA, 2006**) which have been extensively reported to have anti-oxidant activities (**YAO *et al*., 2004**). The antioxidant activity displayed by leaf extracts of *A. ovata* and *A. mucronulata* in this study supports the reports of **YAO *et al*** (**2004**).

3.5. Conclusions

All seven Rutaceae species examined displayed antioxidant activity though the degree varies from plant to plant even among plants of the same genera. The antioxidant activity of *C. pulchellum* is particularly noteworthy as its antioxidant activity is higher than that of *C. album* which is popularly utilised in South African traditional medicine. The plant *A. mucronulata* which has not been reported to be utilised for medicinal purposed also displayed a good antioxidant activity. The antioxidant activity displayed by the plants used in this study make them potential sources for combating oxidative stress involved in the pathogenesis of skin diseases. It is therefore necessary to investigate these plants for antifungal activity against fungal strains associated with ringworm/eczema since they have shown promising antioxidant activities.

.

CHAPTER 4. ANTIFUNGAL ACTIVITY OF SEVEN RUTACEAE PLANT LEAF EXTRACTS AGAINST *TRICHOPHYTON RUBRUM*, *T. MENTAGROPHYTES* AND *MICROSPORUM GYPSEUM*

4.1. Introduction

Preparations composed of natural compounds have been used for centuries for the treatment of skin conditions and several dermatological disorders such as inflammation, photo-toxicity, psoriasis, atopic dermatitis and alopecia areata (**ABURJAI and NATSHEH, 2003**). It has been evaluated for pharmacological activities such as immunoreactivity, antioxidant, antimicrobial, anti-inflammatory, and antifungal (**LI *et al*., 1988; ROUT *et al*., 2007; SHARKER and SHAHID, 2009**).

Skin diseases have become a global burden especially in Africa where the incidence of skin diseases is worsened as a result of inadequate number of dermatologist and adverse weather condition which supports the manifestation of these diseases and growth of the fungi responsible. The increasing incidence of HIV/AIDS has escalated the occurrence of skin diseases in Africa. In the last thirty years, there has been constant progression of mycoses in immunocompromised patients (**DI SILVERIO *et al.,* 1991**). Most of the fungi responsible for the mycoses are dermatophytes, mainly *Trichophyton mentagrophytes var. mentagrophytes*, *T. mentagrophytes var. interdigitale* and *T. rubrum* (**ANDRÉ and ACHTEN, 1987; EVANS, 1990; MIDGLEY *et al.,* 1994**). The fungi *T. rubrum* causes various superficial infections, which accounts for at least 60 % of dermatophytosis, such as ringworm of the scalp and hair, hand, glabrous skin, nail infections and athlete's foot (**GRÄSER *et al*., 2000**).

In different cultures in Africa and across the world, various plants are used to enhance beauty and treat various skin diseases which include ringworm/eczema. **GEDIYA *et al*.,** (**2011**) identified several plants that are used traditionally to cure skin diseases and as cosmetics and discovered some of the plants have dual use as cosmetics and as cures for skin diseases. *Citrus* a genus of the Rutaceae is popularly known to be often used in the production of skin beauty products and also used in the treatment of skin diseases. **ADEOGUN *et al.,*** (**2014**) found that *Citrus aurantifolia* and *C. medica* leaves/fruits are used for the treatment of skin disease in an ethnobotanical survey of medicinal plants used in the treatment of skin diseases

.

in Abeokuta South Local Government of Ogun State Nigeria. Apart from plants of the genus *Citrus*, other plants from the Rutaceae family are utilized in traditional medicinal systems for the treatment of skin diseases. The aim and objective of this part of the study was to investigate the antifungal activity of the leaf extracts of *Calodendrum capense*, *Coleonema album*, *C. pulchellum*, *Murraya koenigii* and *M. paniculata* against *T. rubrum*, *T. mentagrophytes* and *Microsporum gypseum*.

4.2. Materials and methods

Fresh plant materials (leaves) were sourced from the University of KwaZulu-Natal Botanical Garden. The leaves were dried in an incubator at 40ºC for four days. Dried leaves were ground into powders and stored in air-tight containers at 23±1ºC in the dark. Known quantities of ground leaf samples were extracted non-sequentially using 100% petroleum ether, acetone, methanol, ethanol and water. Different fractions were filtered using a Buchner filter and evaporated to dryness in a vacuum concentrator at 30ºC. The extracts were poured into pill vials, left to dry under a fan at room temperature and stored in a sterile container in a walk-in fridge before use. The dried extracts (25 mg each) were weighed into separate Eppendorfs and dissolved in 1 ml of 70% ethanol, sonicated and then used for antifungal bioassays. *Trichophyton rubrum* (ATCC 28188), *T. mentagrophytes* (ATCC 9533) and *Microsporum gypseum* (ATCC 24102) were purchased from Quantum Biotechnologies, Randburg, Republic of South Africa. They were grown on yeast malt agar (Y3127). A small portion of a seven-day-old culture was scraped with a scapel into McCartney bottles containing yeast malt broth (Sigma Y3752) and incubated at 37ºC for 2 (*M. gypseum*) and 7 days (*T. rubrum* and *T. mentagrophytes*) respectively. The suspension was adjusted using a spectrophotometer to an absorbence of between 0.25 and 0.28 at 530 nm. The fungal inoculum was prepared with $1x10^6$ CFU (Colony Forming Units) in the suspension. Susceptibility testing was carried out using the micro-dilution method. One hundred microliters of distilled water were pipetted into each of the 96 wells in a microtitre plate and a hundred microliters of the diluted leaf extract were added to the 1^{st} well of the column and then serially diluted down the wells. One hundred microliters of the diluted fungal suspension were added to each of the 96 wells. The microtitre plates were subsequently incubated at 37ºC for 48 h after which 50 µl of *p*-iodonitrotetrazolium violet (INT)

.

(Sigma 58030-1G-F) indicator was added to each well to determine growth inhibition. The microtitre wells were observed 30 min to 1 h after the indicator was added. The lowest concentration of clear wells was regarded as the minimum inhibitory concentration (MIC) values. 50 µl of broth was added to each well immediately after the readings were taken. The microtitre wells were incubated at 37ºC for 48 h and then observed in order to determine the minimum fungicidal concentration (MFC). Ketoconazole and griseofulvin (1 mg/ml each) were used as positive control. The assay was repeated at least three times in order to ensure consistency of results.

4.3. Results

Antifungal activity of the leaf extracts is expressed in mg/ml and recorded in Table 4.1. MIC values ≤1 mg/ml is regarded as good; MIC ≤0.5 mg/ml is regarded as remarkable and MIC≤1.5 mg/ml is regarded as moderate. Methanol extract (**0.195 mg/ml**) of *C. pulchellum* and ethanol extract of *M. koenigii* (**0.391 mg/ml**) displayed remarkable antifungal activity against *T. rubrum.* Extracts with good antifungal activities against *T. rubrum* are: *C. album* acetone and methanol extracts (**0.781 mg/ml**); *M. koenigii* acetone and methanol extracts (**0.781 mg/ml**); *M. paniculata* acetone extract (**0.781 mg/ml**); and *C. capense* methanol and ethanol extracts (**1.172 mg/ml**). Methanol extracts of *C. album* and *C. pulchellum* (**0.391 mg/ml)** exhibited the best antifungal activity against *T. mentagrophytes.* Plant extracts with good antifungal activities against *T. mentagrophytes* are*: M. koenigii* P.E extract (**0.781 mg/ml**); *C. capense* methanol extract (**0.781 mg/ml**); and *M. paniculata* P.E and ethanol extracts (**1.022 mg/ml**).

Coleonema pulchellum (**0.049 mg/ml**) and *C. album* methanol (**0.195 mg/ml**) extracts gave the best antifungal activity against *M. gypseum* (Table 4.2). Extracts with good antifungal activities against *M. gypseum* are: *C. album* acetone and ethanol extracts (**0.390 mg/ml**); *M. koenigii* ethanol (**0.391 mg/ml**), methanol (**0.391 mg/ml**), acetone (**0.586 mg/ml**) and P.E (**0.781 mg/ml**) extracts; *C. pulchellum* acetone and ethanol extracts (**0.781 mg/ml**); *C. capense* acetone, methanol and ethanol extracts (**0.781 mg/ml**). A total of 16 extracts out of the 20 extracts from the five plants inhibited the growth of at least one fungal strain with MIC values ranging from **0.049 mg/ml** to **1.022 mg/ml**. The water extracts of all the plants tested gave

.

very poor antifungal activity (results not shown). All the extracts tested displayed fungistatic activity against *T. rubrum*, *T. mentagrophytes* and *M. gypseum*.

.

Table 4.1: Antifungal activity (MIC values expressed as mg/ml) of leaf extracts of C. album, C. pulchellum, M. koenigii, M. paniculata and C. capense against T. rubrum and T. mentagrophytes

Plants	*Trichophyton rubrum*								*Trichophyton mentagrophytes*							
	P. E MIC mg/ml	P. E MFC mg/ml	Acetone MIC mg/ml	Acetone MFC mg/ml	Methanol MIC mg/ml	Methanol MFC mg/ml	Ethanol MIC mg/ml	Ethanol MFC mg/ml	P. E MIC mg/ml	P. E MFC mg/ml	Acetone MIC mg/ml	Acetone MFC mg/ml	Methanol MIC mg/ml	Methanol MFC mg/ml	Ethanol MIC mg/ml	Ethanol MFC mg/ml
C. album	3.125	6.250	**0.781**	6.250	**0.781**	3.125	1.563	3.125	3.125	6.250	**1.172**	3.25	**0.391**	3.125	1.563	6.250
C. pulchellum	3.125	6.250	3.125	3.125	**0.195**	3.125	1.563	6.250	6.25	6.250	3.125	6.250	**0.391**	6.250	1.563	3.125
Murraya koenigii	1.563	6.250	**0.781**	6.250	**0.781**	6.250	**0.391**	6.250	**0.781**	6.250	6.250	6.250	3.125	6.250	6.250	6.250
Murraya paniculata	1.563	6.250	**0.781**	6.250	1.563	6.250	1.563	6.250	**1.022**	6.250	1.563	6.250	1.563	6.250	**1.022**	6.250
C. capense	1.563	6.250	1.563	6.250	**1.172**	3.125	**1.172**	6.250	3.125	6.250	1.563	6.250	**0.781**	6.250	1.563	3.125
Ketoconazole	Greater than 1.0															
Griseofulvin	Greater than 1.0															

.

Table 4.2: Antifungal activity (MIC values expressed as mg/ml) of leaf extracts of C. album, C. pulchellum, M. koenigii, M. paniculata and C. capense against M. gypseum

Plants	*Microsporum gypseum*							
	P. E		Acetone		Methanol		Ethanol	
	MIC mg/ml	MFC mg/ml	MIC mg/ml	MFC mg/ml	MIC mg/ml	MFC mg/ml	MIC mg/ml	MFC mg/ml
C. album	6.250	6.250	**0.391**	3.125	**0.195**	3.125	**0.391**	3.125
C. pulchellum	6.250	6.250	**0.781**	3.125	**0.049**	1.563	**0.781**	3.125
Murraya koenigii	**0.781**	6.250	**0.586**	6.250	**0.391**	6.250	**0.391**	6.250
Murraya paniculata	1.172	6.250	1.172	6.250	1.563	6.250	2.344	6.250
C. capense	6.250	6.250	**0.781**	6.250	**0.781**	3.125	**0.781**	3.125
Ketoconazole	Less than 1.0							
Griseofulvin	Greater than 1.0							

.

4.4. Discussion

Extracts of *C. album* displayed potent and relevant pharmacological activities, with a considerable antifungal activity against several strains responsible for important infectious diseases (**ESTERHUIZEN *et al*., 2006a**). The compounds from *C. album* could be of great pharmaceutical interest for therapeutic application as complementary antifungal agents (**ESTERHUIZEN *et al*., 2006a**). Its sister species *C. pulchellum* contains phenylpropenes, phenylpropanoids and terpenoids which exhibit antimicrobial properties (**BRADER *et al*., 1997**).

In this study, methanolic extract of *C. pulchellum* demonstrated good *in vitro* activity against all the three fungi tested indicating the potency of the plant against skin disease of fungal origin. Hence, *C. pulchellum* (methanolic extract) has good prospects for the formulation of an effective cure for skin disease. Isolated phenylpropenes from *C. pulchellum* had good antimicrobial activity (**BRADER *et al*., 1997**). The authors attributed the observed antimicrobial activity to the reactivity of the phenolic hydroxyl group (**BRADER *et al*., 1997**). The compound responsible for the antifungal activity of the leaf extracts recorded in this study is not known.

n-Hexadecanoic acid a compound isolated from *C. album* has been identified as one of the compounds that possess fungistatic properties against mammalian skin pathogens (**ESTERHUIZEN *et al*., 2006a**). It has been reported to possess antifungal activity against *Trichophyton mentagrophytes*, a zoophilic skin fungus which is responsible for athlete's foot (**WOOD and WELDON, 2002**). In this study, methanolic extract of *C. album* exhibited good activity against *T. mentagrophytes*. Similarly, *Clausena excavata* methanol leaf extract exhibited a remarkable antifungal activity against *T. rubrum* and *T. mentagrophytes* with MIC values of 62.5 µg/ml and 31.2 µg/ml (**GUNTUPALLI *et al.,* 2013)**. The compound, *n*-Hexadecanoic acid could probably be responsible for this activity.

A wide range of coumarin derivatives have been isolated from *C. album* **(DREYER *et al*., 1972; GRAY, 1981)**. Pharmacological activities of *C. album* are attributed to its terpenes and coumarins (and their derivatives). These include: antithrombotic, antiplatelet (**HOULT and PAYA, 1996**) and antimicrobial (**KAYSER and KOLODZIEJ, 1999; LAURIN *et al*., 1999; LIS-BALCHIN and HART, 2002; OKUNADE *et al*., 2004; ESTERHUIZEN *et al.,* 2006a; LIEBENBERG, 2008**) activities. A tincture made from *C. album* and marketed as "Immunat" is widely used

.

as an herbal remedy. Some compounds isolated from this plant have activity against several inflammatory mediators (**ELDEEN and VAN STADEN, 2008**).

As a result of the medicinal and commercial values of this plant, it is amongst the 'highly sort for' South African plants. **FAJINMI *et al*** (**2014**) reported that there is currently an increase in demand for, and utilization of, *C*. *album* in the South African traditional medicinal system. Despite the popularity of *C. album* in the South African traditional medicine, its sister species, *C. pulchellum* has received little or no attention. The two plants, *C. album* and *C. pulchellum* have similar morphological features on the field and can only be distinguished by the colour of their flowers. *Coleonema album* pharmacological activity is well investigated while there is paucity of information on the pharmacological activities and medicinal use of *C. pulchellum*. Many South African natural product companies are now pursuing export markets for *C. album* oil, which may put pressure on the plant's natural populations in the near future (**FAJINMI *et al*., 2013**). Apart from the development of an effective propagation technique and control of harvesting of *C. album* wild populations, the use of another member of the genus with same medicinal value can help reduce the pressure on *C. album* plants and thus prevent the plant from becoming an endangered species in the near future. The result of this study reveals the antifungal activity of *C. pulchellum*. It will be necessary to accord *C. pulchellum* the same attention as *C. album* as it could contain valuable bioactive compounds that can combat several diseases of fungal origin.

Several bioactive compounds such as alkaloids, flavonoids, phenolic compounds, proteins, amino acids, flavonoids, indole alkaloids, spiroquinazoline alkaloids, coumarins and isoflavanoids have been isolated from *M. paniculata* (**GILL *et al.,* 2014**). Its pharmacological activities recorded in the literature include antifungal activity (**LI *et al*., 1988; ROUT *et al*., 2007; SHARKER and SHAHID, 2009**). As a result of the characteristic aroma (**JAIN *et al*., 2012; GILL *et al*., 2014)** of *Murraya koenigii*, it is utilised globally as a spice. It has a high medicinal value (**JAIN *et al*., 2012**) and is utilised in the production of cosmetics. Herbs and spices have been utilized to maintain and enhance human beauty for centuries and there is growing scientific evidence that these plants have several active ingredients with the ability to calm or smooth the skin and ultimately heal, restore and shield the skin (**GEDIYA *et al*., 2011**). Some of the extracts of *M. koenigii* and *M. paniculata* used in

.

this study displayed antifungal activity against the skin fungal strains tested and thus justify their use in formulations for skin care and cosmetics.

The Cape Chestnut (*C. capense*) seed and bark have been reported to be used for skin and hair care. The bark is used traditionally as an ingredient in the production of skin ointment (**VAN WYK and GERICKE, 2000; NOTTEN, 2001**) and is sold at traditional medicine markets (**NOTTEN, 2001**). **MOKOKA *et al*.,** (**2010**) found that *C. capense* had promising antifungal activity. Lupeol a purified compound isolated from the leaves of *C. capense* exhibited antifungal activity and negligible detectable cytotoxicity (**SAKONG, 2012**). The compound lupeol has been isolated from other plant species and is known to exhibit antimicrobial and anti-inflammatory activities (**SAKONG, 2012**). The antifungal activity of *C. capense* recorded in this study could probably be as a result of the presence of lupeol and other antimicrobial compounds.

4.5. Conclusions

The result of antifungal activity of the plants used in this study reveals the potency of the plants against the skin disease causing fungal strains tested. The plants however displayed fungistatic activity. It is therefore necessary to isolate and test compounds from the plants that could probably display a fungicidal activity. Also, essential oil derived from the leaves of the plants need to be investigated for antifungal activity as essential oil has been reported to contain secondary metabolites which include terpenes and have been reported in scientific literature to exhibit fungicidal activity against several fungal strains.

.

CHAPTER 5. ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS DERIVED FROM RUTACEAE PLANT LEAVES AGAINST THREE SKIN DISEASE FUNGAL STRAINS

5.1. Introduction

Harmless fungi are always present on the surface of the skin and infection occurs only when these organisms gain access into the body (**TABASSUM and HAMDANI, 2014**). These fungi generally live in the dead, top layer of skin cells on moist areas of the human body (**TABASSUM and VIDYASAGAR, 2013**). They are capable of penetrating into the cells and subsequently cause itching, swelling, blistering and scaling (**PING-HSIEN *et al*., 2007**). The infections they cause are superficial as they affect the skin, hair, nails and include skin disease such as athlete's foot and ringworm (**TABASSUM and HAMDANI, 2014**). Globally, millions of people are affected by superficial fungal infections (**TABASSUM and VIDYASAGAR, 2013**). Skin disease is a health problem that affect all ages from the neonates to the elderly and cause harm in a number of ways (**TABASSUM and HAMDANI, 2014**). Several skin diseases such as ringworm caused by dermatophytes are common in the tropical and semi-tropical areas of the world (**TABASSUM and VIDYASAGAR, 2013**).

Essential oils are used throughout the world for the treatment of various conditions which include skin conditions, and have fewer side effects compared to synthetic drugs (**TAVARES *et al*., 2008**). Essential oils may be effective in the treatment of skin problems (**PEARLSTINE, 2006**). The antimicrobial action of essential oils is also beneficial in addressing problems related to the skin (**PEARLSTINE, 2006**). Some skin conditions that tend to be responsive to the use of essential oils include eczema and other fungal infections (**BUCKLE, 2003**). Various volatile oils from plants have been reported to have medicinal values for skin treatment (**LIMA *et al.,* 1993**).

Essential oils may be helpful in the treatment of a chronic eczematous lesion (**BLAMEY, 2000**). An eczematous lesion showed significant healing on the 6^{th} day of treatment with a combination of tea tree (*Melaleuca alternifolia*), lemon (*Citrus limonum*), bergamot (*Citrus aurantium*), lavender (*Lavandula angustifolia*) and niaouli

.

(*Melaleuca viridiflora*) oils (**BLAMEY, 2000**). The aim and objective of this part of the study was to investigate the antifungal activity of essential oil volatiles of *Agathosma mucronulata*, *A. ovata*, *Calodendrum capense*, *Coleonema album*, *C. pulchellum*, *Murraya koenigii* and *M. paniculata* against *T. rubrum* and *T. mentagrophytes.*

5.2. Materials and methods

Leaves of *Agathosma mucronulata*, *A. ovata*, *Coleonema album*, *C. pulchellum* were sourced (in September) from the South African National Biodiversity Institute, Kirstenbosch*. Calodendrum capense*, *M. koenigii* and *M. paniculata* leaves were sourced from the University of KwaZulu-Natal botanical garden, Pietermaritzburg. The leaves were freeze-dried and stored in sealed plastic bags. Essential oil was extracted from 20 g each of freeze dried leaves using a soxhlet apparatus. The extraction of oil was done according to the Czech Pharmacopeia. *Trichophyton rubrum* (ATCC 28188) and *T. mentagrophytes* (ATCC 9533) were grown in yeast malt broth for one week and the fungal suspension was subcultured on fresh yeast malt agar for one week. The plates had a good mycelia growth from which 0.3 cm diameter of mycelia was taken. The agar supporting the mycelia was sliced off and the mycelia were placed on fresh agar plates. Filter paper was placed on the upper lid of the fresh agar petri dishes. The filter paper was impregnated with 20 µl of essential oil in such a way that the mycelia placed on the agar plate were directly exposed to the essential oil volatiles. Plates with similar diameter (0.3 cm) of *T. rubrum* and *T. mentagrophytes* but without essential oil were used as controls. The plates were incubated for a week and the diameter of the mycelia was recorded. A formula, Fungal Growth Index (FGI) was used to calculate the percentage increase in the size of the mycelia in order to be able to determine the extent of the inhibition of the fungi by the EO volatiles.

$$FGI = \frac{(\text{Final diameter of mycelia} - \text{Initial diameter of mycelia})}{\text{Initial diameter of mycelia}} \times 100$$

The mycelia from each petri dish were subcultured on fresh agar plates and incubated for another seven days at 37ºC in order to determine if the treatments have a fungistatic or fungicidal effect.

.

5.3. Results

The essential oil of *A. ovata* and *A. mucronulata* totally inhibited the growth of *T. rubrum* but did not have a total inhibition on *T. mentagrophytes* (Table 5.1). The *T. mentagrophytes* mycelia exposed to volatiles from 20 µl of *A. ovata* and *A. mucronulata* essential oil gave rise to **100** and **133.33%** FGI respectively (Table 5.2). These inhibitions are moderate as compared with the control that gave FGI of **233.33%.**

The essential oil volatiles from both *C. album* and *C. pulchellum* inhibited the growth of *T. rubrum in vitro* with final mycelia diameter of **0.3** and FGI of **0%** (Table 5.3 and 5.4) each. Essential oil volatiles of *C. album* showed total inhibition on both fungi of *T. rubrum* and *T. mentagrophytes* (Table 5.3). However, *C. pulchellum* essential oil volatiles did not yield total inhibition on the growth of *T. mentagrophytes* mycelia as the final mycelia diameter of *T. mentagrophytes* treated with the essential oil volatiles was **0.5 cm** resulting in a **66.67%** FGI (Table 5.4). Overall, essential oil of both *C. album* and *C. pulchellum* inhibited the growth of the fungi *T. rubrum* and *T. mentagrophytes* compared to the final diameter of the mycelia controls of **2.0** and **1.0 cm** respectively.

Essential oil volatiles of both *M. koenigii* and *M. paniculata* inhibited mycelia growth of *T. rubrum* and *T. mentagrophytes* compared to the control (Table 5.5). However, antifungal activity of *M. koenigii* essential oil volatiles is low compared to *M. paniculata* essential oil volatiles. The FGI of *M. paniculata* essential oil volatiles against both *T. rubrum* and against *T. mentagrophytes* was **0%** (Table 5.6) indicating a total inhibition of mycelia growth of both fungi. The essential oil volatiles of *M. koenigii* resulted in **366%** FGI for *T. rubrum* and **200%** for *T. mentagrophytes* respectively. Hence, *M. paniculata* essential oil volatiles displayed fungistatic and fungicidal effects while *M. koenigii* essential oil volatiles displayed fungistatic effect against both *T. rubrum* and *T. mentagrophytes*.

.

Table 5.1: The effect of essential oil (20 µl) volatiles of A. mucronulata, *A. ovata and C. capense on mycelia growth of T. rubrum and T. mentagrophytes after 7 days of incubation at 37ºC*

Fungus tested	*A. mucronulata* oil	*A. ovata* oil	*C. capense* oil	Control	Starting material
Trichophyton rubrum diameter (cm)	0.3	0.3	0.3	2.0	0.3
Trichophyton mentagrophytes diameter (cm)	0.6	0.7	-	1.0	0.3

Table 5.2: Effect of A. mucronulata, A. ovata C. capense essential oil volatiles on Fungal Growth Index (FGI) (%) of T. rubrum and T. mentagrophytes as compared to the control

Fungus tested	*A. mucronulata* oil FGI (%)	*A. ovata* oil FGI (%)	*C. capense* oil FGI (%)	Control FGI (%)
Trichophyton rubrum diameter (cm)	0	0	0	566.67
Trichophyton mentagrophytes diameter (cm)	100	133.3	-	233.33

.

Table 5.3: The effect of essential oil (20 µl) volatiles of C. album and C. pulchellum on mycelia growth of T. rubrum and T. mentagrophytes after 7 days of incubation at 37ºC

Fungus tested	*C. album* oil	*C. pulchellum* oil	Control	Starting material
Trichophyton rubrum diameter (cm)	0.3	0.3	2.0	0.3
Trichophyton mentagrophytes diameter (cm)	0.3	0.5	1.0	0.3

Table 5.4: Effect of C. album and C. pulchellum essential oil volatiles on Fungal Growth Index (FGI) (%) of T. rubrum and T. mentagrophytes as compared to the control

Fungus tested	*C. album* oil FGI (%)	*C. pulchellum* oil FGI (%)	Control FGI (%)
Trichophyton rubrum diameter (cm)	0	0	566.67
Trichophyton mentagrophytes diameter (cm)	0	66.67	233.33

.

Table 5.5: The effect of essential oil (20 µl) volatiles of Murraya koenigii and M. paniculata on mycelia growth of T. rubrum and T. mentagrophytes after 7 days of incubation at 37ºC

Fungus tested	*Murraya koenigii* oil	*M. paniculata* oil	Control	Starting material
Trichophyton rubrum diameter (cm)	1.4	0.3	2.0	0.3
Trichophyton mentagrophytes diameter (cm)	0.9	0.3	1.0	0.3

Table 5.6: Effect of M. koenigii and M. paniculata essential oil volatiles on Fungal Growth Index (FGI) (%) of T. rubrum and T. mentagrophytes as compared to the control

Fungus tested	*M. koenigii* essential oil FGI (%)	*M. paniculata* essential oil FGI (%)	Control FGI (%)
Trichophyton rubrum diameter (cm)	366	0	566.67
Trichophyton mentagrophytes diameter (cm)	200	0	233.33

.

5.4. Discussion

Essential oils are known to have several properties which are of great importance in skin health (**RHIND, 2012)**. Such properties include skin barrier function, maintenance and restoration of texture and hydration levels, reduction of inflammation, stimulation of cell regeneration, prevention or control of infections and allergic responses and alleviation of itching (**RHIND, 2012)**. '*Buchu*' (*Agathosma species*) has been an indispensable part of the San and Khoi traditional healing culture in the Cape and is still being used for this purpose throughout South Africa (**MOOLLA *et al.,* 2007**). The San people used aromatic plants lubricated with fat to keep their skin soft and moist in the desert climate, and as an antifungal agent to promote good health through the uptake of aromatic substances by the skin (**SIMPSON, 1998**). The result of this study supports the use of these plants as skin antifungal agents.

Agathosma ovata is still used for medicinal purposes amongst the Cape people (**VAN WYK and GERICKE, 2000**). The antifungal activity of the essential oil of *A. ovata* displayed in this study justifies its use for medicinal purposes. There is paucity of information on the antimicrobial activity of *A. mucronulata* in scientific literature. According to **JODAMUS and NOTTEN** (**2002**), *A. mucronulata* is not a medicinal plant. Based on the result of this study, the plant should be utilized for medicinal purpose just like some notable members of the *Agathosma* genus. A plant that is yet to be utilized as a cure for diseases could be a storehouse of several therapeutic substances and could possess remarkable antimicrobial activity.

Essential oil derived from *C. album* is incorporated into several skin care products including '*Savane'*, an organic anti-aging lift serum which costs up to $100 per 30 ml. The result of this study shows its ability to also inhibit the growth of skin disease fungal strains thus having a dual effect on the skin. The result of this study shows the therapeutic value of *C. pulchellum* essential oil and can thus be used as an alternative to *C. album* in skin care formulations. Antimicrobial activities of essential oil have been attributed mainly to its volatile components such as monoterpenes which in many cases are the main composition of essential oil. The genus *Coleonema* has been reported to have an abundance of terpenes and other metabolites (such as coumarins) and could be at the root of the activity displayed in this study.

.

The oil of *M. paniculata* is utilized externally to treat bruises, eruption and incorporated in the soap and perfume industry (**PRAJAPATI *et al.,* 2003**). The essential oil of *M. koenigii* and *M. paniculata* have been reported to be used as a spice, cosmetics and a cure for skin diseases in different parts of the world. The antifungal activity of *M. koenigii* and *M. paniculata* recorded in this study could be responsible for their effectiveness in the treatment of skin diseases and their use in skincare. *Murraya paniculata* has been reported to contain caryophyllene oxide which has antifungal activities (**YANG *et al*., 1999**). The essential oil derived from *M. koenigii* is utilized in the soap, cosmetic and aromatherapy industry (**RAO *et al*., 2011**).

The essential oil from leaves of *M. koenigii* showed antifungal activity against *A. niger, A. fumigatus, C. albicans, C. tropicalis,* and *M. gypseum* (**SAINI and REDDY, 2013**). In this study, *M. koenigii* leaf extracts gave remarkable antifungal activity against *M. gypseum*. However, the essential oil of *M. koenigii* displayed low activity against the two skin fungal strains tested while that of *M. paniculata* gave remarkable activity. The efficacy of essential oil varies and depends solely on the type and concentration of the oil, and the tested microbial strain (**PUROHIT and KAPSNER, 1994; MANGENA and MUYIMA, 1999**). Essential oil from several species of the family Rutaceae has been reported to exhibit antifungal activity *in vitro* at various degrees.

The essential oil extracted from the epicarp of *Citrus sinensis* (L.) Osbeck exhibited absolute fungitoxicity against ten post-harvest pathogens (**SHARMA and TRIPATHI, 2006**). **BALAKUMAR *et al.,*** (**2011**) reported the potent antifungal activity of the essential oil of *Aegle marmelos* (Correa) leaves against the clinical isolates of dermatophytes. The seeds of *C. capense* are crushed and boiled to obtain oil that is suitable for soap production (**NOTTEN, 2001; LALL and KISHORE, 2014**). Essential oil derived from Cape Chestnut seed is popularly known as *Yangu* oil (**RAMOROKA and MAPUNYA, 2006**) and has natural UV protection. The leaves and bark of *C. capense* is used as a facial mask and in soap preparations (**MAPUNYA *et al*., 2012**). The result of the antifungal activity of the essential oil of *C. capense* leaves in this study highlight its therapeutic value and can thus be used on the skin just like the oil from the seeds of the plant. However, the cytotoxicity profile of the leaves need to be analysed for it to be certified safe for use on the skin.

.

5.5. Conclusions

The essential oil volatiles from all the species tested in this study displayed antifungal activity against *T. rubrum* and *T. mentagrophytes*. The degree of antifungal activity displayed however varies from one plant to the other and between plants of the same genus. The essential oil volatiles which exhibited fungicidal effects against the fungi are particularly noteworthy. The essential oil of all the plant species which exhibited fungicidal activity can be combined to formulate an effective ointment/antifungal that can combat skin diseases associated with *T. rubrum*. The essential oil used in this study can provide a novel source of antifungals for the treatment of skin diseases. It is necessary to analyse the essential oil for the presence of bioactive compounds as it will help to deduce the compounds that could be responsible for the antifungal activity displayed in this study.

.

CHAPTER 6. ANALYSIS OF THE ESSENTIAL OIL COMPONENTS OF SEVEN RUTACEAE SPECIES

6.1. Introduction

Essential oils are composed of volatile, natural, compounds with a strong scent, formed by aromatic plants as part of their secondary metabolites (**BURTS, 2004**). They are produced by many parts of the plants such as flowers, buds, seeds, leaves, twigs, bark, wood, fruit and roots (**BAKKALI *et al*., 2008**). In nature, these compounds play a crucial role in the protection of the plants as antibacterial, antiviral, antifungal and insecticidal agents (**RAJENDRAN *et al.,* 2014**).

Essential oils are valuable products of natural origin utilised as raw materials in the production of perfumes, cosmetics, aromatherapy oils, medicines, phytotherapy oils, spices and nutraceuticals (**BUCHBAUER, 2000**). Essential oils are well known for their various therapeutic properties and are used in the treatment of a variety of infections caused by both pathogenic and non-pathogenic microorganisms (**HAMID *et al*., 2011**). The increasing importance of essential oil in pharmaceuticals, aromatherapy oils, and their beneficial role in cosmetics (**RAJENDRAN *et al.,* 2014**) has increased the need to analyse the essential oil components of a variety of plants. Essential oils are primarily composed of mono- and sesquiterpenes and aromatic polypropanoids (**RAJENDRAN *et al.,* 2014**).

The monoterpenes are known to comprise 25 different classes, sesquiterpenes are composed of 147 classes and 118 classes of diterpenes exist (**BAŞER and DEMIRCI, 2007**). The term “terpene” was coined by Kekulé in 1880, as they were first discovered in turpentine (**MOUSSAIEFF *et al.,* 2008**). A single terpene (monoterpene) is formed from two isoprene units connected head to tail (**SADGROVE and JONES, 2015**). Hemi- (1 isoprene), mono- (2 isoprenes), sesqui- (3 isoprenes) and di- (4 isoprenes) terpenes are the most common essential oil components, followed by the non-terpenoid group, phenylpropanoids (**SADGROVE and JONES, 2015**).

In the earlier literature the term “terpene” was often used to describe terpenoid compounds which include oxygenated terpenes (**SELL, 2010**). However, in modern terminology “terpene” is used to describe monoterpene hydrocarbons (two isoprenes with only carbon and hydrogen) (**SELL, 2010**). In the family Rutaceae,

.

essential oil is often composed of mono- and sesquiterpenes (**RAJENDRAN *et al.*, 2014**). The aim of this part of the study was to analyse the components of essential oil derived from the leaves of *Agathosma mucronulata*, *A. ovata*, *Calodendrum capense*, *Coleonema album*, *C. pulchellum*, *Murraya koenigii* and *M. paniculata.*

6.2. Materials and methods

Leaves of *Agathosma mucronulata*, *A. ovata*, *Coleonema album*, *C. pulchellum* were sourced (in September) from the South African National Biodiversity Institute, Kirstenbosch*. Calodendrum capense*, *M. koenigii* and *M. paniculata* leaves were sourced from the University of KwaZulu-Natal botanical garden, Pietermaritzburg. The leaves were freeze-dried and stored in sealed plastic bags. Essential oil was extracted from 20 g each of freeze-dried leaves using a soxhlet apparatus. The extraction of oil was done according to the Czech Pharmacopeia. The essential oil derived was subjected to analysis using Gas Chromatography Mass Spectrometry (GCMS) (Agilent 5975C). Sample injection volume was 1 uL at 50°C for 15 min, increase to 250°C - 2°C / min, hold at 250°C for 15 min

- injection at 220°C
- column HP-5, 30 µm x 250 mm x 0,25 µm
- carrier gas He 1ml / min
- MS detection

The percentages of the major constituents (≥1.5%) were recorded individually while the minor constituents (≤1.5%) were recorded as ‘others’.

6.3. Results

Agathosma ovata leaves essential oil yielded forty-three components with β-myrcene (**22.03%**), 1,6-octadiene-3-ol, 3, 7-dimethyl-2-aminobenzoate (**18.75%**), trifluoroacetyl-lavandulol (**12.97%**), trans-β-ocimene (**11.81%**), β-ocimene (**5.65%**), bicyclogermacrene (**5.10%**), β-phellandrene (**3.47%**), thujone (**2.07%**), β-pinene (**1.88%**) and caryophyllene (**1.74%**) as the major compounds (**Table 6.1**). The major components of *A. mucronulata* leaves essential oil are: β-myrcene (**34.05%**), β-pinene (**18.24%**), caryophyllene (**7.4%**), (1S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (**6.09%**), D-limonene (**6.06%**), eucalyptol (**5.46%**), bicyclogermacrene (**5.38%**), trans-β-ocimene (**4.21%**), 2,4,4-trimethyl-2-vinyl-guaia-1(10),11-diene (**3.00%)** and

.

β-ocimene (**2.36%**) of the thirthy-three components (**Table 6.1**). Compounds found in essential oil of both *A. ovata* and *A. mucronulata* are trans-β-ocimene, β-ocimene, caryophyllene, β-myrcene and bicyclogermacrene.

The essential oil of *C. capense* leaves is composed of sixty- three compounds of which cis-β-farnesene (**44.30%**), naphtalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis) (**4.88%**), β-phellandrene (**4.07%**), 1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-,[S-(E,E)] (**3.68%**), cis-β-farnesene (**3.29%**), β-bourbonene (**2.87%**), caryophyllene (**2.86%**), gamma-muurolene (**4.01%**), bicyclo[5.2.0],nonane,4-methylene (**2.03%**), isolongifolene, 9,10-dehydro (**1.64%**), trans-α-bergamotene (**1.59%**) and trans-β-ocimene (**1.53%**) are the major components (**Table 6.4**).

Caryophyllene (**24.91%**), trans-β-ocimene (**9.49%**), β-myrcene (**7.96%**) and octahydro-7-methyl-3-methylene-4-(1-methylethyl)-β-copaene (**7.53%**) are the major components of *C. album* essential oil while bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene (**32.04%**), (+)-3-carene (**10.88%**), β-ocimene (**9.46%**), gamma-elemene (**8.95%**) and α-pinene (**7.43%**) are the major components of *C. pulchellum* essential oil. The components of the oil greater than **1.5%**, found in both essential oil of *C. album* and *C. pulchellum* are caryophyllene, bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene, α-pinene, gamma-elemene and carene (**Table 6.2**). However, the common bioactive compounds exist in both essential oil in different quantities.

Essential oil of *M. koenigii* yielded a total of twenty-three components. The major components of the essential oil are: caryophyllene (**42.54%**), (4aR-trans)-guaia-9, 11-diene (**23.35%**), alpha-guaiene (**10.71%**) and humulene (**6.93%**). The sister species, *M. paniculata* essential oil yielded forty-two components of which 1, 6, 10-dodecatriene,7,11-dimethyl-3-methylene (**38.62%**), 1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl) (**19.0%**), caryophyllene (**10.63%**), Gamma-elemene (**8.55%**) and bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl) (**5.42%**) are the major components. Among components greater than **1.5%**, there are two compounds common to both species (**Table 6.3**). These compounds are: caryophyllene and bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl). However, the two compounds are present in different quantities in the essential oil of the two plants.

Table 6.1: List of major compounds present in the essential oils of Agathosma ovata and A. mucronulata

Agathosma ovata		*Agathosma mucronulata*	
Compound	%	Compound	%
β-Myrcene	22.03	β-Myrcene	34.05
1,6-Octadiene-3-ol, 3, 7-dimethyl-2-aminobenzoate	18.75	β-Pinene	18.24
Trifluoroacetyl-lavandulol	12.97	Caryophyllene	7.4
Trans-β-Ocimene	11.81	(1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	6.09
β- Ocimene	5.65	D-Limonene	6.06
Bicyclo germacrene	5.10	Eucalyptol	5.46
β-Phellandrene	3.47	Bicyclo germacrene	5.38
D-Limonene	2.79	Trans-β-Ocimene	4.21
Thujone	2.07	2,4,4-trimethyl-2-vinyl-Guaia-1(10),11-diene	3.00
β-Pinene	1.88	β- Ocimene	2.36
Caryophyllene	1.74	Others	7.75
Others	11.74		

Table 6.2: List of major compounds present in the essential oils of C. album and C. pulchellum

Coleonema album		*Coleonema pulchellum*	
Compound	%	Compound	%
Caryophyllene	24.91	Bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene	32.04
Trans-β-Ocimene	9.49	(+)-3-Carene	10.88
β-Myrcene	7.96	β-Ocimene	9.46
Octahydro-7-methyl-3-methylene-4-(1-methylethyl)-β-copaene	7.53	Gamma-Elemene	8.95
β-Pinene	7.00	α-Pinene	7.43
(+)-3-Carene	5.98	Caryophyllene	6.98
Bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene	5.97	β-Pinene	5.36
Bicyclogermacrene	5.11	β-Myrcene	4.47
A-Terpineol	3.69	(+)-4-Carene	3.76
Eucalyptol	2.58	β-Phellandrene	3.73
1,3,6-Octatriene,3,7-dimethyl-, (Z) -β-Ocimene	2.25	α-Phellandrene	1.60
Gamma-Elemene	1.84	Others	5.34
Others	15.69		

Table 6.3: List of major compounds present in the essential oils of M. koenigii and M. paniculata

M. koenigii		*M. paniculata*	
Compound	%	Compound	%
Caryophyllene	42.54	1, 6, 10-Dodecatriene,7,11-dimethyl-3-methylene	38.62
(4aR-trans)-Guaia-9,11-diene	23.35	1,6-cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)	19.0
Alpha-Guaiene	10.71	Caryophyllene	10.63
Humulene	6.93	Gamma-Elemene	8.55
Beta-phellandrene	2.66	Bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl)	5.42
Bicyclo[3.1.1]hept-2-ene,2,6-dimethyl-6-(4-methyl-3-pentenyl)	2.22	Cis-beta-Farnesene	3.12
Naphthalene,decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)	2.10	Beta-Bisabolene	1.86
Beta-Pinene	1.62	Naphthalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S-Cis)	1.56
Others	7.87	Others	11.24

Table 6.4: List of major compounds present in C. capense leaf essential oil

Compound	%
Cis-β-Farnesene	44.30
Naphtalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis)	4.88
1,6-Cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-,[S-(E,E)]	3.68
Cis-β-Farnesene	3.29
β-Bourbonene	2.87
Caryophyllene	2.86
β-Phellandrene	4.07
Gamma-Muurolene	4.01
Bicyclo[5.2.0],nonane,4-methylene	2.03
Isolongifolene,9,10-dehydro	1.64
Trans-α-Bergamotene	1.59
Trans-β-Ocimene	1.53
Others	23.25

6.4. Discussion

According to **MOOLLA** (**2006**), sabinene was the most dominant component ranging between 25.6% and 44.4% in all the ten samples of *A. ovata* oil analysed. However, β-myrcene is the major component of the essential oil of *A. ovata* analysed in this study. This could be as a result of the fact that the chemical composition of *A. ovata* essential oil is subject to seasonal variation as reported by **MOOLLA** (**2006**). The similar components found in all the ten samples of *A. ovata* essential oil analysed by **MOOLLA** (**2006**) include: sabinene, *p*-cymene, β-pinene, α-pinene, α-thujene, myrcene, limonene, linalool and terpinen-4-ol. In this study, myrcene, limonene and β-pinene also form part of the major components of the essential oil while, *p*-cymene and terpinen-4-ol were part of the components in the minor (others) category. It is however not know which of the components are responsible for the antifungal activity of *A. ovata* essential oil in this study. The results of **MOOLLA** (**2006**) revealed that the antimicrobial activity of the essential oil of *A. ovata* may not depend on the level of one component but rather the ratio of several components.

Globally, there is a growing need to discover a reservoir of medicinal plants that are yet to be fully exploited for their medicinal and antioxidant activities. *Agathosma mucronulata* leaves used in this study displayed remarkable antifungal (against *T. rubrum*) and antioxidant activity and it is the first time its medicinal value has been recorded in scientific literature. Herbs which have no specific nutritional value can also be an important source of antioxidants (**WARREN, 1999**). In this study, *A. mucronulata* essential oil is composed of compounds such as limonene, myrcene and caryophyllene which have been reported previously in literature to exhibit antifungal activity. The antifungal compound caryophyllene is also present in the essential oil of *C. capense*.

The major components of *C. album* oil are β-phellandrene (29.1%) and myrcene (20.5%) among the 43 components characterized (**BAŞER *et al*., 2006**). However, the major components of the *C. album* oil analysed in this study are caryophyllene (24.91%), trans-β-ocimene (9.49%), β-myrcene (7.96%) and octahydro-7-methyl-3-methylene-4-(1-methylethyl)-β-copaene (7.53%). The difference could be as a result of the age of plant used and the season of harvest. Essential oil from leaves of *C. pulchellum* sourced from Australia was found to consist mainly (50%) of monoterpene hydrocarbons (**BROPHY and LASSAK, 1986**). In the three oil samples of *C. pulchellum* analysed by **BAŞER *et al*., (2006**),

monoterpenes dominated with 92.2%, 81.4%, and 97.0% in plant samples from the Witwatersrand Botanical Garden, Schoenmakerskop (Port Elizabeth) and RAU Garden (Johannesburg) respectively. β-phellandrene (17.7%), β-pinene (11.8%), linalool (11.8%) and α-pinene (9.7%) were the main components in the Witwatersrand Botanical Garden sample; α-pinene (16.8%), sabinene (15.1%), β-pinene (11.6%) and linalool (10.5%) were the predominant components in the Schoenmakerskop essential oil sample and in the RAU gardens sample oil, α-pinene (17.4%), β-pinene (13.6%), β-phellandrene (10.3%) and linalool (10.0%) were the main constituents (**BAŞER *et al*., 2006**).

α-pinene (7.43%) also formed one of the major components of the *C. pulchellum* essential oil analysed in this study. However, bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene (32.04%) formed one-third of the oil component composition which deviates from what was discovered by **BAŞER *et al***., (**2006**). The difference could be as a result of the age and season of harvest of the plant material and the geographical location of the plants. Monoterpene composition of the oil of South African *C. pulchellum* was more than that of the Australian sample (**BROPHY and LASSAK, 1986**). Monoterpenes were the major components of the oils analysed by **BAŞER *et al*.,** (**2006**). The various reports of essential oil analysis available in scientific literature reveals the crucial role climatic and physiological factors play in the chemical composition of essential oil. This explains why the results of essential oil analysis of these species in the present study differ from what has been reported by other researchers.

Essential oil is a complex mixture of naturally occurring compounds mainly monoterpenes and sesquiterpenes and is considered as an alternative natural antimicrobial agent (**CHEE *et al.,* 2009**). The plant *M. paniculata* has been reported to contain caryophyllene oxide which has antifungal activities (**YANG *et al*., 1999**). In this study, caryophyllene is recorded as one of the major components in both essential oil of *M. koenigii* and *M. paniculata* (**Table 6.3**). This compound is reported as one of the major components of essential oil of *M. koenigii* (**GOPALAN *et al.,* 1984; CHOWDHURY *et al*., 2008**) and *M. paniculata* (**CHOWDHURY *et al*., 2008; RODRÍGUEZ *et al*., 2012**) in most of the literature available. Hence, caryophyllene could be responsible for the antifungal activity displayed by the plants as recorded in this study.

6.5. Conclusions

The array of compounds in each of the essential oils of the plants analysed in this study have been recorded in various scientific publications to exhibit antimicrobial activity. The compounds could have acted synergistically to bring about the antifungal activity recorded in chapter 5 of this study. It is not certain if essential oil purchased from organic product companies will comprise the same compounds and exhibit the same level of antifungal activity. The mode of action of the essential oil volatiles on the mycelia growth is also not known. It is therefore necessary to investigate the antifungal activity of essential oil derived from two highly utilised Rutaceae plants and their mode of action on skin disease causing fungal strains.

CHAPTER 7. ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS OF *AGATHOSMA BETULINA* AND *COLEONEMA ALBUM* AND ITS EFFECT ON THE MORPHOLOGY OF *T. RUBRUM* AND *T. MENTAGROPHYTES*

7.1. Introduction

The use of essential oil to enhance skin beauty is an ancient practice. In this age of civilisation, essential oils have diverse application, among which is the production of organic beauty products. Hence, if essential oil could enhance skin beauty, it can also have therapeutic effects on the skin. Essential oils extracted from several plants have been of interest as a result of their potential use as a replacement of synthetic antifungal agents. Essential oils are produced by blossoms, leaves and fruits of different plants and stored in special tissues such as glandular hairs, oil cells and oil ducts (**WALTERS and ROBERTS, 2008)**. Essential oil is a complex, aromatic, volatile mixture of several compounds with diverse chemical structures (**WALTERS and ROBERTS, 2008)**.

It is generally believed that the biological activity of an essential oil is the result of both its active and inactive substances (**WALTERS and ROBERTS, 2008)**. The inactive components may have an influence on resorption, skin penetration, rate of reaction, or bioavailability of the active compounds while several active components may have synergistic effects (**WALTERS and ROBERTS, 2008)**. Essential oils mainly consist of monoterpenes and sesquiterpenes, and are the lipophilic secondary metabolites of plants, which are derived from mevalonate and isopentenyl pyrophosphate (**SPURGEON and PORTER, 1981**). Essential oil occurs widely in nature with a diversity of terpenoid structures in plants and are generally responsible for the plant distinctive scent (**PARK *et al*., 2009**).

Scents emitted from essential oil-bearing organs of plants are often referred to as volatiles which are important as scents in plants as they are responsible for the attraction of insects to pollinate flowers (**ROWAN, 2011**). Plants release volatiles from their roots, fruit, flowers and leaves (**FROST *et al*., 2008; DICKE *et al*., 2009**). Members of the family Rutaceae are known for their distinctive scent and the presence of oil glands on the reverse side of their leaves. Essential oil derived from several Rutaceae species have been incorporated into skin care products. The South African indigenous plants *Coleonema album* and *Agathosma betulina* are

among the most popularly used medicinal plant in the family Rutaceae in the South African traditional medicine system. This has resulted to the local cultivation of these plants by farmers, indigenous nurseries and National conservation bodies. Essential oil of *C. album* and *Agathosma betulina* are marketed by several local and international natural product companies. *Buchu*, *Agathosma betulina* oil is used for medicinal and cosmetic purposes (**VAN WYK *et al*., 1997**). Globally, *Buchu* is highly sought after for its essential oil which has a high diosphenol content (**MOOLLA *et al*., 2007**). **MOOLLA and VILJOEN** (**2008**) reported that a kilograme of *A. betulina* oil sells on the international market for about 700 Euros.

The biological activities of essential oils have been extensively researched and several reports exist in the scientific literature. However, the growth and morphological changes in the structure of dermatophytes exposed to essential oil volatiles/scents is not well documented. The aim of the study was to: investigate the effect of 'commercial' *A. betulina* and *C. album* essential oil on *Trichophyton rubrum* and *T. mentagrophytes* growth and morphology, and analyse the compounds present in the essential oil of the two plants.

7.2. Materials and methods

7.2.1. Antifungal activity of essential oil volatiles and essential oil analysis

Essential oil of *C. album* (EOCM10) and *A. betulina* (EOBUCB10) were purchased from Still Pure, Cilliers Street, Western Cape. Yeast Malt broth (Sigma Y3752) and Yeast Malt agar (Y3127) were purchased from Sigma, *Trichophyton rubrum* (ATCC 28188) and *T. mentagrophytes* (ATCC 9533) were purchased from Quantum Biotechnologies, Randburg, South Africa. *Agathosma betulina* and *C. album* oil (10 µl each) was subjected to analysis using GCMS (same as in section 6.2 of chapter 6). The percentages of the major constituents (≥1%) were recorded individually while the minor constituents (≤1%) were recorded as 'others' *Trichophyton rubrum* and *T. mentagrophytes* were grown in yeast malt broth for one week and the fungal suspension was subcultured on fresh yeast malt agar for one week. The plate showed good mycelia growth from which 1.4 cm diameter of mycelia was taken using an autoclaved pill vial. The agar supporting the mycelia was sliced off and the mycelia were placed on fresh agar plates. Filter paper was placed on the upper lid of the fresh agar petri dishes. The filter paper was impregnated with 10, 20

and 40 µl of essential oil in such a way that the mycelia placed on the agar plate are directly exposed to the essential oil volatiles. A plate with similar diameter (1.4 cm) of *Trichophyton rubrum* and *T. mentagrophytes* but without essential oil was used as control. The plates were incubated for a week. The diameter of the mycelia was recorded after 7 days of incubation and the Fungal Growth Index (FGI) was calculated.

$$FGI = \frac{\text{Final diameter of mycelia} - \text{Initial diameter of mycelia}}{\text{Initial diameter of mycelia}} \times 100$$

The mycelia from each petri dish were subcultured on fresh agar plates and incubated for another seven days at the same temperature in order to determine if the treatments have a fungistatic or fungicidal effect.

7.2.2. Effect of essential oil volatiles on morphology of fungal mycelia

Sections from the initial mycelia 1.4 cm used, and the outgrowth after one week was prepared for viewing under scanning electron microscope (SEM) (Zeiss EVO LS15, Carl Zeiss, Germany). Sections were fixed in 3% buffered glutaraldehyde overnight, washed twice in 0.05 M of sodium cacodylate buffer, dehydrated in 10, 30, 50, 70 and 90% ethanol for 10 min each and 3 times in 100% ethanol for 10 mins. Samples were subjected to critical point drying using a Quorum K850 Critical Point Dryer, United Kingdom, mounted on SEM stubs and sputter coated with gold using Eiko IB-3 Ion Sputter Coater, Japan. Dried coated samples were viewed with the SEM using 2000 and 5000 magnification.

7.3. Results

7.3.1. Antifungal activity of essential oil volatiles and essential oil analysis

There was inhibition/reduction of fungal growth in all the plates exposed to essential oil volatiles. However, the rates of inhibition varied between the fungal species, oil tested and the different volumes tested. The volatiles from *A. betulina* oil has a stronger inhibitory effect than *C. album* oil volatiles; with the highest inhibition recorded in 40 µl. Susceptibility to essential oil volatiles is more pronounced in *T. rubrum* than in *T. mentagrophytes* (**Table 7.1 and Fig 7.1**). The best inhibition was

recorded in *T. rubrum* exposed to volatiles from 40 µl of *A. betulina* oil (**Table 7.1**) with a fungal growth index of **0%**, indicating its fungicidal activity (**Table 7.2**). The same volume of *A. betulina* oil had a fungistatic effect on *T. mentagrophytes* with a FGI of **7%** (**Table7. 2**).

Mycelia from *T. rubrum* exposed to 40 µl essential oil volatiles did not grow when subcultured on a fresh plate of agar. This is an indication that the volatiles totally degraded the hyphae and spores and hence has a fungicidal effect. However, all the other petri dishes subcultured produced mycelia after 7 days of incubation. Essential oil analysis of *A. betulina* and *C. album* using GCMS revealed the presence of terpenes. The major components of the essential oil of *A. betulina* is 1-menthone (**36.35%**), D-limonene (**29.84%**) and 6-methoxy-3(2H)-pyridazinone (**8.35%**) while that of *C. album* is β-pinene (**28.92%**), (1R)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (**27.37%**) and β-phellandrene (**22.70%**) (**Table 7.3**). The essential oils from both plant species is composed mainly of volatile monoterpenes.

7.3.2. Effect of essential oil volatiles on morphology of fungi mycelia

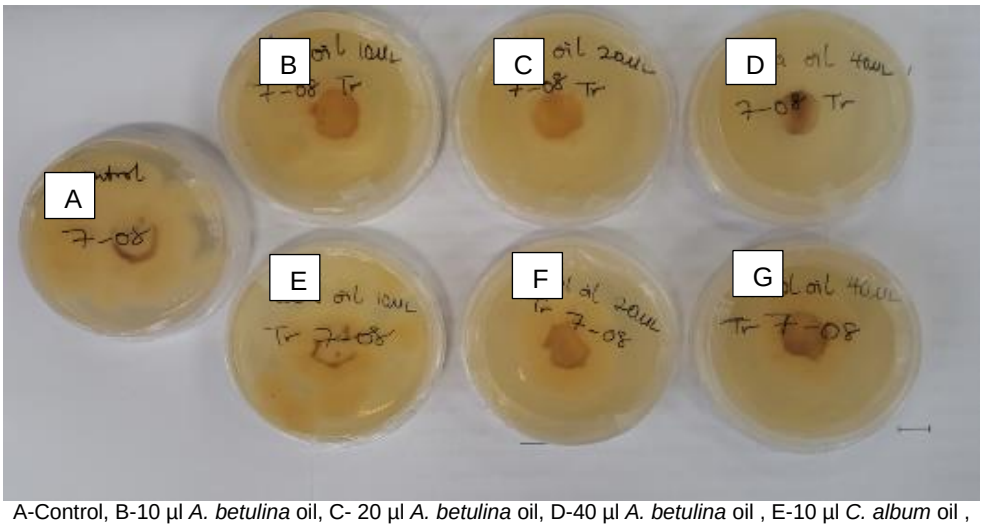
The mode of action of the essential oil volatiles was revealed using an electron microscope. The volatiles inhibited fungal growth by inhibiting the production of spores (**Figures 7.3 and 7.4**). The best mode of action recorded (40 µl *A. betulina* oil) revealed the total destruction of hyphae and spores (**Figures 7.3 and 7.5**) of *T. rubrum*. This total destruction is irreversible as the subcultured mycelia did not grow after 7 days of incubation on fresh agar, thus indicating the fungicidal action of the essential oil volatiles.

Table 7.1: Effect of A. betulina (Aga oil) and C. album essential oil (Col oil) volatiles from different volumes on mycelia growth/diameter of T. rubrum and T. mentagrophytes as compared to the starting material and control

Fungus tested	10 µl		20 µl		40 µl		Starting material	Control
	Col oil	Aga oil	Col oil	Aga oil	Col oil	Aga oil		
Trichophyton rubrum diameter (cm)	3.75	2.75	3.55	1.9	3.4	1.4	1.4	3.9
Trichophyton mentagrophytes diameter (cm)	4.05	2.9	3.68	2.55	3.28	1.50	1.4	5.05

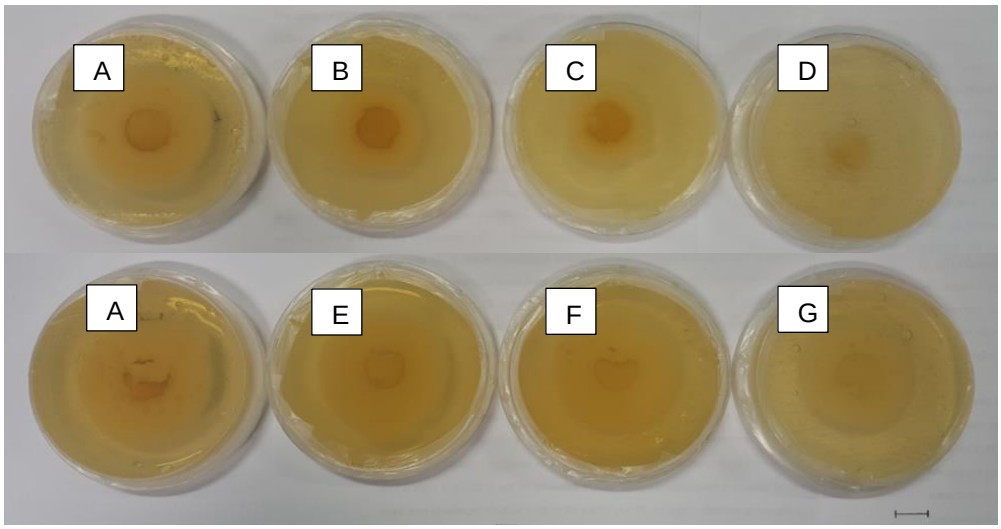
Table 7.2: Effect of A. betulina and C. album essential oil volatiles on Fungal Growth Index (%) of T. rubrum and T. mentagrophytes as compared to the control

Fungus tested	10 µl FGI (%)		20 µl FGI (%)		40 µl FGI (%)		Control FGI (%)
	Col oil	Aga oil	Col oil	Aga oil	Col oil	Aga oil	
Trichophyton rubrum	167	96	153	36	143	0	179
Trichophyton mentagrophytes	189	107	163	82	134	7	261



A-Control, B-10 µl *A. betulina* oil, C- 20 µl *A. betulina* oil, D-40 µl *A. betulina* oil , E-10 µl *C. album* oil , F-20 µl *C. album* oil, G-40 µl *C. album* oil

Figure 7.1: Effect of A. betulina and C. album essential oil volatiles on mycelia growth/diameter of Trichophyton rubrum on yeast malt agar after 7 days

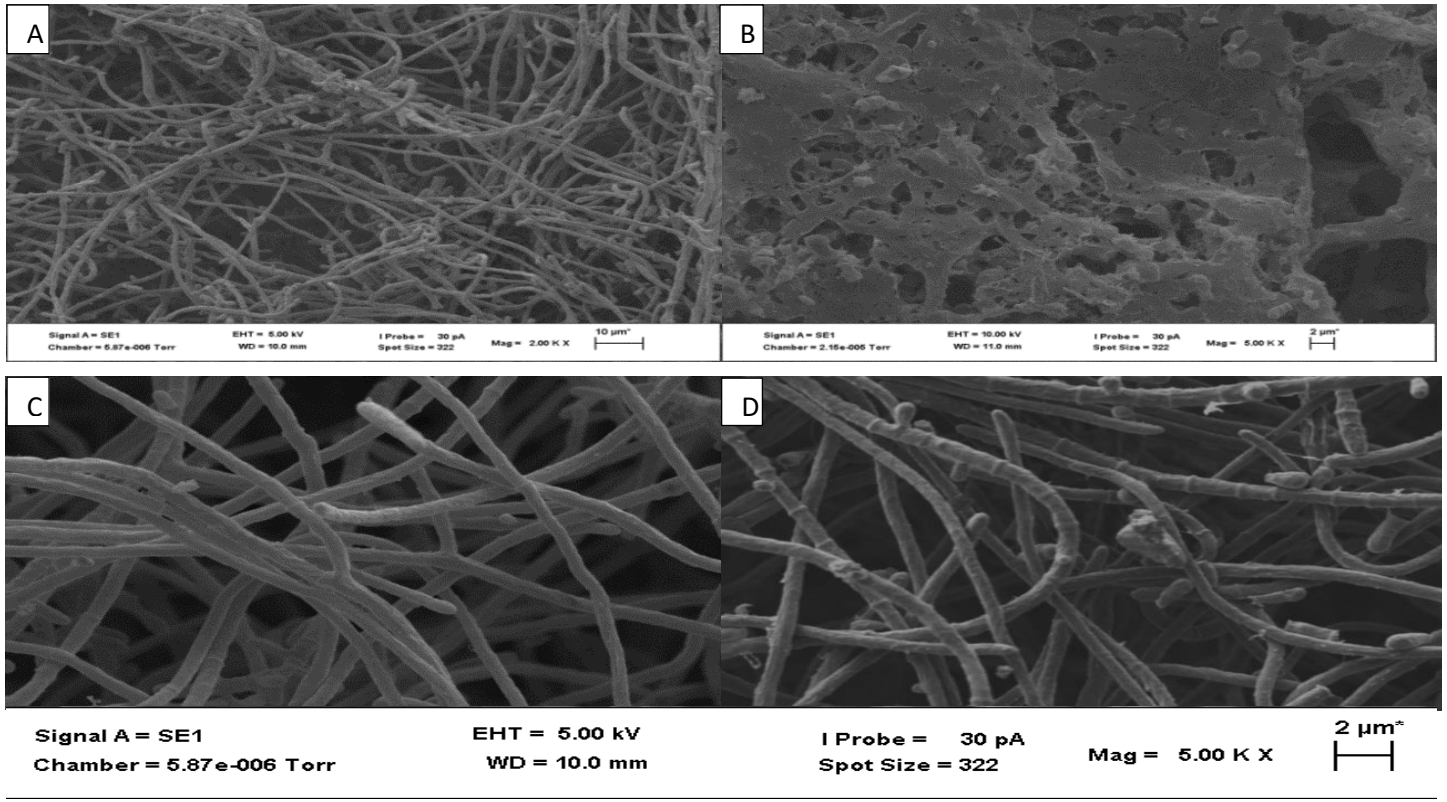


A-Control, B-10 µl *A. betulina* oil, C- 20 µl *A. betulina* oil, D-40 µl *A. betulina* oil, E-Control, F-10 µl *C. album* oil, G-20 µl *C. album* oil, H-40 µl *C. album* oil

Figure 7.2: Effect of A. betulina and C. album essential oil volatiles on mycelia growth/diameter of T. mentagrophytes on yeast malt agar after 7 days

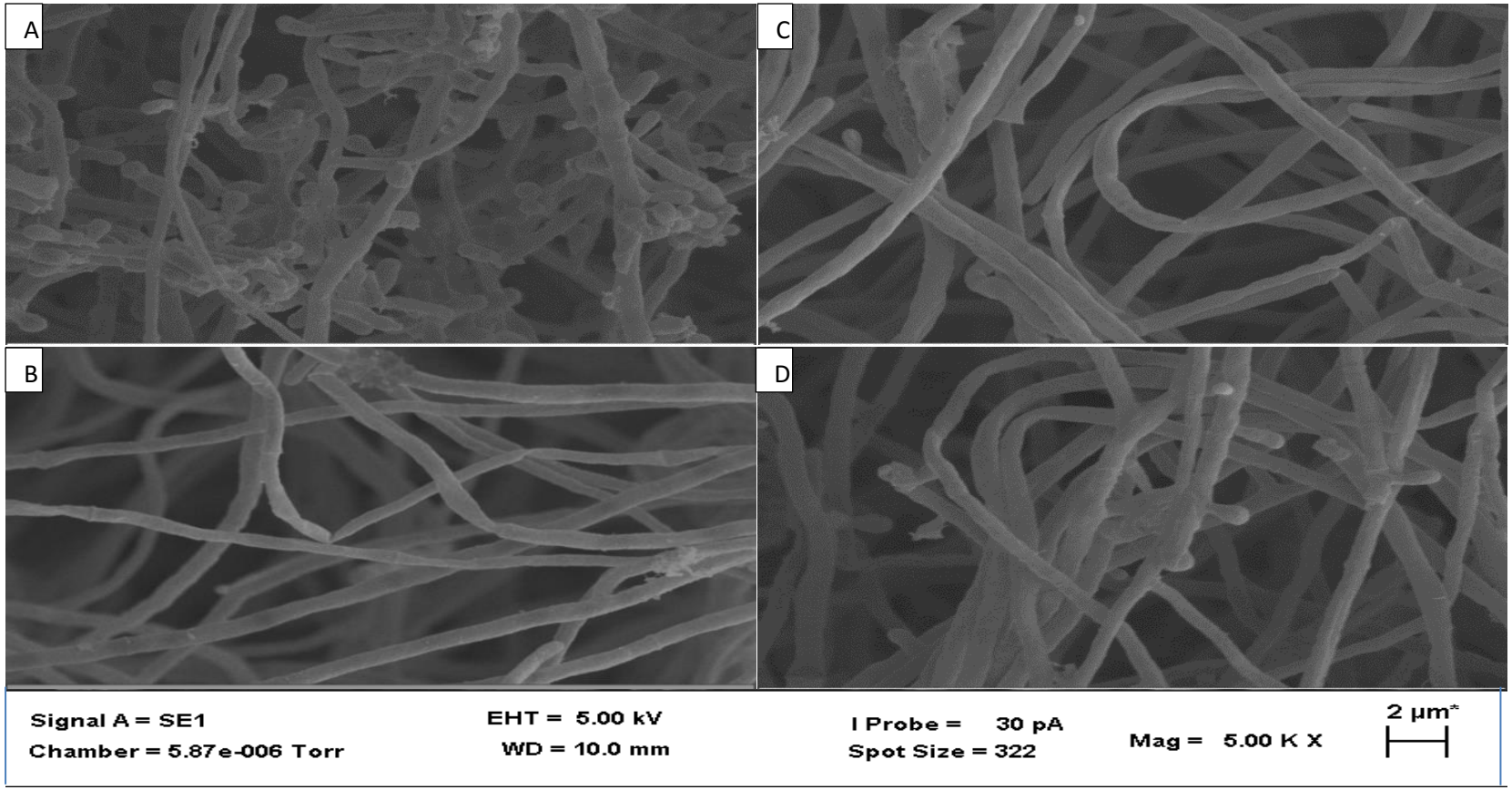
Table 7.3: List of compounds in commercial A. betulina and C. album essential oils

Agathosma betulina		*Coleonema album*	
Compound	(%)	Compound	(%)
1-Mentone	36.35	β-Pinene	28.92
D-Limonene	29.84	(1R)-2,6,6-Trimethyl bicyclo[3.1.1]hept-2-ene	27.37
6-Methoxy-3(2H)-pyridazinone	8.35	β-Phellandrene	22.70
1,4,4-trimethyl-3-Ethoxy-4-methoxyphenol	7.25	Trans-β-Ocimene	7.14
Eucalyptol	4.43	β-Ocimene	4.27
Cyclohexanone,2-(2-methylpropylidene	3.05	Gamma-Terpinene	1.44
Pulegone	2.97	(+)-4-Carene	1.37
β-Myrcene	2.25	Eucalyptol	1.35
(1R)-2,6,6-Trimethyl bicyclo[3.1.1]hept-2-ene	1.33	p-Cymene	1.09
Others	4.18	Others	4.35



A-Control, B-40 µl *A. betulina* oil, C- 20 µl *A. betulina* oil, D-40 µl *C. album* oil

Figure 7.3: Effect of A. betulina and C. album essential oil volatiles on T. rubrum morphology



A- Control, B-40 µl *A. betulina* oil, C-20 µl *A. betulina* oil, D-40 µl *C. album* oil

Figure 7.4: Effect of A. betulina and C. album essential oil volatiles on morphology of T. mentagrophytes

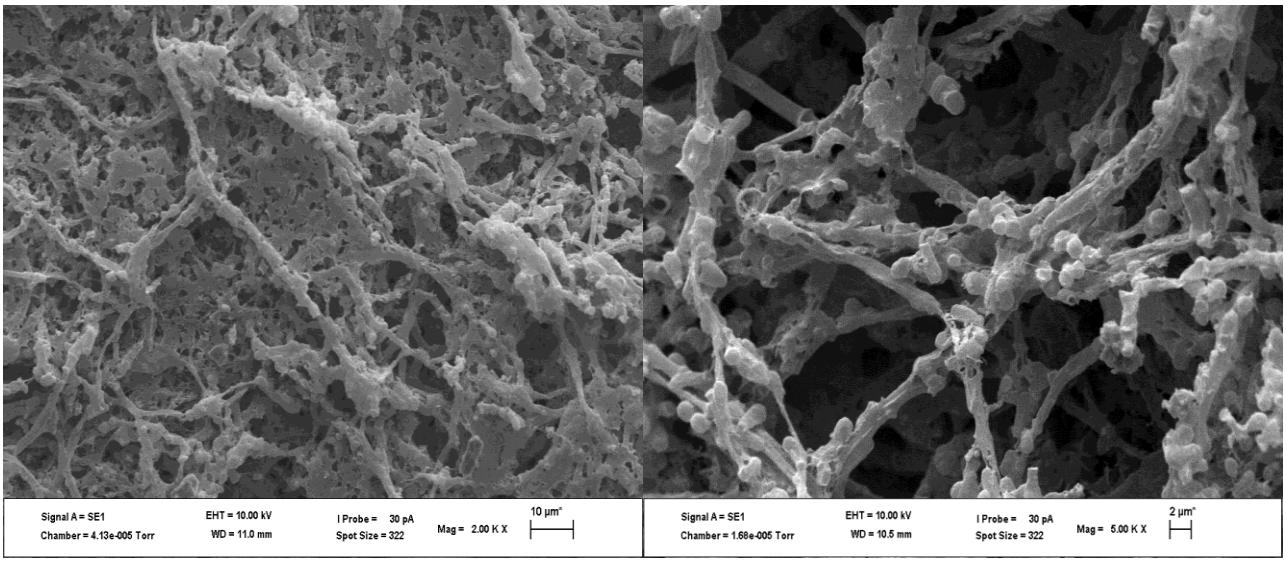


Figure 7.5: Alteration of T. rubrum morphology by A. betulina essential oil (40 µl) volatiles

7.4. Discussion

Essential oils can have a practical use in the inhibition of dermatophyte growth (**PEREIRA *et al*., 2011)**. *Trichophyton* species produce hyphae which are capable of penetrating the innermost skin layer and cause damage in the host (**ZURITA and HAY, 1987; GUPTA *et al*., 2003**). Hence, researchers are investigating the potential of essential oils in inhibiting mycelial growth of pathogenic fungi as a result of their importance in the manifestation of mycosis (**PEREIRA *et al*., 2011)**.

In this study, the essential oil volatiles inhibited the production of spores on the hyphae. The spores are asexual reproductive structures which can initiate the development of skin disease under favourable conditions and are capable of transmitting the infections to other individuals. In *T. rubrum*, asexual sporulation is an important means of propagation and the conidia produced asexually are regarded as the primary cause of skin disease in humans (**LENG *et al*., 2008**). The conidia which are a primary means of dispersion also provide a “safe house” for the filamentous fungal genome during adverse environmental conditions (**LENG *et al*., 2008**). Asexual sporulation remains a vital means of reproduction for the fungus *T. rubrum* (**LENG *et al*., 2008**). Under favourable conditions, *T. rubrum* produces several pear-shaped or club-shaped microconidia, which are assumed to be the primary cause of skin and nail infections in humans (**KANE, 1997; GUPTA *et al*., 2003**).

In many filamentous fungi, the production of arthrospores and microconidia occurs regularly during the asexual cycle and can be differentiated using their morphology and development (**COELHO *et al*., 2008**). Microconidia are produced on the ends of conidiophores which are formed laterally from the hyphae, while arthrospores are formed by the fragmentation of hyphae resulting from prolonged cultivation (**HOFF *et al*., 2005**). Arthroconidia are genetically programmed disarticulation of septate hyphae (**RASHID, 2001**) which forms both short-chained, spherical or oval single arthrospores (**HASHIMOTO and BLUMENTHAL, 1977**) and is considered the major cause of infections in the host (**GUPTA *et al*., 2003**).

Fungal spores (conidia) can lodge in the furrows and crevices of dry, cracked skin and subsequently penetrate under the top layers of the epidermis. When individuals with skin diseases scratch the area affected, the spores are released unto the fingers, nails, palm and surfaces such as bedsheets, carpets, blankets, clothing and couch. When fingers and nails with spores are used to touch other parts of the body such as the head, neck, groin, feet and face that were not previously infected,

the spores bring about skin disease under favourable condition for fungal growth. The spores released onto surfaces remain there until they are transferred to an individual with a low or compromised immune system and then cause skin disease. Hence, skin diseases can be transferred from one member of the family to the other through fungal spores.

Spores are metabolically active cells enhanced with lipid-containing vacuoles and intracellular organs and thus have the ability to be resistant to some antifungal agents and adverse environmental conditions (**YAZDANPARAST and BARTON, 2006**). However, in spite of their importance in pathogenesis and physiology of skin disease, the conidia of *T. rubrum* remain little studied (**LENG *et al*., 2008**). The studies on *T. rubrum* focus mainly on issues such as epidemiology, clinical case reports, strain relatedness, and drug susceptibilities (**KANE, 1997**). In this study, the inhibitory effect of essential oil volatiles on the production and its deleterious effect on the morphology of conidia/spores were demonstrated. This study reveals the ability of essential oil volatiles to stop the transmission of skin disease and reinfection of treated individuals.

The components of essential oils are important as their qualitative and quantitative composition determine the characteristics of the oils, which in turn could be responsible for its antimicrobial potentials (**DUGO *et al*., 2000**). Various methods have been used to analyse the antimicrobial properties of essential oils. The antimicrobial effect of essential oils is determined by *in vitro* screening methods, usually that of the disc diffusion method which involves the placing of an impregnated (with antimicrobial agent) filter disc on the surface of inoculated agar plates and inhibition of growth is observed (**BAUER *et al*., 1966; DEANS and RITCHIE, 1987; SMITH-PALMER *et al*., 1998; SKANDAMIS and NYCHAS, 2001**; **WANNISSORN *et al*., 2005; FISHER and PHILLIPS, 2006**). In the late 1950s, this method was adopted for the screening of essential oil vapours by placing the filter disc on the lid of the petri dish (**MARUZZELLA and SICURELLA, 1960**).

Another factor to consider when determining the MIC of an essential oil is that the absolute concentration of inhibition can be between the lowest MIC and the next concentration in which growth is observed (**FISHER and PHILLIPS, 2008**). Minimum inhibitory doses (MID), which is the minimum dose of the essential oil volatiles to inhibit growth is determined by a parallel method to the MIC (**FISHER and PHILLIPS, 2008**). This is carried out in a sealed container by either placing the

essential oil on the surface of the container to give a slow evaporation or an impregnated disc to give a faster evaporation (**INOUYE *et al*., 2003; FISHER and PHILLIPS, 2006**). The incubation temperature can have an effect on the microbial inhibition when using this method as the MID value is dependent on the evaporation rate of the volatile components (**FISHER and PHILLIPS, 2008**). Another method of assessing antimicrobial activity is to make a well in the inoculated agar and add the test substance (**DEANS and RITCHIE, 1987; DORMAN and DEANS, 2000**). None of these screening methods is quantitative as they only give an indication of the essential oils having antimicrobial properties (**FISHER and PHILLIPS, 2008**).

In this study, the agar plate method was used in the presence of the fungi mycelia. This method was adopted in order to mimic the presence and full manifestation of skin disease and thus test if the essential oil volatiles will inhibit the spread of the disease or growth of the fungus and its reproductive structures or otherwise. The majority of the methods reported in the literature involve subculturing of the fungal suspension on fresh agar plates and introducing the essential oil volatiles to the plate by placing an impregnated disc near the middle of the agar on the upper lid of the petri dish. These methods demonstrate the introduction of the essential oil/antifungal and the disease or fungi at the same time. However, in clinical practice and traditional medicine, an antimicrobial/antifungal is only prescribed when there is an existing disease or infection and there is already a full manifestation of the skin disease in which there is the presence of adequate reproductive structures (spores) capable of aggravating the degree of the disease.

The antimicrobial effect of essential oils vapour has been assessed using the direct disc diffusion method and the outcome of the assessment indicated that only the water-soluble components diffused across the agar while the re-deposition of the vapourised components on the surface of the agar accounted for the remainder of the inhibition (**FISHER and PHILLIPS 2008**). The Minimum Inhibitory Concentrations (MICs) of essential oils can be assessed by two protocols (**FISHER and PHILLIPS, 2008**). Visible growth can be observed using the agar dilution method; while optical density, absorbance or viable counts are measured in addition to visible growth observed using the broth dilution method (**BURT, 2004**). The MIC is determined as the lowest concentration at which growth is inhibited. The main problem with determination of the level of antimicrobial activities of essential oils in this way is their hydrophobic nature which makes them insoluble in water-based media (**FISHER and**

PHILLIPS, 2008). This problem can be solved by the use of emulsifiers such as Tween 20 or 80 alone or in combination with solvents such as acetone, polyethylene glycol or ethanol (**TASSOU *et al*., 2000; BURT, 2004; FISHER and PHILLIPS, 2006**). However, the presence of surfactants has a major influence on the results of antimicrobial studies (**MATSUZAKI *et al*, 2013**). These reasons prompted the use of fungi mycelia and essential oil in the form of volatiles in this study.

Essential oils are a complex mixture of naturally occurring compounds mainly monoterpenes and sesquiterpenes and are considered alternative natural antimicrobial agents (**CHEE *et al.,* 2009**). Evaporation of essential oils results from external factors such as temperature, humidity, concentration and pressure (**AUMO *et al*., 2006**) i.e. the diffusion across a homogenous membrane increases with an increase in temperature (**CLARYS *et al*., 1998**) and hence determines the level of antimicrobial activity *in vitro*. Volatile components of essential oils have been reported to exhibit antifungal effects (**JAIN and AGRAWAL, 2002**). A naturally occurring monoterpene, limonene has been detected in essential oil of several plants (**PEPELJNAK *et al*., 2005; SONBOLI *et al*., 2006**). Limonene is a volatile, colourless liquid with an intense aroma of oranges (**CHEE *et al.,* 2009**). Studies have shown that terpenes and terpenoids may have antioxidant and antimicrobial properties against pathogenic fungi, including dermatophytes (**HAMMER *et al*., 2000; PAULI, 2006; BARCHIESI *et al*., 2008; PALMEIRA-DE-OLIVEIRA *et al*., 2009**).

The antifungals used clinically in the therapy of skin diseases vary in their mode of action. Mode of action involves: direct membrane damage, disruption of microtubules and inhibition of mitosis, inhibition of other processes within the fungal cell such as protein, glucan and ergosterol synthesis (**MURRAY *et al*., 2013**). Damage to microorganism membranes, collapse of the proton pump, cytoplasm granulation and break down of the electron transport chain are some events possibly related to the antifungal property of essential oils (**SIKKEMA *et al*., 1995; COX *et al*., 2000**). Several records in the scientific literature worldwide reported that macromolecules found in fungi of which functionality is related to growth, survival, cell morphogenesis and virulence, are identified as promising targets for new antifungal agents (**ODDS *et al*., 2003**). However, there is little study related to the investigation of compounds, which are responsible for the bioactivities of essential oils, and its exact mechanism of the antimicrobial action (**PARK *et al*., 2009**).

In this study, *A. betulina* essential oil analysis revealed two monoterpenes as the most abundant compounds. Menthone and limonene have been reported to have antimicrobial activities *in vitro*. Menthone had *in vitro* antibacterial activity against *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (**ISCAN *et al*., 2002; SCHELZ *et al*., 2006**). Menthone showed antifungal activity against *Botrytis cinerea* and is the major antifungal agent in *Schizonepeta tenuifolia* (**LEE *et al*., 2007**). Hence, the presence of menthone in the *A. betulina* essential oil used in this study could be the reason for its remarkable antifungal activity.

Limonene is a volatile compound (**RANGANNA *et al*., 1983; POURBAFRANI *et al*., 2010**) with antifungal activity (**OMRAN *et al*., 2011**).It prevented the growth of fungal mycelium within 2 h (**MATSUOKA, 1990**). Limonene had fungicidal effects on *T. rubrum*, induced changes in its cell membrane integrity and metabolic activity *in vitro* (**CHEE *et al.,* 2009**). Fungicidal agents are more preferred as compared to fungistatic agents for the treatment of fungal infections as the utilization of fungistatic agents could lead to recurrence of the infection (**CHEE *et al.,* 2009**). In this study, *A. betulina* essential oil (40 µl) volatiles displayed fungicidal effects on *T. rubrum* and hence could offer an effective antifungal of natural origin. Its effect on *T. mentagrophytes* is however fungistatic. The variation in the susceptibility of microorganisms to essential oils could be as a result of the variation in the cell wall structure, lipid and protein composition of the cytoplasmic membrane (**WALTERS and ROBERTS, 2008**)**.**

The essential oil components of *C. album* reported in this Chapter is different from the profile of the *C. album* essential oil reported in the previous Chapter (Chapter 6). This could be as a result of the difference in sources of the oil as the oil analysed in this Chapter was purchased from an organic product company in South Africa while the sample used in the previous Chapter was derived from plants sourced from the South African National Botanical Garden in Kirstenbosch. The source of the *C. album* leaves from which the essential oil used in this Chapter was sourced is not known.

Harvesting related factors such as season of harvest, geographical location and age of the plant at the time of harvest play a crucial role on the essential oil yield and most importantly the composition of the bioactive compounds present in the

essential oil (**PREEDY, 2015**). Cavacrol, the major component of oregano essential oil was higher during the drier and warmer season (70.75-84.88%) while several other compounds accumulated at higher levels during the wetter and colder season along with very low levels of cavacrol (56.46-75.12%) (**KARAMANOS and SOTIROPOULOU, 2013**). In the essential oil sourced from typically cultivated *Origanum vulgare* (subspecies *hirtum*), thymol and y-terpinene were the main components while the commercial sample showed a different profile as carvacrol (>77%) was the main component (**BONFANTI *et al*., 2012**).

BAŞER *et al*., (**2006**) analysed the essential oil of *C. pulchellum* sourced from 3 different geographical areas within South Africa and there was slight similarity in the major components but there were differences in the levels of the similar components. Monoterpenes dominated in all the oil samples tested. Several small scale industries use essential oil purchased from commercial companies for the production of creams, lotions, soap, body wash and ointments. The antifungal activity and bioactive compounds of commercial essential oil was investigated in this study in order to compare the antifungal activity and presence of active compounds to the essential oil extracted from plants sourced from the garden to the essential oil purchased from the commercial companies. Another reason was also to ensure that the essential oil purchased commercially contains active ingredients with activities that will make its use by the populace worthwhile.

7.5. Conclusions

The laboratory prepared *C. album* EO gave a remarkable and higher antifungal activity(Chapter 5) as compared with the commercial EO (of the same species) used in this part of the study. The essential oil volatiles of *A. betulina* had good inhibitory effect on the mycelia growth of *T. rubrum* and *T. mentagrophytes*. The fungicidal effect of the essential oil volatiles on *T. rubrum* is noteworthy. The mode of action of *A. betulina* essential oil volatiles on the growth of *T. rubrum* mycelia is through the inhibition of spore growth and production; alteration of the morphology by total destruction of the hyphae and spores. Essential oil induced different actions on the mycelia at different concentrations. The major components of the essential oil of *A. betulina*, 1-menthone (36.35%) and D-limonene (29.84%) are probably responsible for this activity as they have been previously reported to

possess antimicrobial activity. The remarkable activity recorded in this study give an insight to the potential of *A. betulina* essential oil as a skin antifungal.

CHAPTER 8. GENERAL DISCUSSION

A combination of inflammatory conditions and an overwhelmed antioxidant system could result in pathological levels of ROS and thus, oxidative stress which further exacerbates the disease state (**WAGENER *et al*., 2013**). Oxidative stress plays a crucial role of in pathogenesis of dermatologic diseases (**SIVARANJANI *et al*., 2013**; **KRUK and DUCHNIK, 2014**) and affects the defensive systems against ROS/RNS (**KRUK and DUCHNIK (2014**). ROS and RNS are produced during normal cellular metabolism, and can be either beneficial or harmful to the organism (**HALLIWELL and GUTTERIDGE, 1999; TROUBA *et al*., 2002; VALKO *et al.,* 2006**). Antioxidants block the damaging effects of ROS and can inhibit and/or reverse several processes that contribute to epidermal toxicity and disease (**TROUBA *et al*., 2002**). Eczema or atopic dermatitis' refers to several persistent skin conditions with various hallmarks such as chronic relapsing form of skin inflammation (**SIVARANJANI *et al*., 2013; WAGENER *et al*., 2013**), disturbance of the epidermal-barrier function (**SHEU *et al*., 1997; SHEU *et al*., 1998; RAMOS-E-SILVA, and JACQUES, 2012**) which at its climax results in dry skin (**SIVARANJANI *et al*., 2013**).

Eczema is a chronic inflammatory skin disease which infects individuals globally. In most people with eczema, the development of skin inflammation occurs first before any skin lesions become visible during the manifestation of the disease (**SIVARANJANI *et al*., 2013**). The skin is continuously subjected to ROS formation through UV irradiation and environmental exposure; and is rich in antioxidant systems with the ability to lower the ROS levels (**WAGENER *et al*., 2013**). Oxidative stress leads to the production of oxidation products, such as 4-hydroxy-2-nonenal or malonaldehyde (**MEFFERT *et al*., 1976**), which denature proteins, alter apoptosis, and cause the release of pro-inflammatory mediators, such as cytokines, which can be critical in the induction of inflammatory skin diseases (**MEFFERT *et al*., 1976**). A decrease in inflammation and an improved rate of recovery could be achieved in the treatment of inflammatory skin conditions by targeting oxidative stress (**WAGENER *et al*., 2013**).

Vitamin E and vitamin A are lipophilic antioxidants while vitamin C is a water soluble vitamin (**SIVARANJANI *et al*., 2013**). Vitamin E acts as a chain breaking antioxidant by removing free radicals and it inhibits their peroxidative effects on

unsaturated lipids of the cell membrane (**HAMID *et al*., 2010**). Vitamin C acts to neutralize free radicals through the donation of H+ ions to free radical R, (**HOSSAIN and ASADA, 1985**). Vitamin C not only neutralizes the alkoxyl, hydroxyl, and peroxyl radicals by donating H+; ascorbate (a form of vitamin C) can also neutralize the radical forms of some other antioxidants such as vitamin E and GSH (**BUETTNER and JURKIEWICZ, 1996**). Beta-carotenes display effective scavenging activity against alkoxyl and peroxyl radicals (**DEBAJYOTHI, 2008**). People deficient in essential nutrients such as beta-carotene, the vitamin B complex, C and E often suffer from the drying of the skin (**TABASSUM and HAMDANI, 2014**). In the treatment of atopic dermatitis, the use of natural antioxidants like vitamins A, C and E in combination with corticosteroids and immunosuppressant is a valuable addition to the conventional therapy (**SIVARANJANI *et al*., 2013**).

In traditional medicine, plant essential oil, extracts, tinctures and formulations are used as a source of antioxidants in the place of beta-carotene and vitamins. These whilst inhibiting the growth of the fungi responsible for the skin disease also restore skin health by combating inflammation which results from oxidative stress, restores the skin barrier function and improve skin texture. A combination of all these functions restores the skin health to normality and in the process adds to skin beauty. However, a very important factor to consider is the degree of antifungal activity of the plant essential oil, extract, tincture or formulation. A fungicidal activity results in better treatment of skin disease as compared to a fungistatic activity as fungistatic activity results in prolonged use of an antifungal and can also lead to resistance of the organism which is a major problem often encountered in clinical treatment of skin diseases. Some of the essential oil tested in this study exhibited remarkable fungicidal action.

The incidence of resistance to antifungal agents may be increasing (**DENNING, 1995; JOHNSON *et al*., 1995; WALSH *et al*., 2000; DODGSON *et al*., 2004; PUJOL *et al*., 2004; BLIGNAUT *et al*., 2005**) with drug resistant fungal strains particularly common causative pathogens of infection in high-risk patient groups, such as HIV/AIDS patients (**JOHNSON *et al*., 1995; PANKHURST, 2001**). The reports of recurrences of infections are usually attributed to the discontinuation of therapy (**SOARES *et al.,* 2013**). The frequent use of azoles (especially fluconazole) in the treatment and prevention of fungal infections has led to reports of emerging resistance to antifungal agents (**MURRAY *et al*., 2013**). The resistance to azoles has

been reported in 32% of symptomatic and in 14% of asymptomatic patients (**REVANKAR *et al*., 1996; MAENZA *et al*., 1997; MARTINS *et al*., 1997**). The prevalence correlates with the amount of cluster of differentiation 4 (CD_4) cells, the fungal load, and the duration and doses of therapy (**MAENZA *et al*., 1996**). **VUFFRAY *et al*., 1994**, showed that resistance can also occur in patients with high single doses of fluconazole. **PELLETIER *et al.,*** (**2000**) documented resistance of *C. albicans* to clotrimazole in HIV-infected children. Antifungal resistance genes are not transmissible from cell to cell in fungi (**MURRAY *et al*., 2013**).

The biochemical mechanisms that may be responsible for drug resistance in fungus involves a decrease in drug uptake, structural alterations in the target site and an increase in drug efflux or in intracellular target levels, gene amplification, gene transfer, gene deletion, point mutations, loss of cis -and trans-acting regulatory elements and transcriptional activation (**KAMAI *et al.*, 2004**). Fungal resistance to antifungal treatment develops slowly and involves the emergence of intrinsically resistant species or a gradual and stepwise alteration of the functions and structures of the fungal cell that leads to resistance to an agent which the species have been prior exposed to (**MURRAY *et al*., 2013**).

The use of immune-modulating drugs, concomitant infections such as HIV and the nutritional status of the host are more crucial factors that determine the outcome of the infection than the ability of an antifungal to inhibit or totally destroy the infecting organism (**MURRAY *et al.,* 2013**). The *in vitro* resistance of an isolate can be classified as either intrinsic or acquired (**SOARES *et al.,* 2013**). Intrinsic resistance allows all normal members of a species to tolerate a particular drug while acquired resistance means that a resistant strain emerges from a population that was previously drug-sensitive (**KAMAI *et al.*, 2004**). The clinical resistance to griseofulvin, or relapses after treatment, is common in dermatophytoses (**SOARES *et al.,* 2013**).

Fluconazole and itraconazole are still not the perfect antifungals, since they have some non-negligible drug interactions with such drugs that are used in chemotherapy or with AIDS treatment. These interactions can result in a decrease in azole concentration or even an increase in toxicity (**ALBENGRES *et al.,* 1998**). At present, antibiotics and antifungals play a vital role in the control of fungal diseases (**SOARES *et al.,* 2013**). However, some of these antifungals have shortcomings such as toxicity, fungistatic activity and a limited spectrum of action or resistance

(**GARCÍA-SOSA *et al.*, 2011**). As a result of these shortcomings, there is a need to develop innovative antifungal products of natural origin (**ZIMMERMAM-FRANCO *et al*., 2013**). It is therefore important to find an effective solution to the increasing incidence of skin diseases, most especially the issue of fungi resistance to antibiotics.

Traditional medicinal resources, especially plants; play a major role in the management of skin diseases (**ABBASI *et al*., 2010**). Among the rural people of Maputaland (South Africa), at least 50 remedies derived from 47 plant species are utilized for the treatment of skin disorders (**DE WET *et al*., 2013**). **EGHAREVBA and IKHATUA** (**2008**) investigated the ethno-medical uses of plants used in the treatment of various skin diseases in Ovia North East of Edo State, Nigeria. They discovered that a decoction of *Cassia alata* leaves added to kerosene is applied on the skin to treat eczema and ringworm. *Ficus exasperate* leaves are rubbed on the affected part of the skin and *Vitex doniana* leaf juice is applied on the skin to treat ringworm (**EGHAREVBA and IKHATUA, 2008**).

Chamomile extract, essential oil and its isolated bioactive compounds have been reported to possess anti-inflammatory activity and are thus useful in the treatment of skin inflammation, eczema and the prevention of skin disorders (**CARLE and GOMAA, 1992; HOERMANN and KORTING, 1994; BRUNETON, 1999**). Other plant extracts, essential oil and bioactive compounds used in skin and hair care; and treatment of skin diseases are well documented in scientific literature. Extracts of *Aloe vera* acts to reverse aging by stimulating the synthesis of collagen and elastin fibres (**DAVIS *et al*., 1994**). Aloe is an ingredient in many cosmetics as it heals, softens and ultimately moisturizes the skin (**GEDIYA *et al*., 2011**). A study revealed the effectiveness of extra virgin coconut oil as a moisturizer (**AGERO and VERALLO-ROWELL, 2004**). Coconut oil helped to prevent protein loss during the wet combing of hair when used for fourteen hours prior to combing (**AARTI and MOHILE, 2003**).

Several essential oils have anti-inflammatory activity including bisabolol a compound found in chamomile and the volatile oil of turmeric (**RAO *et al.*, 1982; PUROHIT and KAPSNER, 1994**). Essential oils have various benefits which include shine or conditioning in hair care products, and improvement of skin elasticity **(ABURJAI and NATSHEH, 2003)**. The efficacy of tea tree oil as an antifungal (**NENOFF *et al.*, 1996; HAMMER *et al.*, 1998; WESELER *et al.*, 2002**), antiseptic

(**BUDHIRAJA *et al.*, 1999**), antiviral and topical antibacterial agent (**CARSON and RILEY, 1994; CARSON and RILEY, 1995; CARSON *et al.*, 1995; SHAPIRO *et al.*, 1996; COX *et al.*, 2000**) have been confirmed by scientific studies and clinical trials. In the treatment of dandruff caused by the yeast *Pityrosporum ovale,* tea tree oil is useful as a result of its antifungal properties (**SATCHELL *et al.*, 2002**).

Apart from the plants discussed above, essential oil, plant extracts, herbal formulations and tinctures from several species of the Rutaceae have been incorporated in skin and haircare products and utilized in the treatment of several skin diseases globally. The peel of *Citrus limon* is used to prevent hair loss (**GEDIYA *et al*., 2011**). *Citrus aurantium p*eel is used in the production of soaps and shampoos (**GEDIYA *et al*., 2011**). D-limonene, a compound found in all citrus fruit peel oils is used in a wide range of skin cleansing applications (**DELLUTRI, 1986**). Some of the Rutaceae family plants used in traditional medicine as a cure for skin diseases have also been found to inhibit the growth of skin disease fungal strains *in vitro*. The results of this study give credence to the use of some of the plants studied for the treatment of skin diseases of fungal origin and their incorporation in skincare products.

CHAPTER 9. CONCLUSIONS AND RECOMMENDATIONS

The improvement of previously used antibiotics and the introduction of a new range of antibiotics for the treatment of skin diseases of fungal origin have not been able to significantly solve the problem of increasing incidence of skin disease resistance to antibiotics. Hence, the search for an effective skin ointment or antifungal of natural origin could present a lasting solution to the shortcomings of antibiotics. Natural products could offer a source of low cost, effective and chemically diverse compounds with the ability to subdue resistant skin disease fungal strains.

An antifungal from natural origin can be effective in the treatment of eczema/ringworm often referred to as atopic dermatitis if it can address the biological processes involved in the pathogenesis and manifestation of the disease. These include inflammation, oxidative stress and total inhibition of the fungal strains involved. Inflammation of the skin can be dealt with by addressing the issue of oxidative stress as inflammation arises as a result of oxidative stress and most natural compounds with antioxidant activity are known to also exhibit anti-inflammatory activity. Hence, targeting oxidative stress in a chronic inflammatory skin condition such as atopic dermatitis may improve the disease state by reducing inflammation and thus improving recovery.

The leaf extracts of the Rutaceae plants selected for this study displayed antifungal activity (fungistatic) at different degrees. The antifungal activity displayed by some of the plant essential oil volatiles is quite remarkable as they displayed fungicidal activity. The essential oil of all the plants sourced from SANBI and the UKZN garden contain caryophyllene as part of their main components. This compound could probably be one of the major components responsible for the antifungal activity displayed in this study. The activity of the essential oil volatiles against *T. rubrum* is particularly noteworthy as it is responsible for the majority of skin diseases of fungal origin.

Essential oil volatiles of *A. betulina* have remarkable antifungal activity (fungicidal) against *T. rubrum*. The essential oil volatiles have the ability to inhibit growth of *T. rubrum*, stop the transmission of skin disease caused by *T. rubrum* from one individual to the other or from one part of the body to another and ultimately deal with resistance or reinfection in individuals with a compromised immune systems such as in HIV/AIDS. Essential oil volatiles of *A. betulina* and other plants used in

this study that exhibited fungicidal activity can therefore be used in the production of sprays that can be used on the body parts where there is skin infection. In addition, they can also be formulated into a product that can be used to fumigate a house/ room of an infected individual or family in order to kill the spores and avoid reinfection. Hence, the result of this study can give impetus to the production of an effective antifungal from natural origin. It will be worthwhile to establish the level of cytotoxicity of the extracts and essential oil used in this study as the skin can easily absorb any substance rubbed onto it. These substances can find their way into other parts of the body. It is therefore necessary to carry out clinical trials to ensure biosafety of substances derived from nature before they are developed into a skincare product.

CHAPTER 10. REFERENCES

AALTO-KORTE K, TURPEINEN M., 1993. Transepidermal water loss and absorption of hydrocortisone in widespread dermatitis. *British Journal of Dermatology*, 128, 633–635

AARTI S, MOHILE RB., 2003. Effect of mineral oil, sunflower oil, and coconut oil on prevention of hair damage. *Journal of Cosmetic Science*, 54, 175–192

ABBASI AM, KHAN MA, AHMAD M, ZAFAR M, JAHAN S, SULTANA S., 2010. Ethnopharmacological application of medicinal plants to cure skin diseases and in folk cosmetics among the tribal communities of North-West Frontier Province, Pakistan. *Journal of Ethnopharmacology,* 128, 322–335

ABDEL-RAHMAN SM, NAHATA MC., 1997. Oral terbinafine: a new antifungal agent. *Annals of Pharmacotherapy*, 31, 445–456

ABDEL-RAHMAN SM, POWELL DA, NAHATA MC., 1998. Efficacy of itraconazole in children with *Trichophyton tonsurans Tinea capitis*. *Journal of American Academy of Dermatology*, 38, 443–446

ABDEL-RAHMAN SM., 2001. Polymorphic exocellular protease expression in clinical isolates of *Trichophyton tonsurans*. *Mycopathologia*, 150, 117–120

ABURJAI T, NATSHEH FM., 2003. Plants used in cosmetics (Review Article), *Phytotherapy Research*, 17, 987–1000

ADEBAJO AC, AYOOLA OF, IWALEWA EO, AKINDAHUNSI AA, OMISORE NO, ADEWUNMI CO, ADENOWO TK., 2006. Anti-trichomonal, biochemical and toxicological activities of methanolic extract and some carbazole alkaloids isolated from the leaves of *Murraya koenigii* growing in Nigeria. *Phytomedicine*, 13, 4, 246–254

ADEBAJO AC, REISCH J., 2000. Minor furocoumarins of *Murraya koenigii*. *Fitoterapia,* 71, 3, 334-337

ADEOGUN II, FAWIBE OO, AJIBOYE AA, AGBOOLA DA., 2014. Ethnobotanical survey of medicinal plants used in the treatment of skin diseases in Abeokuta South Local Government of Ogun State Nigeria. *Asian Journal of Pharmaceutical Technology and Innovation*, 02, 08

AGERO AL, VERALLO-ROWELL VM., 2004. A randomized double-blind controlled trial comparing extra virgin coconut oil with mineral oil as a moisturizer for mild to moderate xerosis. *Dermatitis,* 15, 3, 109–16

AHMED A., 2001. Cytotoxic potentialities of essential oils. *Mansoura Journal of Pharmaceutical Sciences*, 17, 38–50

AKCAGLAR S, ENER B, TOKER SC, EDIZ B, TUNALI S, TORE O., 2011. A comparative study of dermatophyte infections in Bursa, Turkey. *Medical Mycology,* 49, 602–607

AL BURTAMANI SK, FATOPE MO, MARWAH RG, ONIFADE AK, ALSAIDI SH., 2005. Chemical composition, antibacterial and antifungal activities of the essential oil of *Haplophyllum tuberculatum* from Oman. *Journal of Ethnopharmacology,* 96, 107–112

ALBENGRES E, LE LOUET H, TILLEMENT JP., 1998. "Systemic antifungal agents. Drug interactions of clinical significance". *Drug Safety*, 18, 2, 83–97

ALEXANDER M., 2001. Aromatherapy & immunity: How the use of essential oil aids immune potentiality: Part 3 immune responses to inflammation and essential oils useful in inhibiting them. *International Journal of Aromatherapy*, 11, 4, 220–224

ALLARDICE P., 1992. The scented garden. A complete guide to growing and using fragrant plants. *Collins Angus and Robertson Publishers*, Pymble, Australia

ALY R., 1994. Ecology and epidemiology of dermatophyte infections. *Journal of American Academy of Dermatology,* 31, 21–25

ANCEREWICZ J, MIGLAVACA E, CARRUPT PA, TESTA B, BREE F, ZININ R, TILLEMENT JP, LABIDELLE P, GOYOT SD, CHAUVET-MONGES AM, CREVENT A, LE RIDANT A., 1998. Structure property relationship of trimetadizine derivatives and model compounds as potential antioxidants. *Free Radical Biology and Medicine*, 25, 1, 113–120

ANDRÉ J, ACHTEN G., 1987. Onychomychosis. *International Journal of Dermatology*, 26, 481–490

APPGS, 2013. The psychological and social impact of skin diseases on people's lives. *All Party Parliamentary Group on Skin*, 9

ARBAB IA, ABDUL AB, ASPOLLAH M, ABDULLAH R, ABDELWAHAB SI, IBRAHIM MY, ALI LZ, 2012. A review of traditional uses, phytochemical and pharmacological aspects of selected members of *Clausena* genus (Rutaceae). *Journal of Medicinal Plants Research*, 6, 38, 5107–5118

ARBAB IA, ABDUL AB, ASPOLLAH M, ABDULLAH R, ABDELWAHAB SI, MOHAN S, ABDELMAGEED AHA., 2011. *Clausena excavata* Burm. f.

(Rutaceae): A review of its traditional uses, pharmacological and phytochemical properties. *Journal of Medicinal Plants Research*, 5, 33, 7177–7184

ARNAO MB., 2000. Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practice case. *Trends in Food Science and Technology*, 11, 11, 419–421

ARNDT CA, WALSH TJ, MCCULLY CL, BALIS FM, PIZZO PA, POPLACK DG., 1988. Fluconazole penetration into cerebrospinal fluid: implications for treating fungal infections of the central nervous system. *The Journal of Infectious Diseases*, 157, 1, 178–180

ARUNA K, SIVARAMAKRISHNAN VM., 1996. Anticarcinogenic effects of the essential oils from cumin, poppy and basil. *Phytotherapy Research*, 10, 577–580

ASWAL BS, BHAKUNI DS, GOEL AK, KAR K, MEHROTRA BN, MUKHERJEE KC., 1984. Screening of Indian plants for biological activity Part X. *Indian Journal of Experimental Biology*, 22, 312–332

ATRAIDE DD, AKPA MR, GEORGE IO., 2011. The pattern of skin disorders in a Nigerian tertiary hospital. *Journal of Public Health Epidemiology*, 3, 177–181

AUMO J, WARNA J, SALMI T, MURZIN DY., 2006. Interaction of kinetics and internal diffusion in complex catalytic three-phase reactions: activity and selectivity in citral hydrogenation. *Chemical Engineering Science,* 61, 2, 814

AUST O, SIES H, STAHL W, POLIDORI MC., 2001. Analysis of lipophilic antioxidants in human serum and tissues: tocopherols and carotenoids. *Journal of Chromatography A*, 936, 1-2, 83–93

AYYANAR M, IGNACIMUTHU S., 2005. Traditional knowledge of Kani tribals in outhalai of Tirunelveli hills, Tamil Nadu, India. *Journal of Ethnopharmacology*, 102, 246–255

AZIZ SSSA, SUKARI MA, RAHMANI M, KITAJIMA N, AIMI C, AHPANDI NJ., 2010. Coumarins from *Murraya paniculata* (Rutaceae) (Koumarin daripada *Murraya Paniculata* (Rutaceae). *The Malaysian Journal of Analytical Sciences,* 14, 1, 1–5

BAEZA LC, BAILÃO AM, BORGES CL, PEREIRA M, SOARES CM, MENDES-GIANNINI MJ., 2007. cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin. *Microbes and Infection*, 9, 1415–1421

BAFI NF, ARNASON JT, BAKER J, SMITH ML., 2005. Antifungal constituents of northern prickly ash, *Zanthoxylum americanum* mill. *Phytomedicine*, 12*,* 370–377

BAKKALI F, AVER BECK S, AVER BECK D, IDAOMAR M., 2008. Biological effects of essential oils: a review. *Food and Chemical Toxicology*, 46, 446-475

BALAKUMAR S, RAJAN S, THIRUNALASUNDARI T, JEEVA S., 2011. Antifungal activity of *Aegle marmelos* (L.) Correa (Rutaceae) leaf extract on dermatophytes. *Journal of Tropical Medicine*, 1, 309–312

BALDRIDGE JR, THOMASHOW MF, HINRICHS DJ., 1988. Induction of immunity with a virulent *Listeria monocytogenes* 19113 depends on bacterial replication. *Infection and Immunity*, 56, 2109–2113

BALFOUR JA, FAULDS D., 1992. Terbinafine: A review of its pharmacodynamics and pharmacokinetic properties, and therapeutic potential in superficial mycoses. *Drugs*, 43, 259–284

BANG K, LUND M, WU K, MOGENSEN SC, THESTRUP-PEDERSEN K., 2001. CD_4+CD_8+ (thymocyte-like) T lymphocytes present in blood and skin from patients with atopic dermatitis suggest immune dysregulation. *British Journal of Dermatology*, 144, 6, 1140–1147

BARANOVA Z, KOZAK M, BILEK J., 2003. Zoophilic dermatomycoses in a family caused by *Trichophyton mentagrophytes* var. *quincheanum* - A case report. *Acta Veterinaria Brno*, 72, 311–314

BARATTA MT, DORMAN HJD, DEANS SG, FIGUEIREDO AC, BARROSO JG, RUBERT OG., 1998. Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour and Fragrance Journal*, 13, 235–244

BARCHIESI F, SILVESTRI C, ARZENI D, GANZETTI G, CASTELLETTI S, SIMONETTI O, CIRIONI O, KAMYSZ W, KAMYSZ E, SPREGHINI E., 2008. *In vitro* susceptibility of dermatophytes to conventional and alternative antifungal agents. *Medical Mycology,* 47, 321–326

BARIK BR, DEY AK, CHATTERJEE A., 1983. Murrayatin, a coumarin from *Murraya exotica*. *Phytochemistry*, 22, 10, 2273–2275

BARONE JA, MOSKOVITZ BL, GUARNIERI J, HASSELL AE, COLAIZZI JL, BIERMAN RH, JESSEN L., 1998. Enhanced bioavailability of itraconazole in hydroxypropyl-β- cyclodextrin solution versus capsules in healthy volunteers. *Antimicrobial Agents and Chemotherapy*, 42, 7, 1862–1865

BARONI A, BUOMMINO E, DE GREGORIO V, RUOCCO E, RUOCCO V, WOLF R., 2012. Structure and function of the epidermis related to barrier properties. *Clinics in Dermatology*, 30, 257–262

BAŞER KHC, DEMIRCI B, ÖZEK T, VILJOEN AM, VICTOR JE., 2006. Composition of the essential oils of five *Coleonema s*pecies from South Africa. *Journal of Essential Oil Research*, 18, 26–29

BAŞER KHC, DEMIRCI F., 2007. Chemistry of essential oils. *Flavours and Fragrances,* 43–86

BASKARAN P, MOYO M, VAN STADEN J., 2014. *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. *South African Journal of Botany,* 90, 74–79

BAUER WA, KIRBY WM, SHERRIS JC, TURCK C., 1966. Antibiotic susceptability testing by a standardised single disc method. *American Journal of Clinical Pathology*, 45, 4, 493–496

BAYLAC S, RACINE P., 2003. Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. *International Journal of Aromatherapy*, 13, 2-3, 138–142

BEGUM R, RAHMAN MS, CHOWDHURY S, RAHMAN MM, GIBBONS S, RASHID MA, 2010. A new 7-oxygenated coumarin from *Clausena suffruticosa*. *Fitoterapia*, 81, 656–658

BHAGRA S, GANJU SA, SOOD A, GULERIA RC, KANGA AK., 2013. *Microsporum gypseum* dermatophytosis in a patient of acquired immunodeficiency syndrome: A rare case report. *Indian Journal of Medical Microbiology*, 31, 3, 295–298

BICKERS DR, ATHAR M., 2006. Oxidative stress in the pathogenesis of skin disease. *Journal of Investigative Dermatology*, 126, 12, 2565–2575

BLAMEY C., 2000. Case history of infected eczema treated with essential oils. *Alternative Therapies*, 1, 11–14

BLIGNAUT E, MOLEPO J, PUJOL C, SOLL DR, PFALLER MA., 2005. Claderelated amphotericin B resistance among South African *Candida albicans* isolates. *Diagnostic Microbiology and Infectious Disease*, 53, 29–31

BLOCK G, PATTERSON B., 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer,* 18, 1–29

BOND P, GOLDBLATT P., 1984. Plants of the Cape Flora: A descriptive catalogue. *South African Journal of Botany*, 13

BONFANTI C, JANNI R, MAZZAGLIA A, LANZA CM, NAPOLI EM, RUBERTO G., 2012. Emerging cultivation of oregano in Sicily: sensory evaluation of plants and chemical composition of essential oils. *Industrial Crops and Products*, 35, 160–165

BOOGAERTS MA, MAERTENS J, VAN DER GEEST R, BOSLY A, MICHAUX JL, VAN HOOF A, CLEEREN M, WOSTENBORGHS R, DE BEULE K., 2001. Pharmacokinetics and safety of a 7-day administration of intravenous itraconazole followed by a 14-day administration of itraconazole oral solution in patients with hematologic malignancy. *Antimicrobial Agents and Chemotherapy*, 45, 3, 981–985

BORELLI D, BRAN JL, FUENTES J, LEGENDRE R, LEIDERMAN E, LEVINE HB, RESTREPO A, STEVENS DA., 1979. Ketoconazole, an oral antifungal: laboratory and clinical assessment of imidazole drugs. *Postgraduate Medical Journal*, 55, 657–661

BORGMANN S, NIKLAS DM, KLARE I, ZABEL LT, BUCHENAU P, AUTENRIETH, IB, HEEG P., 2004. Two episodes of vancomycin-resistant *Enterococcus faecium* outbreaks caused by two genetically different clones in a newborn intensive care unit. *International Journal of Hygiene and Environmental Health*, 207, 4, 386–389

BOUWSTRA JA, PILGRIM K, PONEC M., 2006. Structure of the skin barrier. In: **ELIAS PM, FEINGOLD KR., 2006.** Skin barrier. *Taylor and Francis*, New York, 65–95

BRACONI D, BERNARDINI G, SANTUCCI A., 2010. Post-genomics and skin inflammation (review article). *Mediators of Inflammation*, 1–12

BRADER G, BACHER M, HOFER O, GREGER H., 1997. Prenylated phenylpropenes from *Coleonema pulchellum* with antimicrobial activity. *Phytochemistry*, 45, 1207–1212

BRAMMER KW, FARROW PR, FAULKNER JK., 1990. Pharmacokinetics and tissue penetration of fluconazole in humans. *Reviews of Infectious Diseases*, 12, 3, 318–326

BRAND-WILLIAMS W, CUVELIER ME, BERSET C., 1995. Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology*, 28, 25–30

BRASS C, GALGIANI JN, BLASCHKE TF., 1982. Disposition of ketoconazole, an oral antifungal, in humans. *Antimicrobial Agents and Chemotherapy*, 21, 1, 151–158

BRAZIL, 2006. Ministry of health SdC, technology and strategic inputs. National policy of medicinal plants and herbal medicine. *Pharmaceuticals DDA Editor*, Brasilia

BRIGANTI S, PICARDO M., 2003. Antioxidant activity, lipid peroxidation and skin diseases. What's new? *Journal of the European Academy of Dermatology and Venereology*, 17, 6, 663–669

BROPHY JJ, LASSAK EV., 1986. Volatile leaf oil of *Coleonema pulchellum* Williams (Rutaceae). *Flavour and Fragrance Journal*, 1, 155–157

BRUNETON J. 1999. Pharmacognosy, phytochemistry, medicinal plants. *Lavoisier Publishing*, Paris

BUCHBAUER G., 2000. The detailed analysis of essential oils leads to the understanding of their properties. *Perfumer Flavorist*, 25, 64–67

BUCKLE J., 2003. Clinical aromatherapy: Essential oils in practice. *Churchill Livingstone*, London

BUDHIRAJA S, CULLUM E, SIOUTIS S, EVANGELISTA L, HABANOTA T., 1999. Biological activity of *Melaleuca alternifolia* (tea tree) oil component, terpene-4-ol, in human myelocytic cell line HL-60. *Journal of Manipulative and Physiological Therapeutics*, 22, 447–453

BUETTNER GR, JURKIEWICZ BA., 1996. Catalytic metals, ascorbate and free radicals: combinations to avoid. *Radiation Research,* 145, 532–541

BURGESS MA, BODEY GP., 1972. Clotrimazole (Bay b 5097): *in vitro* and clinical pharmacological studies. *Antimicrobial Agents and Chemotherapy*, 2, 6, 423–426

BURT S., 2004. Essential oils: their antibacterial properties and potential applications in foods: A review. *International Journal of Food Microbiology*, 94, 3, 223–253

BUTLER MS., 2005. Natural products to drugs: natural product derived compounds in clinical trials. *Natural Product Reports*, 22, 162–195

CABAÑES FJ., 2000. Emerging mycotoxins: introduction. *Revista Iberoamericana de Micología,* 17, 61–62

CANDI E, SCHMIDT R, MELINO G., 2005. The cornified envelope: a model of cell death in the skin. *Nature Reviews Molecular Cell Biology*, 6, 4, 328–340

CANTRELL CL, SCHRADER KK, MAMONOV LK, SITPAEVA GT, KUSTOVA TS, DUNBAR C, WEDGE DE., 2005. Isolation and identification of antifungal and antialgal alkaloids from *Haplophyllum sieversii*. *Journal of Agricultural and Food Chemistry*, 53*,* 7741

CAO G, ALESSIO HM, CULTER R., 1993. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine*, 14, 303–311

CAO G, PRIOR RL., 1999. The measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology*, 299, 50–62

CAO G, SOFIC ER, PRIOR RL., 1996. Antioxidant capacity of tea and common vegetables. *Journal of Agriculture and Food Chemistry,* 44, 3426–3431

CAO G, VERDON CP, WU AHB, WANG H, PRIOR R L., 1995. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clinical Chemistry*, 41, 1738–1744

CARLE R, GOMAA K. 1992. Chamomile: a pharmacological and clinical profile. *Drugs Today*, 28, 559–565

CARRILLO-MUÑOZ AJ, GIUSIANO G, EZKURRA PA, QUINDÓS G., 2006. Antifungal agents: mode of action in yeast cells. *Revista Espanola de Quimioterapia*, 19, 2, 130–139

CARSON CF, COOKSON BD, FARRELLY HD, RILEY TV., 1995. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. *Journal of Antimicrobial Chemotherapy*, 35, 421–424

CARSON CF, RILEY TV., 1994. Susceptibility of *Propionibacterium acnes* to the essential oil of *Melaleuca alternifolia*. *Letters in Applied Microbiology*, 19, 24–25

CARSON CF, RILEY TV., 1995. Anti-microbial activity of the major components of the essential oil of *Melaleuca alternifolia*. *Journal of Applied Bacteriology*, 78, 264–269

CATALÁ A., 2009. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids*, 157, 1, 1–11

CHAKRABORTY A, CHOWDHURY BK, BHATTACHARYYA P., 1995. Clausenol and clausenine—two carbazole alkaloids from *Clausena anisata*. *Phytochemistry*, 40, 295–298

CHASE MW, MORTON CM, KALLUNKI JA., 1999. Phylogenetic relationships of Rutaceae: a cladistic analysis of the subfamilies using evidence from *rbcl* and *atpb* sequence variation1. *American Journal of Botany*, 86, 8, 1191–1199

CHEE HY, KIM H, LEE MH., 2009. *In vitro* antifungal activity of limonene against *Trichophyton rubrum*. *Mycobiology,* 37, 3, 243-246

CHEESEMAN KH, SLATER TF., 1993. An introduction to free radical biochemistry. *British Medical Bulletin.* 49, 481–493

CHHABRA SC., MAHUNNAH RLA, MSHIU EN., 1991. Plants used in traditional medicine in eastern Tanzania. V. Angiosperms (Passifloraceae to Sapindaceae). *Journal of Ethnopharmacology*, 33, 143–157

CHI CC, WANG SH, CHOU MC., 2005. The causative pathogens of onychomycosis in Southern Taiwan. *Mycoses*, 48, 413–20

CHIN YW, BALUNAS MJ, CHAI HB, KINGHORN AD., 2006. Drug discovery from natural sources. *AAPS Journal*, 8, 239–253

CHIOU C, GROLL A, WALSH T., 2000. New drugs and novel targets for treatment of invasive fungal infections in patients with cancer. *Oncologist*, 5, 2, 120–135

CHISOLM GM, STEINBERG D., 2000. The oxidative modification hypothesis of atherogenesis: An overview. *Free Radicals in Biology and Medicine*, 8, 1815–1826

CHO J, HWANG T, CHANG T, LIM Y, SUNG P, LEE T, CHEN J., 2012. New coumarins and anti-inflammatory constituents from *Zanthoxylum avicennae*. *Food Chemistry*, 135, 17–23

CHOWDHURY JU, BHUIYAN NI, YUSUF M., 2008. Chemical composition of the leaf essential oils of *Murraya koenigii* (L.) Spreng and *Murraya paniculata* (L.) Jack. *Bangladesh Journal of Pharmacology*, 3, 59-63

CLARKE S., 2008. Essential chemistry for aromatherapy. 2nd Edition, *Elsevier,* New York

CLARYS P, ALEWAETERS K, JADOUL A, BAREL A, MANADAS RO, PREAT V., 1998. *In vitro* percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers. *European Journal of Pharmaceutics and Biopharmaceutics*, 46, 3, 279

COELHO LM, AQUINO-FERREIRA R, CLAUDIA M, MAFFEI L and MARTINEZ-ROSSI NM., 2008. *In vitro* antifungal drug susceptibilities of dermatophytes microconidia and arthroconidia. *Journal of Antimicrobial Chemotherapy*, 62, 758–761

COETZEE C, JEFTHAS E, REINTEN E., 1999. Indigenous plant genetic resources of South Africa. In: **JANICK J**, Perspectives on new crops and new uses*. ASHS Press*, Alexandria

CONTASSOT E, BEER HD, FRENCH LE., 2012. Interleukin-1, inflammasomes, autoinflammation and the skin. *Swiss Medical Weekly,* 142

COOPOOSAMY RM, NAIDOO KK., 2011. Assessing the potential of *Tetradenia riparia* in treatment of common skin conditions in rural communities of South Africa. *African Journal of Microbiology Research*, 5, 19, 2942–2945

CORRÊA MP., 1969. Dicionário de Plantas úteis do Brasil e das exóticas cultivadas. *Imprensa Nacional*, Rio de Janeiro, 4, 374

COWLING RM, HOLMES PM, REBELO AG., 1992. Plant diversity and endemism. In: **COWLING RM,** The ecology of fynbos: nutrients, fire and diversity*. Oxford University Press*, Cape Town

COX SD, MANN CM, MARKHAM JL, BELL HC, GUSTAFON JE, WARMINGTON JR, WYLLIE SG., 2000. The mode of antimicrobial action of essential oil of *Melaleuca alternifolia* (tea tree oil). *Journal of Applied Microbiology,* 88, 1, 170–175

CVETNIC Z, VLADIMIR S., 2004. Antimicrobial activity of grapefruit seed and pulp ethanolic extract. *Acta Pharmaceutica,* 54, 243

D'ANTUONO A, BARDAZZI F, ANDALOU F., 2001. Unusual manifestations of dermatophytoses. *International Journal of Dermatology*, 40, 164–166

DARLENSKI R, FLUHR JW., 2012. Influence of skin type, race, sex, and anatomic location on epidermal barrier function. *Clinics in Dermatology*, 30, 269–273

DARR D, FRIDOVICH I, 1994. Free radicals in cutaneous biology. *Journal of Investigative Dermatology*, 102, 671–675

DAVIS RH, LEITNER MG, RUSSO JM, BYRNE ME., 1994. Wound healing: Oral and topical activity of *Aloe vera*. *Journal of the American Podiatric Medical Association*, 79, 559–562

DE GROOT AC, FROSCH PJA., 1997. Adverse reactions to fragrances. A clinical review. *Contact Dermatitis*. 36, 2, 57–86

DE WET H, NCIKI S, VAN VUUREN SF., 2013. Medicinal plants used for the treatment of various skin disorders by a rural community in northern Maputaland, South Africa. *Journal of Ethnobiology and Ethnomedicine*, **DOI:** 10.1186/1746-4269-9-51

DEACON JW., 1998. Introduction to modern mycology. 3a Edition, *Blackwell*, Oxford

DEANS SG, RITCHIE G., 1987. Antibacterial properties of plant essential oils. *International Journal of Food Microbiology*, 5, 2, 165–180

DEANS SG, SVOBODA KP, GUNDIDZA M, BRECHANY EY., 1992. Essential oil profiles of several temperate and tropical aromatic plants: their antimicrobial and antioxidant activities. *Acta Horticultural*, 306, 229–232

DEBAJYOTHI D., 2008. Biochemistry. 13th Edition, *Academic Publishers*, Kolkatta

DEIGNER HP, HERMETTER A., 2008. Oxidized phospholipids: emerging lipid mediators in pathophysiology. *Current Opinion in Lipidology*, 19, 3, 289–294

DEKIC BR, RADULOVIC NS, DEKIC VS, VUKICEVIC RD, PALIC RM., 2010. Synthesis and antimicrobial activity of new 4-heteroarylamino coumarin derivatives containing nitrogen and sulfur as heteroatoms. *Molecules*, 15, 2246–2256

DEL GIUDICE P, YVES P., 2002. The widespread use of skin lightening creams in Senegal: A persistent public health problem in West Africa. *International Journal of Dermatology*, 41, 2, 69–72

DELLUTRI J., 1986. All purpose cleaner containing D-limonene. U.S. Patent 3pp US4620937

DEMMIG-ADAMS B, ADAMS WW., 2002. Antioxidants in photosynthesis and human nutrition. *Science*, 298, 2149–2153

DENNING DW., 1995. Can we prevent azole resistance in fungi? *Lancet*, 346, 454–455

DI SANTO R., 2010. Natural products as antifungal agents against clinically relevant pathogens. *Natural Product Reports*,

DI SILVERIO A, BRAZELLI V, BRANDOZZI G, BARBARINI G, MACCABRUNI A, SACCHI S., 1991. Prevalence of dermatophytes and yeast (*Candida spp.*, *Malassezia furfur*) in HIV patients. A study of former drug addicts. *Mycopathologia*, 114, 103–107

DICKE M, VAN LOON JJA, SOLER R., 2009. Chemical complexity of volatiles from plants induced by multiple attack. *Nature Chemical Biology*, *5*, 317–324

DIOGO HC, SARPIERI A, MELHEM M, PIRES MC., 2010. Avaliação do método de disco-difusão paradeterminação da eficácia da terbinafina *in vitro* em agentes de micoses superficiais e subcutâneas. *Anais Brasileiros de Dermatologia*, 85, 324–330

DISMUKES WE, CLOUD G, BOWLES C, 1985. Treatment of blastomycosis and histoplasmosis with ketoconazole: results of a prospective randomized clinical trial. *Annals of Internal Medicine*, 103, 6, 861–872

DODGSON AR, DODGSON KJ, PUJOL C, PFALLER MA, SOLL DR., 2004. Clade-specific flucytosine resistance is due to a single nucleotide change in the FUR1 gene of Candida albicans. *Antimicrobial Agents and Chemotherapy*, 48, 2223–2227

DOE PT, ASIEDU A, ACHEAMPONG JW, ROWLAND PAYNE CM., 2001. Skin diseases in Ghana and the UK. *International Journal of Dermatology*, 40, 323–326

DORMAN HJD, DEANS SG., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, 88, 308–316

DRAGLAND S, SENOO H, WAKE K, HOLTE K, BLOMHOFF R., 2003. Several culinary and medicinal herbs are important sources of dietary antioxidants. *Journal of Nutrition*, 133, 1286–1290

DREHER D, JUNOD AE., 1996. Role of oxygen free radicals in cancer development. *European Journal of Cancer*, 32, 30–38

DREYER DL, PICKERING MV, COHAN P., 1972. Distribution of limnoids in the Rutaceae. *Phytochemistry*, 11, 705–713

DUGO P, MONDELLO L, DUGO L, STANCANELLI R, DUGO G., 2000. LC-MS for the identification of oxygen heterocyclic compounds in citrus essential oils. *Journal of Pharmaceutical and Biomedical Analysis*, 24, 1, 147–154

DUH PD, TU YY, YEN GC., 1999. Antioxidants activity of aqueous extract of Harnjyur (*Chrysanthemum morifolium* Ramat). *Lebensmittel-Wissenschaft und Technologie*, 32, 269–277

DUNCAN SH, LEITCH EC, STANLEY KN, RICHARDSON AJ, LAVEN RA, FLINT HJ, STEWART CS., 2004. Effects of esculin and esculetin on the survival of *Escherichia coli* O157 in human faecal slurries, continuous-flow simulations of the rumen and colon and in calves. *British Journal of Nutrition*, 91, 749–755

DURACKOWA Z., 2010. Some current sights into oxidative stress. *Physiological Research*, 59, 459–469

EASTWOOD MA., 1999. Interaction of dietary antioxidants *in vivo*: how fruit and vegetables prevent disease? *Quaterly Journal of Medicine,* 92, 527–530

EGAN D, O'KENNEDY R, MORAN E, COX D, PROSSER E, THORNES RD., 1990. The pharmacology, metabolism, analysis and applications of coumarin and coumarin-related compounds. *Drug Metabolism Review*, 22, 503–529

EGAN D, O'KENNEDY R., 1992. Rapid and sensitive determination of coumarin and 7-hydroxycoumarin and its glucuronide conjugate in urine and plasma by high performance liquid chromatography. *Journal of Chromatography B*, 582, 137–143

EGHAREVBA KA, IKHATUA MI., 2008. Ethno-medical uses of plants in the treatment of various skin diseases in Ovia North East, Edo State, Nigeria. *Research Journal of Agriculture and Biological Sciences*, 4, 1, 58–64

ELDEEN IMS, VAN STADEN J., 2008. Anti-inflammatory and mycobacterial activity of leaf extracts of *Coleonema album*. *South African Journal of Botany*, 74, 345–347

ELDER K, BAKER DJ, RIBES JA., 2005. Infections, infertility and assisted reproduction. *Cambridge University Press*, United Kingdom

ELMETS CA., 1994. Management of common superficial fungal infections in patients with AIDS. *Journal of the American Academy of Dermatology*, 31, 60–63

ERBAGCI Z., 2004. Topical therapy for dermatophytoses: should corticosteroids be included? *American Journal of Clinical Dermatology*, 5, 375–384

ESTERHUIZEN LL, MEYER R, DUBERY IA., 2006a. Antimicrobial compounds from *Coleonema album* (Rutaceae). *Zeitschrift für Naturforschung C*, 61, 489–498

ESTERHUIZEN LL, MEYER R, DUBERY I., 2006b. Antioxidant activity of metabolites from *Coleonema album* (Rutaceae). *Natural Product Communication*, 1, 367–375

EVANS EGV., 1990. Nail dermatophytes: the nature and scale of the problem. *Journal of Dermatological Treatment*, 1, 2, 47–48

FAJINMI OO, AMOO SO, FINNIE JF, VAN STADEN J., 2014. Optimization of *in vitro* propagation of *Coleonema album*, a highly utilized medicinal and ornamental plant. *South African Journal of Botany*, 94, 9–13

FAJINMI OO, KULKARNI MG, FINNIE JF, VAN STADEN J., 2013. Factors influencing seed germination of *Coleonema album* – an aromatic and medicinal plant. *Seed Science and Technology*, 41, 303–309

FARAH CS, ASHMAN RB, CHALLACOMBE SJ., 2000. Oral candidosis. *Clinics in Dermatology*, 18, 553–62

FELDMEYER L, WERNER S, FRENCH LE, BEER HD*.*, 2010. Interleukin-1, inflammasomes and the skin. *European Journal of Cell Biology*, 89, 638–644

FEO VD, SIMONEA FD, SENATOREB F., 2002. Potential allelochemicals from the essential oil of *Ruta graveolens. Phytochemistry*, 61, 573–578

FERNANDES CN, LAMY F, AKITI T, BARREIROS MG., 1998. *Microsporum gypseum* infection in Aids patient: A case report. *Journal Anais Brasileiros de Dermatologia*, 73, 39v41

FERNÁNDEZ-TORRES B, CABANES FJ, CARRILLO-MUN˜OZ AJ, ESTEBAN AJ, INZA I, ABARCA L, GUARRO J., 2002. Collaborative study of optimal antifungal susceptibility testing conditions for dermatophytes. *Journal of Clinical Microbiology*, 40, 3999–4003

FERNÁNDEZ-TORRES B, CARRILLO AJ, MARTÍN E, DEL PALACIO A, MOORE MK, VALVERDE A, SERRANO M, GUARRO J., 2001. *In vitro* activities of 10 antifungal drugs against 508 dermatophyte strains. *Antimicrobial Agents and Chemotherapy*, 45, 2524–2528

FERNANDEZ-TORRES B, INZA I, GUARRO J., 2003. Comparison of *in vitro* antifungal susceptibilities of conidia and hyphae of dermatophytes with thick-wall macroconidia. *Antimicrobial Agents and Chemotherapy*, 47, 3371–3372

FIGUEROA JI, FULLER LC, ABRAHA A, HAY RJ., 1998. Dermatology in southwestern Ethiopia: Rationale for a community approach. *International Journal of Dermatology*, 37, 752–758

FILIPPINI R, PIOVAN A, INNOCENTI G, CANIATO R, CAPPELLETTI EM., 1998. Production of coumarin compounds by *Haplophyllum patavinum in vivo* and *in vitro*. *Phytochemistry*, 49, 8, 2337–2340

FINN G, KENEALY E, CREAVEN B, EGAN D., 2002. *In vitro* cytotoxic potential and mechanism of action of selected coumarins, using human renal cell lines. *Cancer Letters,* 183, 61–68

FISH F, GRAY AI, WATERMAN PG., 1975. Alkaloids, coumarins, triterpenes, and a flavonone from the root of *Zanthoxylum dipetalum*. *Phytochemistry*, 14, 9, 2073–2076

FISHER K, PHILLIPS C., 2006. The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus in vitro* and in food systems. *Journal of Applied Microbiology*, 101, 6, 1232–1240

FISHER K, PHILLIPS C., 2008. Potential antimicrobial uses of essential oils in food: is Citrus the answer? *Trends in Food Science & Technology*, 19,156–164

FLAMINI G, TEBANO M, CIONI P., 2007. Volatiles emission patterns of different plant organs and pollen of *Citrus limon*. *Analytica Chimica Acta*, 589, 120–124

FOCHO DA, NDAM WT, FONGE BA., 2009. Medicinal plants of Aguambu-Bamumbu in the Lebialem Highland, SouthWest Province of Cameroon. *African Journal of Pharmacy and Pharmacology*, 3, 1–13

FREEDBERG IM, EISEN AZ, WOLFF K, AUSTEN KF, GOLDSMITH LA, KATZ S., 2003. In: **FITZPATICK TB,** Dermatology in general medicine. 6th Edition, *McGraw-Hill*, New York

FROMTLING RA., 1988. Overview of medically important antifungal azole derivatives. *Clinical Microbiology Reviews*, 1, 2, 187–217

FROST CJ, MESCHER MC, DERVINIS C, DAVIS JM, CARLSON JE, DE MORAES CM., 2008. Priming defense genes and metabolites in hybrid poplar by the green leaf volatile *cis*-3-hexenyl acetate. *New Phytology*, 180, 722–733

FUCHS E, RAGHAVAN S., 2002. Getting under the skin of epidermal morphogenesis. *Nature Reviews Genetics,* 3, 199–209

FYFE L, ARMSTRONG F, STEWART J. 1998. Inhibition of *Listeria monocytogenes* and *Salmonella enteriditis* by combination of plant oils and derivatives of benzoic acid: the development of synergistic antimicrobial combinations. *International Journal of Antimicrobial Agents*, 9, 195–199

GALATI EM, MONFORTE MT, KIRJAVAINEN S, FORESTIERI AM, TROVATO A, TRIPODO MM., 1994. Biological effects of hesperidin, a citrus flavonoid (Note I): antiinflammatory and analgesic activity. *Farmaco*, 40, 709–712

GARCÍA-SOSA K, SÁNCHEZ-MEDINA A, ÁLVAREZ SL, ZACCHINO S, VEITCH NC, SIMÁ-POLANCO P, PEÑA-RODRIGUEZ LM., 2011. Antifungal activity of

sakurasosaponin from the root extract of *Jacquinia flammea*. *Natural Product Research*, 25, 1185–1189

GAZZANI G, PAPETTI A, MASSOLINI G, DAGLIA M., 1998. Anti- and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. *Journal of Agriculture and Food Chemistry,* 46, 4122

GEDIYA SK, MISTRY RB, PATEL UK, BLESSY M, JAIN HN., 2011. Herbal plants used as a cosmetics. *Journal of Natural Products and Plant Resources*, 1, 1, 24–32

GIBBS S., 1996. Skin disease and socioeconomic conditions in rural Africa: Tanzania. *International Journal of Dermatology*, 35, 633–639

GILL NS, KAUR N, ARORA R., 2014. An overview on: *Murraya paniculata* LINN. *International Journal of Institutional Pharmacy and Life Sciences,* 4, 1–11

GLASER R, HARDER J, LANGE H, BARTELS J, CHRISTOPHERS E, SCHRODER JM., 2005. Antimicrobial psoriasin (S100A7) protects human skin from *Escherichia coli* infection. *Nature Immunology*, 6, 57–64

GLOBAL INFORMATION AND ADVICE ON HIV AND AIDS, 2016. HIV and AIDS in South Africa. https://www.avert.org/professionals/hiv-around-world/sub-saharan-africa/south-africa

GOLDBLATT P, MANNING JC., 2002. Plant diversity of the Cape region of southern Africa. *Annals of Missouri Botanical Garden*, 89, 281–302

GOLDBLATT P., 1978. An analysis of the flora of southern Africa: Its characteristics, relationships, and origins. *Annals of Missouri Botanical Garden*, 65, 369–436

GOLDBLATT P., 1997. Floristic diversity in the Cape Flora of South Africa. *Biodiversity and Conservation*, 6, 359–377

GONZALEZ-TRUJANO ME, CARRERA D, VENTURA-MARTINEZ R, CEDILLO-PORTUGAL E, NAVARRETE A., 2006. Neuropharmacological profile of an ethanol extract of *Ruta chalepensis* L. in mice. *Journal of Ethnopharmacology*, 106, 129–135

GOODMAN DS, TELPLITZ ED, WILSHNER A, KLEIN RS, BURK PG, HERSHENBAUM E., 1987. Prevalence of cutaneous disease in patients with acquired immunodeficiency syndrome (AIDS) or AIDS related complex. *Journal of the American Academy of Dermatology*, 17, 210–220

GOPALAN C, RAMA SHASTRI BV, BALASUBRAMANIAN SC., 1984. Nutritive value of Indian Foods. *ICMR*, New Delhi, 66,117

GRÄSER Y, KUIJPERS AFA, PRERSBER W, DE HOOG GS, 2000. Molecular Taxonomy of the *Trichophyton rubrum* Complex. *Journal of Clinical Microbiology,* 38, 9, 3329–3336

GRAY AI., 1981. New coumarins from *Coleonema album. Phytochemistry*, 20, 1711–1713

GUARRERA PM., 1999. Traditional antihelmintic, antiparasitic and repellent uses of plants in Central Italy. *Journal of Ethobotany*, 68, 183–192

GUNATHILAKE R, SCHMUTH M, SCHARSCHMIDT TC, GRUBER R, GRABHER D, LESLIE KS, MAURER TA, MAURO TM, ELIAS PM., 2010. Epidermal barrier dysfunction in non-atopic HIV: evidence for an “inside-to-outside” pathogenesis. *Journal of Investigative Dermatology*, 130, 1185–1188

GUNAYDIN K, SAVCI S., 2005. Phytochemical studies on *Ruta chalepensis* (Lam.) *Natural Product Research*, 19, 203–210

GUNTUPALLI C, RAMAIAH M, KUMAR GS., 2013. RP-HPLC analysis and antimicrobial screening of *Clausena excavata* BURM. F. (Rutaceae). *International Journal of Phytotherapy*, 3, 2, 91-97

GUPTA AK, ADAM P, DLOVA N, LYNDE CW, HOFSTADER S, MORAR N, ABOOBAKER J, SUMMERBELL RC., 2001. Therapeutic options for the treatment of *Tinea capitis* caused by *Trichophyton species*: griseofulvin vs the new oral antifungal agents, terbinafine, itraconazole, and fluconazole. *Pediatric Dermatology*, 18, 433–438

GUPTA AK, AHMAD I, PORRETTA M, SUMMERBELL RC., 2003. Arthroconidial formation in *Trichophyton raubitschekii*. *Mycoses*, 46, 8, 322–328

GURGEL LA, SIDRIM JJ, MARTINS DT, CECHINEL FILHO V, RAO VS., 2005. *In vitro* antifungal activity of dragon’s blood from *Croton urucurana* against dermatophytes. *Journal of Ethnopharmacology*, 97, 409–412

GUTTERIDGE JMC., 1988. Lipid peroxidation: some problems and concepts. In: **HALLIWELL B,** Oxygen radicals and tissue injury. *FASEB*, USA

HAILAT N, BATINHEH Z, LAFI S, RAWEILY E, AQEL M, AL-KATIB M, HANASH S., 1995. Effect of *Nigella sativa* volatile oil on Jurkat T cell leukemia polypeptides. *International Journal of Pharmacognosy*, 33, 16–20

HALLIWELL B, ARUOMA OI., 1991. DNA damage by oxygen derived species. Its mechanism and measurement in mammalian systems. *FEBS Letters*, 281, 9–19

HALLIWELL B, GUTTERIDGE JMC, CROSS CE., 1992. Free-radicals, antioxidants, and human diseases-Where are we now? *Journal of Laboratory and Clinical Medicine*, 119, 598–620

HALLIWELL B, GUTTERIDGE JMC., 1999. Free radicals in biology and medicine. 3rd Edition, *Oxford University Press,* United Kingdom

HAMID AA, AIYELAAGBE O, USMAN LA, AMEEN OM, LAWAL A., 2010. Antioxidants: Its medicinal and pharmacological applications. *African Journal of Pure and Applied Chemistry,* 4, 8, 142–51

HAMID AA, AIYELAAGBE OO, USMAN LA., 2011. Essential oils: its medicinal and pharmacological uses. *International Journal of Current Research*, 33, 86–98

HAMMER A, CARSON F, RILEY V., 1998. *In vitro* activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida spp*. *Journal of Antimicrobial Chemotherapy*, 42, 591–595

HAMMER KA, CARSON CF, RILEY TV., 2000. *Melaleuca alternifolia* (tea tree) oil inhibits germ tube formation by *Candida albicans*. *Medical Mycology,* 38, 354–361

HARUNA A, SOAD SZM, HASSAN NM, RAMLI NKCM., 2011*. In vitro* study of antifungal activity of *Entada spiralis* Ridl crude extract against dermatophytes of superficial skin disease. *Revelation and Science,* 1, 1, 57–61

HARVEY AL., 2007. Natural products as a screening resource. *Current Opinion in Chemical Biology*, 11, 480–484

HASHIMOTO T, BLUMENTHAL HJ., 1977. Factors affecting germination of *Trichophyton mentagrophytes* arthrospores. *Infection and Immunity*, 18, 479–86

HAVLICKOVA B, CZAIKA VA, FRIEDRICH M., 2008. Epidemiological trends in skin mycoses worldwide. *Mycoses*, 51, 4, 2–15

HAY R, BENDECK SE, CHEN S, ESTRADA R, HADDIX A, MCLEOD T, MAHE A., 2006. Skin Diseases. In: Disease Control Priorities in Developing Countries. 2nd Edition, *Oxford University Press*, New York

HAY R, MARKS R., 2004. The International Foundation for Dermatology: An exemplar of the increasingly diverse activities of the International League of Dermatological Societies. *British Journal of Dermatology*, 150, 747–749

HAY RJ, ESTRADA R, ALARCON H, CHAVEZ G, LOPEZ LF, PAREDES S, ANDERSSON N., 1994. Wastage of family income on skin disease in Mexico. *British Medical Journal,* 309, 848

HAYASHI K, KAMIYA M, HAYASHI T. 1995. Virucidal effects of the steam distillate from *Houttuynia cordata* and its components on HSV-1, influenza virus and HIV. *Planta Medica*, 61, 237–241

HAZRA A, DOLLIMORE D, ALEXANDER K., 2002. Thermal analysis of evaporation of compounds used in aromatherapy using thermogravimetry. *Thermochimica Acta*, 392–393, 221–229

HE HP, SHEN YM, CHEN ST, HE YN, HAO XJ., 2006. Dimeric coumarin and phenylpropanoids from *Clausena lenis*. *Helvetica Chimica Acta*, 89, 2836–2840

HE HP, SHEN YM, HE YN, YANG XS, ZHU WM, HAO XJ., 2000. O-terpenoidal coumarins from *Clausena excavata*. *Heterocycles*, 53, 1807–1810

HEDBERG I, HEDBRERG O, MADATI PJ, MSHIGENI KE, MSHIU EN, SAMUELSSON G, 1983. Inventory of plants used in traditional medicine in Tanzania. II. Plants of the families Dilleniaceae–Opiliaceae. *Journal of Ethnopharmacology*, 9, 105–127

HEEL RC, BROGDEN RN, PAKES GE., 1980. Miconazole: a preliminary review of its therapeutic efficacy in systemic fungal infections. *Drugs*, 19, 1, 7–30

HEERES J, BACKX LJJ, MOSTMANS JH, VAN CUTSEM J., 1979. Antimycotic imidazoles. Part 4. Synthesis and antifungal activity of ketoconazole, a new potent orally active broad-spectrum antifungal agent. *Journal of Medicinal Chemistry*, 22, 8, 1003–1005

HEIM KE, TAGLIAFERRO AR, BOBILYA DJ., 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *Journal of Nutritional Biochemistry*, 13, 572–584

HEINECKE JW., 1998. Oxidants and antioxidants in the pathogenesis of artherosclerosis: Implications for the oxidized low density lipoprotein hypothesis. *Atherosclerosis*, 141, 1–15

HITCHCOCK CA., 1991. Cytochrome P-450–dependent 14 a-sterol demethylase of *Candida albicans* and its interaction with azole antifungals. *Biochemical Society Transactions*, 19, 782–787

HO KM, WONG KH., 2001. Dermatologic manifestations in HIV disease. In: **CHAN KCW, WONG KH, LEE SS.** *HIV Manual*, 231–245

HOERMANN HP, KORTING HC. 1994. Evidence for the efficacy and safety of topical herbal drugs in dermatology: Part I: Antiinflammatory agents. *Phytomedicine*, 1, 161–171

HOFF B, SCHMITT EK, KUCK U., 2005. CPCR1 but not its interacting transcription factor AcFKH1 controls fungal arthrospore formation in *Acremonium chrysogenum*. *Molecular Microbiology*, 56, 1220–1233

HOLMES PM, REBELO AG, DORSE C, WOOD J., 2012. Can Cape Town's unique biodiversity be saved? Balancing conservation imperatives and development needs. *Ecology and Society,* 17, 2, 28

HOLMSTED B, WASSEN SH, SCHULTES RE., 1975. Jaborandi: An interdisciplinary appraisal. *Journal of Ethnopharmacology,* 1, 3–21

HONG PHE, YUE MS, GUO YZUO, WEI MZHU, XIAO SY, XIAO J., 2000. Two new O-terpenoidal coumarins, excavacoumarin A and B from *Clausena excavata*. *Chinese Chemical Letters*, 11

HONGPING H, YUEMAO S, YINENG H., 2000. Six new O-terpenoidal coumarins, excavacoumarins B-G from *Clausena excavata*. *Heterocycles,* 53, 2067

HORSBURGH CR, KIRKPATRICK CH., 1983. Long-term therapy of chronic mucocutaneous candidiasis with ketoconazole: experience with twenty-one patients. *American Journal of Medicine*, 74, 1, 23–29

HOSSAIN MA, KOZI ASADA., 1985. Monodehydroascorbate reductase from cucumber is a flavin adenine dinucleotide enzyme. *The Journal of Biological Chemistry,* 260, 24, 12920–1226

HOULT JRS, PAYA M., 1996. Pharmacological and biochemical action of simple coumarins: Natural products with therapeutic potential. *General Pharmacology*, 27, 713–722

HOWELL MD, KIM BE, GAO P, GRANT AV, BOGUNIEWICZ M, DEBENEDETTO A, SCHNEIDER L, BECK LA, BARNES KC, LEUNG DY., 2009. Cytokine modulation of atopic dermatitis filaggrin skin expression. *Journal of Allergy and Clinical Immunology*, 124, 3, 2, 7–12

HU J, MCKOY K, PAPIER A, KLAUS S, RYAN T, GROSSMAN H, MASENGA EJ, SETHI A, CRAFT N., 2011. Dermatology and HIV/AIDS in Africa. *Journal of Global Infectious Diseases*, 3, 3, 275–280

INOUYE S, ABE S, YAMAGUCHI H, ASAKURA M., 2003. Comparative study of antimicrobial and cytotoxic effects of selected essential oils by gaseous and solution contacts. *International Journal of Aromatherapy*, 13, 33–41

ISCAN G, KIRIMER N, KURKCUOGLU M, BAŞER KHC, DEMIRCI F., 2002. Antimicrobial screening of *Mentha* piperita essential oils. *Journal of Agricultural and Food Chemistry*, 50, 3943–3946

JAIN PK, JOSHI H., 2012. Coumarin: chemical and pharmacological profile. *Journal of Applied Pharmaceutical Science*, 2, 6, 236–240

JAIN SK, AGRAWAL SC., 2002. Fungistatic activity of some perfumes against otomycotic pathogens. *Mycoses,* 45, 88–90

JAIN V, MOMIN M, LADDHA K., 2012. *Murraya koenigii:* an updated review. *International Journal of Ayurvedic and Herbal Medicine*, 2, 4, 607–627

JOBANPUTRA R, BACHMANN M., 2000. The effect of skin diseases on quality of life in patients from different social and ethnic groups in Cape Town, South Africa. *International Journal of Dermatology,* 39, 826–831

JODAMUS N, NOTTEN A., 2002. *Agathosma mucronulata* Sond. http://www.plantzafrica.com/plantab/agathosmucro.htm

JOHNSON EM, WARNOCK DW, LUKER J, PORTER SR, SCULLY C., 1995. Emergence of azole drug-resistance in Candida species from HIV-infected patients receiving prolonged fluconazole therapy for oral candidiasis. *Journal of Antimicrobial Chemotherapy*, 35, 103–114

JOSEPH-HORNE T, HOLLOMON DW., 1997. Molecular mechanisms of azole resistance in fungi. *FEMS Microbiology Letters*, 149, 141–9

KALIA YN, PIROT F, GUY RH., 1996. Homogeneous transport in a heterogeneous membrane: water diffusion across human stratum corneum *in vivo*. *Biophysical Journal*, 71, 2692–2700

KAMAI Y, MAEBASHI K, KUDOH M, MAKIMURA K, NAKA W, UCHIDA K, YAMAGUCHI H., 2004. Characterization of mechanisms of fluconazole resistance in a *Candida albicans* isolate from a Japanese patient with chronic mucocutaneous candidiasis. *Microbiology and Immunology*, 48, 937–943

KAMAL GM, ANWAR F, HUSSAIN AI, SARRI N, ASHRAF MY., 2011. Yield and chemical composition of Citrus essential oils as affected by drying pretreatment of peels. *International Food Research Journal*, 18, 4, 1275–1282

KANE J., 1997. Laboratory handbook of dermatophytes: a clinical guide and laboratory handbook of dermatophytes and other filamentous fungi from skin, hair, and nails. *Star Pub*, Belmont, Canada, 344

KAPOOR VP., 2005. Herbal cosmetics for skin and haircare. *Natural Product Radiance*, 4, 4, 309

KAR A., 2003. Pharmacognosy and pharmacobiotechnology. *New Age International Publishers*, Mumbai, India

KARAMANOS AJ, SOTIROPOULOU DEK., 2013. Field studies of nitrogen application on Greek Oregano (*Origanum vulgare*) essential oil during two cultivation seasons. *Industrial Crops and Products*, 46, 246–252

KATZ M, POULSEN BJ., 1971. In: **BRODIE BB, GILETTE J**, Handbook of experimental pharmacology. *Springer Verlag*, Berlin

KATZUNG GB, SILVA P, MUNDIM FD, VOOEUX PJ., 1998. Basic and clinical pharmacology. *Editora Guanabara Koogan*, Rio de Janeiro, Brazil

KAUR R, KASHYAP B, BHALLA P., 2008. Onychomycosis-epidemiology, Diagnosis and management. *Indian Journal of Medical Microbiology,* 26, 108–116

KAYSER O, KOLODZIEJ H., 1999. Antibacterial activity of simple coumarins: Structural requirements for biological activity. *Zeitschrift für Naturforschung C*, 54c, 169–174

KONGKATHIP B, SUTTHIPRABHA S, YOOSOOK C, MONGKOLSOOK Y, KONGKATHIP N., 2010. Determination of a pyranocoumarin and three carbazole compounds in *Clausena excavata* by RP-HPLC. *Journal of Chromatographic Science,* 48, 445–449

KOROLKOVAS A., 1996. *Dicionário Terapêutico Guanabara*. *Guanabara-Koogan*, Rio de Janeiro, 202–203

KOSTOVA I., 2006. Synthetic and natural coumarins as antioxidants. *Mini Reviews in Medicinal Chemistry*, 6, 365–374

KRUK J, DUCHNIK E., 2014. Oxidative stress and skin diseases: possible role of physical activity. *Asian Pacific Organization for Cancer Prevention*, 15, 2, 561–568

KUBO M, MATSUDA H., 1992. Anti-allergic agents containing hesperidin. *Patent Japan Kokai Tokkyo Koho,* 64295, 428

KUMAR R, SAHA A, SAHA D., 2012. A new antifungal coumarin from *Clausena excavata*. *Fitoterapia*, 83, 230–233

LA CROIX IF., 1984. Scented plants in Southern Africa. *Macmillan South African*, Johannesburg

LACY A, O'KENNEDY R., 2004. Studies on coumarins and coumarin-related compounds to determine their therapeutic role in the treatment of cancer, current pharmaceutical design*. Bentham Science Publishers Ltd,* 10, 3797–3811

LALL N, KISHORE N., 2014. Are plants used for skin care in South Africa fully explored? *Journal of Ethnopharmacology*, 153, 1, 61–84

LAPHOOKHIEO S, SRIPISUT T, PRAWAT U, KARALAI C., 2009. A new coumarin from *Clausena excavata*. *Heterocycles*, 78, 2115–2119

LARSON RA., 1988. The antioxidants of higher plants. *Phytochemistry,* 27, 969–978

LAURIN P, FERROUD D, KLICH M, DUPUIS-HAMELIN C, MAUVAIS P, LASSAIGNE P, BONNEFOY A, MUSICKI B., 1999. Synthesis and *in vitro* evaluation of novel highly potent coumarin inhibitors of gyrase B. *Bioorganic and Medicinal Chemistry Letters*, 19, 2079–2084

LEE SO, PARK I, CHOI GJ, LIM HK, JANGKS, CHO KY, SHIN SG, KIM JC., 2007. fumigant activity of essential oils and components of *Illicium verum* and *Schizonepeta tenuifolia* against *Botrytis cinerea* and *Colletotrichum gloeosporioides*. *Journal of Microbiology and Biotechnology*, 17, 9, 1568–1572

LENG W, LIU T, RUI LI, YANG J, WEI C, ZHANG W, JIN Q., 2008. Proteomic profile of dormant *Trichophyton rubrum* conidia. *BMC Genomics*, 9, 303

LEWIS JH, ZIMMERMAN HJ, BENSON GD, ISHAK KG., 1984. Hepatic injury associated with ketoconazole therapy. Analysis of 33 cases. *Gastroenterology*, 86, 3, 503–513

LI Q, ZHU LF, BUT PPH, KONG YC, CHANG HT, WATERMAN PG., 1988. Monoterpene and Sesquiterpene rich oils from the leaves of Murraya species: Chemotaxonomic significance. *Biochemical Systematics and Ecology*, 16, 491–494

LIEBENBERG L., 2008. Evaluation of biologically active compounds in *Coleonema album. Masters Degree Dissertation*, University of Johanessburg

LIM EK, ASHFORD DA, HOU B, JACKSON RG, BOWLES DJ., 2004. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. *Biotechnology and Bioengineering*, 87, 623–631

LIMA EO, GOMPERTZ OF, GIESBRECHT AM AND PAULO MQ., 1993. *In vitro* antifungal activity of essential oil from officinal plants against dermatophytes. *Mycoses*, 36, 9-10, 333–336

LIS-BALCHIN M, HART S., 2002. *Coleonema album*: studies of the pharmacological action on smooth muscle *in vitro* and antimicrobial action of its essential oil. *Phytotherapy Research*, 16, 292–294

LÜ ZR, KIM WS, CHO IH, PARK D, BHAK J, SHI L, ZHOU HW, LEE DY, PARK YD, YANG JM, ZOU F., 2009. DNA microarray analyses and interactomic predictions for atopic dermatitis. *Journal of Dermatological Science*, 55, 2, 123–125

MABBERLEY DJ., 2008. Mabberley's plant-book: A portable dictionary of plants. 3rd Edition, *Cambridge University Press*, United Kingdom

MACÊDO DP, NEVES RP, NEVES RP, MAGALHÃES MC, SOUZA-MOTTA CM, QUEIROZ LA., 2005. Pathogenic aspects of *Epidermophyton floccosum* Langeron et Milochevich as a possible ethological agent of *Tinea capitis*. *Brazilian Journal of Microbiology*, 36, 36–37

MACOWAN P., 1893. Buchus of the Cape. *Agricultural Journal of the Cape of Good Hope*, 146

MACURA AB., 1993. Dermatophyte infections. *International Journal of Dermatology*, 32, 313–23

MADISON KC., 2003. Barrier function of the skin: "la raison d'etre" of the epidermis. *Journal of Investigative Dermatology*, 121, 231–41

MAENZA JR, KERULY JC, MOORE RD, CHAISSON RE, MERZ WG, GALLANT JE., 1996. Risk factors for fluconazole-resistant candidiasis in human

immunodeficiency virus–infected patients. *Journal of Infectious Diseases*, 173, 219–225

MAENZA JR, MERZ WG, ROMAGNOLI MJ, KERULY JC, MOORE RD, GALLANT JE., 1997. Infection due to fluconazole-resistant *Candida* in patients with AIDS: prevalence and microbiology. *Clinical Infectious Diseases*, 24, 28–34

MAERTENS JA., 2004. History of the development of azole derivatives. *Clinical Microbiology and Infection*, 10, 1, 1–10

MAKI-ARVELA P, KUMAR N, ERANEN K, SALMI T, MURZIN DY., 2006. Inverse temperature dependence due to catalyst deactivation in liquid phase citral hydrogenation over Pt/Al2O3. *Chemical Engineering Journal*, 122, 3, 127

MALE O., 1990. The significance of mycology in medicine. In: **HAWKSWORTH DL,** Frontiers in mycology. *CAB International*, Wallingford

MANGENA T, MUYIMA NY., 1999. Comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra*, *Pteronia incana* and *Rosmarinus officinalis* on selected bacteria and yeast strains. *Letters in Applied Microbiology*, 28, 291–296

MAPUNYA MB, NIKOLOVA RV, LALL N., 2012. Melanogenesis and antityrosinase activity of selected South African plants. *Evidence-Based Complementary and Alternative Medicine*, 1–6

MARCOS-ARIAS C, ERASO E, MADARIAGA L, QUINDÓS G., 2011. *In vitro* activities of natural products against oral *Candida* isolates from denture wearers. *BMC Complementary and Alternative Medicine,* 11, 119–126

MARENHOLZ I, NICKEL R, RÜSCHENDORF F, SCHULZ F, ESPARZA-GORDILLO J, KERSCHER T, GRÜBER C, LAU S, WORM M, KEIL T, KUREK M, ZALUGA E, WAHN U, LEE YA., 2006. Filaggrin loss-of-function mutations predispose to phenotypes involved in the atopic march. *Journal of Allergy and Clinical Immunology*, 118, 4, 866–871

MARTINEZ-CAYUELA M., 1995. Oxygen free radicals and human disease. *Biochimica*, 77, 147-161

MARTINEZ-ROSSI NM, PERES NT, ROSSI A., 2008. Antifungal resistance mechanisms in dermatophytes. *Mycopathologia*, 166, 369–383

MARTINS MD, LOZANO-CHIU M, REX JH., 1997. Point prevalence of oropharyngeal carriage of fluconazole-resistant *Candida* in human

immunodeficiency virus–infected patients. *Clinical Infectious Diseases*, 25, 843–846

MARUZZELLA JC, SICURELLA NA., 1960. Antibacterial activity of essential oil vapours. *Journal of the American Pharmacists Association,* 49, 692–694

MATSUOKA H, LI Y, TAKEKAWA Y, TERAOKA T., 1990. Evaluation of antifungal volatile compounds on the basis of the elongation rate of a single hypha. *Applied and Environmental Microbiology*, 56, 3779–3784

MATSUZAKI Y, TSUJISAWA T, NISHIHARA T, NAKAMURA M, KAKINOKI Y., 2013. Antifungal activity of chemotype essential oils from rosemary against *Candida albicans*. *Open Journal of Stomatology,* 3, 2, 176–182

MCCLELLAN KJ, WISEMAN LR, MARKHAM A., 1999. Terbinafine. An update of its use in superficial mycoses. *Drugs*, 58,179–202

MCHALE D, KHOPKAR PP, SHERIDAN JB., 1987. Coumarin glycosides from *Citrus* flavedo. *Phytochemistry*, 26, 9, 2547–2549

MEFFERT H, DIEZEL W, SÖNNICHSEN N., 1976. Stable lipid peroxidation products in human skin: detection, ultraviolet light-induced increase, pathogenic importance. *Biologica Experientia*, 32, 11, 1397–1398

MELLO JCPD, MENTZ LA, PETROVICK PR., 2003. Farmacognosia: Da Planta ao Medicamento; Porto Alegre. *EFRGS: Porto Alegre*, Brazil

MERCER DK, ROBERTSON J, WRIGHT K, MILLER L, SMITH S, STEWART CS, O'NEIL DA., 2013. A prodrug approach to the use of coumarins as potential therapeutics for superficial mycoses. *Plos One*, 8, 11, e80760

MIDGLEY G, MOORE MK, COOK JC, PHARN QG., 1994. Mycology of disorders. *Journal of American Academy of Dermatology*, 3, 2, 68–74

MILLER LJ., 1993. Oral pilocarpine for radiation-induced xerostomia. *Cancer Bulletin (Houston)*, 45, 6, 549–550

MIN DB., 1998. Lipid oxidation of edible oil. In: **AKOH K, MIN DB,** Food lipids: chemistry, nutrition and biotechnology. *Marcel Dekkar*, New York

MITCHELL RN, COTRAN RS., 2003. Cell injury, adaptation, and death. In: **KUMAR V, COTRAN RS, ROBBINS SL,** Basic pathology. 7th Edition, *Harcourt (India) Pvt Ltd,* New Delhi

MOKOKA TA, MCGAW LJ, ELOFF JN., 2010. Antifungal efficacy of ten selected South African plant species against *Cryptococcus neoformans*. *Pharmaceutical Biology*, 48, 397–404

MONOD M., 2008. Secreted proteases from dermatophytes. *Mycopathologia*, 166, 285–294

MOOLLA A, VILJOEN AM., 2008. 'Buchu'—*Agathosma betulina* and *Agathosma crenulata* (Rutaceae): a review. *Journal of Ethnopharmacology*, 119, 3, 413–419

MOOLLA A., 2006. A phytochemical and pharmacological investigation of indigenous *Agathosma* species. *MSc Dissertation*, University of Witwatersrand

MOOLLA ASF, VAN VUUREN RL, VAN ZYL, VILJOEN AM., 2007. Biological activity and toxicity profile of 17 *Agathosma* (Rutaceae) species. South African Journal of Botany, 73, 4, 588–592

MOSAM A, IRUSEN EM, KAGORO H, ABOOBAKER J, DLOVA N., 2004. The impact of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) on skin disease in KwaZulu-Natal, South Africa. *International Journal of Dermatology*, 43, 782–783

MOUFIDA S, MARZOUK B., 2003. Biochemical characterization of blood orange, sweet orange, lemon, bergamot and bitter orange. *Phytochemistry*, 62, 8, 1283–1289

MOURE A, CRUZ JM, FRANCO D, DOMINQUEZ JM, SINEIRO J, DOMINQUEZ H, NUNEZ MJ, PARAJO JC., 2001. Natural antioxidants from residual sources. *Food Chemistry*, 72, 145–171

MOUSSAIEFF A, SHEIN NAA, TSENTER J, GRIGORIADIS S, SIMEONIDOU C, ALEXANDROVICH AG, TREMBOVLER V, BEN-NERIAH Y, SCHMITZ ML, FIEBICH BL, MUNOZ E, MECHOULAM R, SHOHAMI E., 2008. Incensole acetate: A novel neuroprotective agent isolated from *Boswellia carterii*. *Journal of Cerebral Blood Flow and Metabolism*, 28, 1341–1352

MURRAY PR, ROSENTHAL KS, PFALLER MA., 2013. Medical Microbiology. *Elsevier Saunders*, Philadelphia, 605–642

MYERS N, MITTERMEIER RA, MITTERMEIER CG, DA FONSECA GAB, KEN J., 2000. Biodiversity hotspots for conservation priorities. *Nature*, 403, 854–856

NAAFS B, MASENGA J, VAN DER WERF TS., 2013. The skin. In: **MABEY D, GILL G, PARRY ELDRYD, WEBER MW**, Principle of medicine in Africa. 4th Edition, *Cambridge University Press*, New York

NARASIMHAN NS, PARADKAR MV, CHITGUPPI VP, KELKAR SL., 1975. Alkaloids of *Murraya koenigii*, Structures of mahanimbine, koenimbine,

mahanine, koenine, koenigine and koenidine. *Journal of Chemistry*, 13, 993–995

NARASIMHAN NS, PARADKAR MV, KELKAR SL., 1970. Alkaloids of *Murraya koenigi*, Structures of mahanine, koenine, koenigine and koenidine. *Indian Journal of Chemistry*, 8, 473–476

NARAYANASWAMY N, BALAKRISHNAN KP., 2011. Evaluation of some medicinal plants for their antioxidant properties. *International Journal of PharmTech Research*, 3, 1, 381–385

NATIONAL AIDS AND STI CONTROL PROGRAM., 2008. Dermatological manifestations of the HIV disease. In: National manual for the management of HIV-related opportunistic infections and conditions. *Baltech Equipments Limited*, Kenya

NEGRI M, SALCI TP, SHINOBU-MESQUITA CS, CAPOCI IRG, SVIDZINSKI TIE, KIOSHIMA ES., 2014. Early state research on antifungal natural products. *Molecules*, 19, 2925–2956

NELSON MM, MARTIN AG, HEFFERMAN MP., 2003. Superficial fungal infections: dermatophytosis, onychomycosis, *Tinea nigra*, piedra. In: **FITZPATRICK TB**, Dermatology in general medicine. 6th Edition, *McGraw-Hill*, New York

NENOFF P, HAUSTEIN UF, BRANDT W., 1996. Antifungal activity of the essential oil of *Melaleuca alternifolia* (tea tree oil) against pathogenic fungi *in vitro*. *Skin Pharmacology*, 9, 388–394

NENOFF P, HERRMANN J, GRASER Y., 2007. *Trichophyton mentagrophytes* sive interdigitale? A dermatophyte in the course of time. *Journal der Deutschen Dermatologischen Gesellschaft*, 5, 198–202

NESS AR, POWLES JW., 1997. Fruit and vegetables, and cardiovascular disease: a review. *International Journal of Epidemiology,* 26, 1–13

NESTLE FO, DI MEGLIO P, QIN JZ, NICKOLOFF BJ., 2009. Skin immune sentinels in health and disease. *Nature Reviews Immunology,* 9, 679–691

NGADJUI BT, MOUNCHEROU SM, AYAFOR JF, SONDENGAM BL, TILLEQUIN F., 1991. Geranyl coumarins from *Clausena anisata*. *Phytochemistry*, 30, 2809–2811

NGUYEN T, BRUNSON D, CRESPI CL, PENMAN BW, WISHNOK JS, TANNENBAUM SR., 1992. DNA damage and mutation in human cells exposed

to nitric oxide *in vitro*. *Proceedings of the National Academy of Sciences, U.S.A*, 89, 3030–3034

NIKI E., 2009. Lipid peroxidation: physiological levels and dual biological effects. *Free Radical Biology and Medicine*, 47, 5, 469–484

NNORUKA EN., 2005. Skin diseases in south-east Nigeria: A current perspective. *International Journal of Dermatology*, 44, 29–33

NOTTEN A., 2001. *Calodendrum capense. Kirstenbosch NBG,* South Africa

ODDS CF., 2003. Antifungal agents: Their diversity and increasing sophistication. *Mycologist*, 17, 51–55

ODDS FC, ALISTAIR JP, GOW B, GOW AR., 2003. Antifungal agents: mechanisms of action. *Trends in Microbiology,* 11, 6, 272–279

OGAWA H, YOSHIIKE T., 1992. Atopic dermatitis: studies of skin permeability and effectiveness of PUVA treatment. *Paediatric Dermatology*, 9, 383–385

OKUNADE AL, ELVIN-LEWIS MPF, LEWIS WH., 2004. Natural antimycobacterial metabolites: current status. *Phytochemistry*, 65, 1017–1032

OLSEN SJ, MUMMANENI V, ROLAN P, NORTON J, GRASELA DM., 2000. Ravuconazole single ascending oral dose study in healthy subjects [abstract]. In: Program and Abstracts of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy (Toronto). *American Society for Clinical Microbiology*, Washington DC

OMRAN SM, MOODIB MA, BAGHER SM, AMIRI N, MOSAVI SJ, MOHAMMAD SA, SAEED GM, MARZIE S, SHIADE J, KHERADI E, SALEHI M., 2011. The effects of limonene and orange peel extracts on some spoilage fungi. *International Journal of Molecular and Clinical Microbiology*, 1, 82–86

OU B, HAMPSCH-WOODILL M, PRIOR RL., 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry,* 49*,* 4619–4626

PALMEIRA-DE-OLIVEIRA A, SALGUEIRO L, PALMEIRA-DE-OLIVEIRA R, MARTINEZ-DE-OLIVEIRA J, PINA-VAZ C, QUEIROZ JA, RODRIGUES AG., 2009. Anti-candida activity of essential oils. *Mini-Reviews in Medicinal Chemistry,* 9, 1292–1305

PALMER CNA, IRVINE AD, TERRON-KWIATKOWSKI A, ZHAO Y, LIAO H, LEE SP, GOUDIE DR, SANDILANDS A, CAMPBELL LE, SMITH FJ, O'REGAN

GM, WATSON RM, CECIL JE, BALE SJ, COMPTON JG, DIGIOVANNA JJ, FLECKMAN P, LEWIS-JONES S, ARSECULERATNE G, SERGEANT A, MUNRO CS, EL HOUATE B, MCELREAVEY K, HALKJAER LB, BISGAARD H, MUKHOPADHYAY S, MCLEAN WH., 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature Genetics*, 38, 4, 441–446

PANDEY A, PANDEY M., 2013. Isolation and characterization of dermatophytes with *Tinea* infections at Gwalior (MP), India. *International Journal of Pharmaceutical Science Invention*, 2, 5–8

PANKHURST C., 2001. Oropharyngeal candidiasis. *Clinical Evidence*, 4, 761–72

PANTHONG K, SRISUD Y, RUKACHAISIRIKUL V, HUTADILOK-TOWATANA N, VORAVUTHIKUNCHAI SP, TEWTRAKUL S., 2013. Benzene, coumarin and quinolinone derivatives from roots of *Citrus hystrix*. *Phytochemistry*, 88, 79–84

PAPPE L., 1857. Florae Capensis medicae prodromus. 2nd Edition, *W. Brittain*, Cape Town

PARK MJ, GWAK KS, YANG I, KIM KW, JEUNG EB, CHANG JW, CHOI IG., 2009. Effect of citral, eugenol, nerolidol and α-terpineol on the ultrastructural changes of *Trichophyton mentagrophytes*. *Fitoterapia*, 80, 290–296

PARTHASARADHI A, AL GUFAI AF., 2004. The pattern of skin diseases in Hail Region, Saudi Arabia. Saudi. *Medical Journal*, 25, 507–510

PATEL S, MEIXNER JA, SMITH MB, MCGINMS MR., 2006. Superficial mycoses and dermatophytes. In: **TYRING SK, LUPI O, HENGGE UR**, Tropical dermatology, *Elsevier Inc,* New York

PAULI A., 2006. Anticandidal low molecular compounds from higher plants with special reference to compounds from essential oils. *Medicinal Research Reviews,* 26, 223–268

PEARLSTINE E., 2006. Skin treatments using essential oils. *Aroma Scents Journal,* Vol. 36(winter)

PELLETIER R, PETER J, ANTIN C, GONZALEZ C, WOOD L, WALSH TJ., 2000. Emergence of resistance of *Candida albicans* to clotrimazole in human immunodeficiency virus-infected children: *in vitro* and clinical correlations. *Journal of Clinical Microbiology*, 38, 4, 1563–1568

PENNYS NS., 1995. Skin manifestations of AIDS. *Martin Dunitz,* London

PEPELJNJAK S, KOSALEC I, KALODERA Z, BLAZEVIC N., 2005. Antimicrobial activity of juniper berry essential oil. *Acta Pharmaceutica*, 55, 417–422

PEREIRA FO, WANDERLEY PA, VIANA FAC, LIMA RB, SOUSA FB, LIMA EO., 2011. Growth inhibition and morphological alterations of *Trichophyton rubrum* induced by essential oil from *Cymbopogon winterianus* Jowitt Ex Bor. *Brazilian Journal of Microbiology*, 42, 233–242

PERES NT, MARANHÃO FC, ROSSI A, MARTINEZ-ROSSI NM., 2010. Dermatophytes: host-pathogen interaction and antifungal resistance. *Journal Anais Brasileiros de Dermatologia*, 85, 657–667

PERFECT JR, DURACK DT, HAMILTON JD, GALLIS HA., 1982. Failure of ketoconazole in cryptococcal meningitis. *Journal of the American Medical Association*, 247, 24, 3349–3351

PHILLIPS EP., 1917. South African Buchu. *South African Journal for Industries*, 1, 55

PILLANS NS., 1910. A preliminary note on Cape Buchus. *Agricultural Journal of the Cape of Good Hope*, 37, 252

PING-HSIEN C, CHI-WEI L, JIA-YING C, MURUGAN M, BOR-JINN S, HUEIH-MIN C., 2007. Antifungal activity of crude extracts and essential oil of *Moringa oleifera* Lam. *Bioresource Technology*, 98, 232–236

PINHEIRO CUB., 1997. Jaborandi (*Pilocarpus* sp., Rutaceae): a wild species and its rapid transformation into a crop. *Economic Botany*, 51, 1, 49–58

PINNAGODA J, TUPKER RA, AGNER T, SERUP J., 1990. Guidelines for transepidermal water loss (TEWL) measurement. A report from the Standardization Group of the European Society of Contact Dermatitis. *Contact Dermatitis*, 22, 164–78

PONT A, WILLIAMS PL, LOOSE DS., 1982. Ketoconazole blocks adrenal steroid synthesis. *Annals of Internal Medicine*, 97, 3, 370–372

POONKOTHAI M, SARAVANAN M., 2008. Antibacterial activity of *Aegle marmelos* against leaf, bark and fruit extracts. *Ancient Science of Life*, 27, 3, 15–18

POTOSKI BA, BROWN J., 2002. The safety of voriconazole. *Clinical Infectious Diseases*, 35, 10, 1273–1275

POURBAFRANI M, FORGAC SG, HORVTH IS, NIKLASSON C., 2010. Production of biofuels, limonene and pectin from citrus wastes. *Bioresource Technology*, 101, 4246–4250

PRAJAPATI ND, PUROHIT SS, SHARMA AK, KUMAR T., 2003. A handbook of medicinal plants. *Agrobios*, Jodhpur, 352–353

PREEDY VR., 2015. Essential oil in food preservation, flavour and safety. *Academic Press*, London, UK

PRIOR RL, XIANLI WU X, SCHAICH K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, 53, 4290–4302

PROESTOS C, BOZIARIS IS, NYCHAS GJE, KOMAITIS M., 2006. Analysis of flavonoids and phenolic acids in Greek aromatic plants: investigation of their antioxidant capacity and antimicrobial activity. *Food Chemistry*, 95, 664–67

PUJOL C, PFALLER MA, SOLL DR., 2004. Flucytosine resistance is restricted to a single genetic clade of *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, 48, 262–6

PUROHIT P, KAPSNER TR., 1994. Natural essential oils; functional benefits. *Cosmetics and Toiletries*, 109, 51–55

QUEIROZ EF, WOLFENDER JL, HOSTETTMANN K., 2009. Modern approaches in the search for new lead antiparasitic compounds from higher plants. *Current Drugs,* 10, 202–211

RAI MK, MARES D., 2003. Plant-derived antimycotics: current trends and future prospects. *Food Products Press*, New York

RAJENDRAN MP PALLAIYAN BB, SELVARAJ N., 2014. Chemical composition, antibacterial and antioxidant profile of essential oil from *Murraya koenigii* (L.) leaves. *Avicenna Journal of Phytomedicine*, 4, 3

RAJKUMAR S, JEBANESAN A., 2010. Chemical composition and larvicidal activity of leaf essential oil from *Clausena dentata* (Willd)M.Roam (Rutaceae) against Chinkungunya vector, *Aedes aegypti* Linn. (Diptera: culicidae). *Journal of Asia – Pacific Entomology,* 13, 2, 107–109

RAJU PV, RAO GR, RAMANI TV, VANDANA S., 2005. Skin disease: clinical indicator of immune status in human immunodeficiency virus (HIV) infection. *International Journal of Dermatology*, 44, 646–649

RAMOROKA R, MAPUNYA TG., 2006. Personal Communication

RAMOS-E-SILVA M, JACQUES CD., 2012. Epidermal barrier function and systemic diseases. *Clinics in Dermatology,* 30, 3, 277–279

RANGANNA S, GOVINDARAJAN VS, RAMANA KV., 1983. Citrus fruits-varieties, chemistry, technology, and quality evaluation. Part II. Chemistry, technology and quality evaluation. A. chemistry. *Critical Reviews in Food Science and Nutrition*, 18, 313–386

RAO BRR, RAJPUT DK, MALLAVARAPU GR., 2011. Chemical diversity in curry leaf (*Murayya koenigii*) essential oil. *Food Chemistry*, 126, 3, 989–994

RAO TS, BASU N, SIDDIQUI HH., 1982. Anti-inflammatory activity of curcumin analogs. *Indian Journal of Medicinal Research*, 75, 574–578

RASHID A., 2001. Arthroconidia as vectors of dermatophytosis. *Cutis*, 67, 23

REVANKAR SG, KIRKPATRICK WR, MCATEE RK, DIB OP, FOTHERGILL AW, REDDING SW, RINALDI MG, PATTERSON TF., 1996. Detection and significance of fluconazole resistance in oropharyngeal candidiasis in human immunodeficiency virus–infected patients. *Journal of Infectous Diseases*, 174, 821–827

RHIND JP., 2012. Aromatherapeutic blending: Essential oils in synergy. *Singing Dragon,* London

RICE-EVANS CA, BURDON RH., 1994. Free radical damage and its control. *Elsevier*, Amsterdam, Netherlands

RIMBACH G, FUCHS J, PACKER L., 2005. Application of nutrigenomics tools to analyze the role of oxidants and antioxidants in gene expression. In: **RIMBACH G, FUCHS J, PACKER L**, Nutrigenomics. *Taylor and Francis Publishers*, United States

RIVEIRO ME, DE KIMPE N, MOGLIONI A, VAZQUEZ R, MONCZOR F, SHAYO C, DAVIO C., 2010. Coumarins: old compounds with novel promising therapeutic perspectives*. Current Medicinal Chemistry*, 17, 1325–1338

ROBERTS DT, EVANS EGV, ALLEN BR., 1990. Pocket picture guides- fungal nail infection. *Gower Medical Publishing*, UK

RODRÍGUEZ EJ, RAMIS-RAMOS G, HEYDEN YV, SIMÓ-ALFONSO EF, LERMA-GARCÍA MJ, SAUCEDO-HERNÁNDEZ Y, MONTEAGUDO U, MORALES Y, HOLGADO B, HERRERO-MARTÍNEZ JM., 2012. Chemical composition, antioxidant properties and antimicrobial activity of the essential oil of *Murraya paniculata* leaves from the mountains of Central Cuba. *Natural Product Communication,* 7, 11, 1527–1530

RODRIGUEZ-TUDELA JL, MARTINEZ-SUAREZ JV, DRONDA F, LAGUNA F, CHAVES F, VALENCIA E., 1995. Correlation of in-vitro susceptibility test results with clinical response: a study of azole therapy in AIDS patients. *Journal of Antimicrobial Chemotherapy*, 35, 793–804

ROJAS JJ, OCHOA VJ, OCAMPO AS, MUÑOZ JF., 2006. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. *BMC Complementary and Alternative Medicine*, 17, 2

ROMANO L, BATTAGLIA F, MASUCCI L, SANGUINETTI M, POSTERARO B, PLOTTI G, ZANETTI S, FADDA G., 2005. *In vitro* activity of bergamot natural essence and furocoumarin-free and distilled extracts, and their associations with boric acid, against clinical yeast isolates. *Journal of Antimicrobial Chemotherapy,* 55, 110–114

ROSENBLATT HM, BYRNE W, AMENT ME, GRAYBILL J, STIEHM ER., 1980. Successful treatment of chronic mucocutaneous candidiasis with ketoconazole. *Journal of Pediatrics*, 97, 657–60

ROUT PK, RAO YR, SREE A, NAIK SN., 2007. Composition of essential oils, concrete, absolute, wan and headspace volatiles of *Murraya paniculata* (Linn.) Jack flowers. *Flavour and Fragrance Journal*, 22, 352–357

ROWAN DD., 2011. Volatile metabolites. A review. *Metabolites*, 1, 41-63

RUDIKOFF D., 2002. The relationship between HIV infection and atopic dermatitis. *Current Allergy and Asthma Reports*, 2, 275–281

SABO JA, ABDEL-RAHMAN SM., 2000. Voriconazole: a new triazole antifungal. *Annals of Pharmacotherapy*, 34, 9, 1032–1043

SADGROVE N, JONES G., 2015. A contemporary introduction to essential oils: chemistry, bioactivity and prospects for Australian agriculture. *Agriculture,* 5, 48–102

SAINI SC, REDDY GBS., 2013. *Murraya koenigii*. IOSR *Journal of Pharmacy and Biological Sciences.* 7, 6, 15–18

SAKONG BM., 2012. Isolation and characterization of compounds from *Calodendrum capense* (Rutaceae) and *Lydenburgia cassinoides* (Celastraceae) for treatment of fungal and bacterial infections in immunocompromised patients. *MSc Dissertation*, University of Pretoria

SANCHEZ-MORENO C, LARRAURI JA, SAURA-CALIXTO F., 1998. A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, 79, 270–276

SANGUINETTI M, POSTERARO B, ROMANO L, BATTAGLIA F, LOPIZZO T, DE CAROLIS E, FADDA G., 2007. *In vitro* activity of *Citrus bergamia* (Bergamot) oil against clinical isolates of dermatophytes. *Journal of Antimicrobial Chemotherapy,* 59, 305–308

SANTOS DA, BARROS MES, HAMDAN JS., 2006. Establishing a method of inoculum preparation for susceptibility testing of *Trichophyton rubrum* and *Trichophyton mentagrophytes. Journal of Clinical Microbiology,* 44, 1, 98

SARADHA JK, RAO BS., 2010. Antibacterial activity of extracts from *Aegle marmelos* against standard pathogenic bacterial strains*. International Journal of PharmTech Research*, 2, 3, 1824–1826

SATCHELL AC, SAURAJEN A, BELL C, BARNETSON RS., 2002. Treatment of dandruff with 5% tea tree oil shampoo. *Journal of American Academy of Dermatology*, 47, 852–855

SATIMIA FT, MCBRIDE SR, LEPPARD B., 1998. Prevalence of skin disease in rural Tanzania and factors influencing the choice of health care, modern or traditional. *Archives of Dermatology*, 134, 1363–1366

SATO K, KRIST S, BUCHBAUER G., 2006. Antimicrobial effect of trans-cinnamaldehyde, (-)-perillaldehyde (-)-citronellal, citral, eugenol and carvacrol on airborne microbes using an airwasher. *Biological Pharmaceutical Bulletin*, 29, 11, 2292–2294

SCHAFER M, FARWANAH H, WILLRODT A, HUEBNER AJ, SANDHOFF K, ROOP D, HOHL D, BLOCH W, WERNER S., 2012. Nrf2 links epidermal barrier function with antioxidant defense. *EMBO Molecular Medicine*, 4, 364–379

SCHAGEN SK, ZAMPELI VA, MAKRANTONAKI E, ZOUBOULIS CC., 2012. Discovering the link between nutrition and skin aging. *Dermato-endocrinology*, 4, 3, 298–307

SCHELZ Z, MOLNAR J, HOHMANN J., 2006. Antimicrobial and antiplasmid activities of essential oils. *Fitoterapia*, 77, 4, 279–85

SCHMELLER W., 1998. Community health workers reduce skin diseases in East African children. *International Journal of Dermatology*, 37, 370–377

SELL C., 2010. Chemistry of essential oils. In: **BAŞER KHC, BUCHBAUER G,** Handbook of essential oils: science, technology, and applications. *CRC Press*, *Taylor and Francis Group*, United Kingdom

SEN CK., 1995. Oxygen toxicity and antioxidants: State of the art. *Indian Journal of Physiology and Pharmacology,* 39, 177–196

SHAPIRO S, MEIER A, GUGGENHEIN B., 1996. The antimicrobial activity of the essential oils and essential oil components towards oral bacteria. *Oral Microbiology and Immunology*, 9, 202–208

SHARKER SMD, SHAHID IJ., 2009. Antinociceptive and bioactivity of leaves of *Murraya paniculata* (L.) Jack, Rutaceae. *Brazilian Journal of Pharmacognosy*, 19, 746–748

SHARMA BC., 2013. Ethnomedicinal plants used against skin diseases by indigenous population of Darjeeling Himalayas, India. *Indian Journal of Fundamental and Applied Life Sciences,* 3, 3, 299–303

SHARMA N, TRIPATHI A., 2006. Fungitoxicity of the essential oil of *Citrus sinensis* on post-harvest pathogens. *World Journal of Microbiology and Biotechnology,* 22, 587–593

SHEU HM, LEE JY, CHAI CY, KUO KW., 1997. Depletion of stratum corneum intercellular lipid lamellae and barrier function abnormalities after long term topical corticosteroids. *British Journal of Dermatology*, 136, 884–90

SHEU HM, LEE JY, KUO KW, TSAI JC., 1998. Permeability barrier abnormality of hairless mouse epidermis after topical corticosteroid: characterization of stratum corneum lipids by ruthenium tetroxide staining and high performance thin-layer chromatography. *Journal of Dermatology*, 25, 281–289

SIDRIM JJC, ROCHA MFG., 2004. Medical mycology light of authors contemporâneos. *Guanabara Koogan*. Rio de Janeiro, Brasil

SIES H., 1991. Oxidative stress. *Academic Press*, San Diego

SIKKEMA J, DE BONT JAM, POOLAN B., 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiology Reviews,* 59, 2, 201–222

SIMÕES CMO, SCHENKEL EP, GOSMANN MG, MELLO JCP, MENTS LA, PETROVICK PR., 2003. Pharmacognosy: The medicinal plant - products of plant origin and development of drugs. 5a Edition, 291–320

SIMPANYA MF., 2000. Dermatophytes: Their taxonomy, ecology and pathogenicity. *Revista Iberoamericana de Micología*, 17, 1–12

SIMPSON D., 1998. Buchu — South Africa's amazing herbal remedy. *Scottish Medical Journal*, 43, 189–191

SIVARANJANI N, RAO VS, RAJEEV G., 2013. Oxidative stress and role of antioxidants in atopic dermatitis. *Journal of Clinical and Diagnostic Research*, 7, 12, 2683–2685

SKANDAMIS PN, NYCHAS GJE., 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *Journal of Applied Microbiology*, 91, 1011–1022

SKARIA BP, JOY PP, MATTHEW S, MATHEW G, JOSEPH A, JOSEPH R., 2007. Aromatic plants. *New India Publishing Agency,* New Delhi

SMITH DC, FORLAND S, BACHANOS E, MATEJKA M, BARRETT V., 2001. Qualitative analysis of citrus fruits extracts by GC/MS: An undergraduate experiment. *Chemical Educator*, 6, 28–31

SMITH-PALMER A, STEWART J, FYFE L., 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology*, 26, 2, 118–122

SOARES LA, DE CÁSSIA J, SARDI O, GULLO FP, DE SOUZA NP, SCORZONI L, LEITE FS, SOARES MJ, GIANNINI M, ALMEIDA AMF., 2013. Anti dermatophytic therapy - Prospects for the discovery of new drugs from natural products. *Brazilian Journal of Microbiology*, 44, 4, 1035–1041

SONBOLI A, BABAKHANI B, MEHRABIAN AR., 2006. Antimicrobial activity of six constituents of essential oil from Salvia. *Zeitschrift für Naturforschung C*, 61, 160–164

SORRELL JM, CAPLAN AI., 2004. Fibroblast heterogeneity: More than skin deep. *Journal of Cell Science,* 117, 667–675

SOUZA MC, SIANI AC, RAMOS MF, MENEZES-DE-LIMA OJ, HENRIQUES MG., 2003. Evaluation of anti-inflammatory activity of essential oils from two Asteraceae species. *Pharmazie,* 58, 8, 582–6

SPURGEON SL, PORTER JW., 1981. Biosynthesis of isoprenoid compounds. *Wiley Interscience*, 3–37

SRUTKOWSKA S, CASPI R, GABIG M, WEGRZYN G., 1999. Detection of DNA replication intermediates after two dimensional agarose gel electrophoresis using a fluorescein- labelled probe. *Analytical Biochemistry*, 269, 221–222

STADTMAN ER., 1994. Protein modification in oxidative stress. In: **PAOLETTI R, SAINUELSSON B, CARAPANO AL, POLI A, RINETTI M.,** Oxidative processes and antioxidants. *Raven Press*, New York

STEVENS DA., 1977. The role of miconazole in systemic fungal infections. *American Review of Respiratory Disease,* 116, 801–806

SUDARAJAN N, AHAMAD H, KUMAR V., 2006. *Cytisus scoparius* Link-A natural antioxidant. *BMC Complementary and Alternative Medicine*, 6, 1–7

SVOBODA K., GREENAWAY RI., 2003. Lemon scented plants. *International Journal of Aromatherapy*, 13, 1, 23–32

SWAIN, 1963. Plant chemical taxonomy. *Academic Press*, London

TABASSUM N, HAMDANI M., 2014. Plants used to treat skin diseases. *Pharmacognosy Reviews*, 8, 15, 52–60

TABASSUM N, VIDYASAGAR GM., 2013. Antifungal investigations on plant essential oils. A review. *International Journal of Pharmacy and Pharmaceutical Sciences,* 5, 2, 19–28

TACHIBANA Y, KIKUZAKI H, LAJIS NH, NAKATANI N., 2003 Comparison of antioxidative properties of carbazole. *Journal of Agriculture and Food Chemistry*, 51, 22, 6461–6467

TAKEMURA Y, KANAO K, KONOSHIMA A, JU-ICHI M, ITO C, FURUKAWA H, TOKUDA H, NISHINO H., 2004. Two new bicoumarins from *Clausena excavata*. *Heterocyclic Chemistry*, 63, 115–122

TAKEMURA Y, NAKAMURA K, HIRUSAWA T, JU M, ITO C, FURUKAWA H., 2000. Four new furanone-coumarins from *Clausena excavata. Notes*, 48 582–584

TANI K, ADACHI M, NAKAMURA Y, KANO R, MAKIMURA K, HASEGAWA A, KANDA N, WATANABE S., 2007. The effect of dermatophytes on cytokine production by human keratinocytes. *Archives of Dermatological Research*, 299, 381–387

TASSOU C., KOUTSOUMANIS K, NYCHAS GJE., 2000. Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. *Food Research International*, 33, 3-4, 273–280

TASSOU CC, DROSINOS EH, NYCHAS GJE., 1995. Effects of essential oil from mint (*Mentha piperita*) on *Salmonella enteritidis* and *Listeria monocytogenes* in

model food systems at 4° and 10 °C. *Journal of Applied Bacteriology*, 78, 593–600

TAVARES AC, GONCALVES MJ, CAVALEIRA C, CRUZ MT, LOPES MC, CANHOTO J., 2008. Essential oil of *Daucus carota* subsp. halophilus: composition, antifungal activity and cytotoxicity. *Journal of Ethnopharmacology*, 119,129–134

TETTENBORN D, 1974. Toxicity of clotrimazole. *Postgraduate Medical Journal,* 50, 1, 17–20

THIELE JJ., 2001. Oxidative targets in the stratum corneum. A new basis for antioxidative strategies. *Skin Pharmacology and Applied Skin Physiology*, 14, 1, 87–91

THUY TT, RIPPERGER H, PORZEL A, SUNG TV, ADAM G., 1999. Coumarins, limonoids and an alkaloid from *Clausena excavata*. *Phytochemistry*, 52, 511–516

TROUBA KJ, HAMADEH HK, AMIN RP, GERMOLEC DR., 2002. Oxidative stress and its role in skin disease. *Antioxidant and Redox Signaling*, 4, 4, 665–673

TSE ICT., 2007*.* Dermatologic manifestations in HIV disease. In: **LEE SS, WU JCY, WONG K,** HIV Manual 2007. *Hong Kong Special Administrative Region Government,* Hong Kong

VALDÉS BSG., 2005. Estructura y actividad de los antifúngicos. *Revista Cubana de Farm,* 39, 1–15

VALDEZ IH, WOLFF A, ATKINSON JC, MACYNSKY AA, FOX PC., 1993. Use of pilocarpine during head and neck radiation therapy to reduce xerostomia and salivary dysfunction. *Cancer*, 71, 5, 1848–1851

VALKO M, RHODES CJ, MONCOL J, IZAKOWIC M, MAZUR M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions*, 160, 1–40

VAN DER MEER JW, KEUNING JJ, SCHEIJGROND HW., 1980. The influence of gastric acidity on the bio-availability of ketoconazole. *The Journal of Antimicrobial Chemotherapy*, 6, 4, 552–554

VAN WYK BE, GERICKE N., 2000. People's plants. A guide to useful plants of Southern Africa. *Briza*, Pretoria, South Africa, 218–219

VAN WYK BE, VAN QUADSHOOM B, GERICKE N., 1997. Medicinal Plants of South Africa. *Briza Publication*, Pretoria, South Africa, 236

VAN DEN BOSSCHE H., 1991. Ergosterol biosynthesis inhibitors. In: **PRASAD R**, *Candida albicans. Springer Verlag*, Berlin

VANDEPUTTE P, FERRARI S, COSTE AT., 2012. Antifungal resistance and new strategies to control fungal infections. *International Journal of Microbiology*, 16, 4236–4260

VEERU P, KISHOR MP, MEENAKSHI M., 2009. Screening of medicinal plant extracts for antioxidant activity. *Journal of Medicinal Plants Research*, 3, 8, 608–612

VELIOGLU YS, MAZZA G, GAO L, OOMAH BD., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agriculture and Food Chemistry,* 46, 10, 4113–4117

VEREY R., 1981. The scented garden; choosing, growing and using the plants that brings fragrance to your life, home and table. *Michael Joseph Limited*, London

VERZERA A, TROZZI A, GAZEA F, CICCIARELLO G, COTRONEO A., 2003. Effects of rootstock on the composition of bergamot (*Citrus bergamia* risso et poiteau) essential oil. *Journal of Agricultural and Food Chemistry,* 51, 206–210

VUFFRAY A, DURUSSEL C, BOERLIN P, BOERLIN-PETZOLD F, BILLE J, GLAUSER MP, CHAVE JP., 1994. Oropharyngeal candidiasis resistant to single-dose therapy with fluconazole in HIV-infected patients [letter]. *AIDS*, 8, 708–709

WAGENER FADTG, CARELS CE, LUNDVIG DMS., 2013. Targeting the redox balance in inflammatory skin conditions: Review. *International Journal of Molecular Sciences*, 14, 9126–9167

WAGH V, SHAIKH S, MAYNALE SST, MHASKE N., 2015. Preparation and evaluation of marigold, liquorice and corange peel extract containing herbal face wash. *World Journal of Pharmaceutical Research*, 4, 12, 1808–1812

WALSH TJ, VIVIANI MA, ARATHOON E, CHIOU C, GHANNOUM M, GROLL AH., 2000. New targets and delivery systems for antifungal therapy. *Medical Mycology*, 38, 1, 335–47

WALTERS KA, ROBERTS SM., 2008. Dermatologic, cosmetic, and cosmetic development. Therapeutic and novel approaches. *Edited Informa Healthcare,* NewYork

WANNISSORN B, JARIKASEM S, SIRIWANGCHAI T, THUBTHIMTHED S., 2005. Antibacterial properties of essential oils from Thai medicinal plants. *Fitoterapia*, 76, 2, 233–236

WARNOCK DW, JOHNSON EM, RICHARDSON MD, VICKERS CF., 1983. Modified response to ketoconazole of *Candida albicans* from a treatment failure [letter]. *Lancet*, 1, 8325, 642–643

WARREN CP., 1999. Antioxidant effects of herbs. *Lancet,* 353**,** 9153, 676

WEBER U., 1876. Ueber die Wikung des *Pilocarpium muriaticum*.*Central Med Wiss*, 44, 409–422

WEIDINGER S, ILLIG T, BAURECHT H, IRVINE AD, RODRIGUEZ E, DIAZ-LACAVA A, KLOPP N, WAGENPFEIL S, ZHAO Y, LIAO H, LEE SP, PALMER CN, JENNECK C, MAINTZ L, HAGEMANN T, BEHRENDT H, RING J, NOTHEN MM, MCLEAN WH, NOVAK N., 2006. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. *Journal of Allergy and Clinical Immunology*, 118, 1, 214–219

WEITZMAN I, SUMMERBELL RC., 1995. The dermatophytes. *Clinical Microbiology Reviews*, 8, 240–259

WELLER R, HUNTERJ, SAVIN J, DAHL M., 2008. The skin and the psche. In: Clinical Dermatology. 4th Edition, *Blackwell Publishing*. USA

WERTZ PW, VAN DEN BERGH B., 1988. The physical, chemical and functional properties of lipids in the skin and other biological barriers. *Chemistry and Physics of Lipids*, 91, 85–96

WERTZ PW., 2000. Lipids and barrier function of the skin. *Acta Dermato-Venereologica*, 208, 7–11

WESELER A, GEISS HK, SALLER R, REICHLING J., 2002. Antifungal effect of Australian tea tree oil on *Malassezia pachydermatis* isolated from canines suffering from cutaneous skin disease. *Schweizer Archiv für Tierheilkunde*, 144, 215–221

WHITE TC, OLIVER BG, GRÄSER Y, HENN MR., 2008. Generating and testing molecular hypotheses in the dermatophytes. *Eukaryotic Cell,* 7, 1238–1245

WIART C., 2006. Medicinal plants of Asia and the Pacific. *Taylor and Francis*, London, 211

WICKRAMARATNE DBM, KUMAR V, BALASUBRAMANIAM S., 1984. Murragleinin, a coumarin from *Murraya gleinei* leaves. *Phytochemistry*, 23, 12, 2964–2966

WILKES GL, BROWN IA, WILDNAUER RH., 1973. The biomechanical properties of skin. *CRC Critical Reviews in Bioengineering*, 1, 453–495

WILLIAMS A., 2003. Transdermal and tropical drug delivery. *Pharmaceutical Press*, London, UK

WILSON RM, DANISHEFSKY SJ., 2006. Small molecule natural products in the discovery of therapeutic agents: the synthesis connection. *Journal of Organic Chemistry,* 71, 8329–8351

WINK DA, KASPRZAK KS, MARAGOS CM, ELESPURU RK, MISRA M, DUNAMS TM, CEBULA TA, KOCH WH, ANDREW AW, ALLEN JS, KEEFER LK., 1991. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science*, 254, 1001–1003

WISEMAN H, HALLIWELL B., 1996. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochemistry*, 313, 17–29

WOJTASZEK P., 1997. Oxidative burst: an early plant response to pathogen infection. *Biochemical Journal*, 322, 681–692

WOLFORD RW, KESTERSON JW, ATTAWAY JA., 1971. Physicochemical properties of citrus essential oils from Florida. *Journal of Agricultural and Food Chemistry*, 19, 6, 1097–1102

WOOD WF, WELDON PJ., 2002. The scent of the reticulate giraffe (*Giraffa camelopardalis reticulata*). *Biochemical Systematics and Ecology*, 30, 913- 917

WOOLLEY DW., 1944. Some new aspects of the relationship of chemical structure to biological activity. *Science,* 100, 2609, 579–583

WORLD HEALTH ORGANIZATION, 2005. Global burden of disease for the Year 2001 by World Bank Region. Disease control priorities project. http://www.fic.nih.gov/dcpp

WU L, WANG X, XU W, FARZANEH F, XU R., 2009. The structure and pharmacological functions of coumarins and their derivatives. *Current Medicinal Chemistry,* 16, 4236–4260

WU T, TIEN H, ARISAWA M, SHIMIZU M, NAOKATA M., 1980. Flavonols and coumarins from the fruit of *Murraya omphalocarpa*. *Phytochemistry*, 19, 10, 2227–2228

WU TS, FURUKAWA H., 1982. Biological and phytochemical investigation of *Clausena excavata. Journal of Natural Products*, 45, 718–720

XIN ZQ, LU JJ, KE CQ, HU CX, LIN LP, YE Y., 2008. Constituents from *Clausena excavata*. *Chemical and Pharmaceutical Bulletin*, 56, 827–830

YANG D, MICHEL L, CHAUMONT JP, CLERC MJ., 1999. Use of caryophyllene oxide as an antifungal agent in an in vitro experimental model of Onychomycosis. *Mycopathologia*, 148, 79–82

YANG EJ, KIM SS, OH TH, BAIK JS, LEE NH, HYUN CG., 2009. Essential oil of citrus fruit waste attenuates LPS-induced nitric oxide production and inhibits the growth of skin pathogens. *International Journal of Agriculture and Biology*, 11, 791–794

YAO LH, JIANG YM, TOMAS-BARBERAN FA, DATTA N, SINGANUSONG R, CHEN SS., 2004. Flavonoids in food and their health benefits. *Plant Foods for Human Nutrition*, 59, 113–122

YASSER AS, NABIL, HO., 2012. Anti-inflammatory new coumarin from the *Ammi majus* L. *Organic and Medicinal Chemistry Letters*, 2, 1–4

YAZDANPARAST SA, BARTON RC., 2006. Arthroconidia production in *Trichophyton rubrum* and a new ex vivo model of onychomycosis. *Journal of Medical Microbiology*, 55, 1577–81

YUNES RA, CALIXTO JB., 2001. Plantas medicinais sob óptica da química medicinal moderna*. Moderna – Chapecó*, Argos

ZIMMERMAM-FRANCO DC, BOLUTARI EB, POLONINI HC, DO CARMO AMR, DAS GRAÇAS M, CHAVES AM, RAPOSO NRB., 2013. Antifungal activity of *Copaifera langsdorffii* Desf Oleoresin against dermatophytes. *Molecules*, 18, 12561–12570

ZURITA J, HAY RJ., 1987. Adherence of dermatophyte microconidia and arthroconidia to human keratinocytes *in vitro*. *Journal of Investigative Dermatology,* 89, 5, 529–534