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

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

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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.

________________________________________
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________________________________________
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.
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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 _____________________
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COLLEGE OF AGRICULTURE ENGINEERING & SCIENCE
DECLARATION 2- PUBLICATIONS

1) Growth inhibition and morphological alteration of \textit{Trichophyton rubrum} and \textit{T. mentagrophytes} by \textit{Agathosma betulina} and \textit{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


CONFERENCE CONTRIBUTIONS FROM THIS THESIS

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

2) Skin healing properties of Rutaceae plants essential oil volatiles.
\textit{O.O. Fajinmi, M.G. Kulkarni, J.F. Finnie and J. Van Staden. RCPGD 15\textsuperscript{th} Annual conference (2015), UKZN, PMB}
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<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Atopic Dermatitis</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>B-PE</td>
<td>B-phycoerythrin</td>
</tr>
<tr>
<td>FGI</td>
<td>Fungal Growth Index</td>
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<tr>
<td>FLG</td>
<td>Filaggrin</td>
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<tr>
<td>GCMS</td>
<td>Gas Chromatography Mass Spectrometry</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid Peroxidation</td>
</tr>
<tr>
<td>MFC</td>
<td>Minimum Fungicidal Concentration</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MID</td>
<td>Minimum Inhibitory Doses</td>
</tr>
<tr>
<td>ORAC</td>
<td>Oxygen Radical Absorbance Capacity</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxidative Species</td>
</tr>
<tr>
<td>SC</td>
<td>Stratum Corneum</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<tr>
<td>TEWL</td>
<td>Trans Epidermal Water Loss</td>
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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%), bicyclic 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%), bicyclic 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 thirly-three components. Compounds found in essential oil of both *A. ovata* and *A. mucronulata* are trans-β-ocimene, β-ocimene, caryophyllene, β-myrcene and bicyclicgermacrene.

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%), gammainmurolene (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-methylethyl)-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-methylethyl)-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 km², 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 eighteenth 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 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, anti-diabetic, immunomodulatory and antiplasmodial activities (ARBAB et al., 2012).

Since *Pilocarpus* species have been confirmed to be a potent stimulant of the secretory system, showing sialagogue, 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 \( \alpha \)- and \( \beta \)-phellandrene, limonene, \( \beta \)-ocimene, \( \beta \)-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 \( \beta \)-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\%), \( \mu \)-cymene (12.1\%), linalool (11.3\%) and \( \alpha \)-pinene (9.1\%) as main constituents. In the oil of *C. calycinum*, 35 compounds were characterized representing 99.4\% of the total volume. \( \beta \)-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 antidermatophytic 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 YOSHIKE, 1992; AALTO-KORTE and TURPEINEN, 1993).
An intact, healthy skin has a highly effective barrier against the ingress of microorganisms 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 28°C 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 CD4 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*, *Coccidioides immitis*, *C. posadasii*, *Paracoccidioides brasiliensis*, and *Penicillium marneffei* (MURRAY et al., 2013). The fungi exist in two morphological forms; yeast or spherules at 37°C and molds at 25°C (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, candidiasis 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,
Household dust may serve as a reservoir of anthropophilic 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
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
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 paronychia, 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 theazole 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).
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 (Fromling, 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 effectiveazole 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; Fromling, 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.
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).
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 (CD4) 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
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 (ZIMMERMAN-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 furanonecoumarins, 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). Trioxigenated 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), girinimbol, girinimbine (ADEBAJO et al., 2006), koinimbine, 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%), beta-caryophyllene (11.81%), spathulenol (10.21%), beta-caryophyllene (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%), delta-elemene (3.57%), alpha-cubebene (2.96%), retinal (1%), alpha-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 alpha- and beta-phellandrene, limonene, beta-ocimene, beta-caryophyllene and myrcene (AL BURTAMANI et al., 2005). Five simple coumarins namely; umbelliferone, scopoletin, 7-isoprenyloxycoumarin, umbelliprenin, and osthelenol, 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-beta-D-glucopyranosyl-1-methylpent-2-enyl)-8,9-dihydro-2H-furo-[2,3-h]-1-benzopyran-2-one and 8-(3-beta-D-glucopyranosyl-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 (AUSTR 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 paradisii 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 monoterpane (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 25°C (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
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 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 microorganismal 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 (SCHAFTER 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<sup>PE</sup> 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<sup>PE</sup> 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][3H,9'][9H]-xanthen]-3-one) as a fluorescent probe and this improved ORAC<sup>FL</sup> 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

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>µmol Trolox Equivalents/g</th>
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<tbody>
<tr>
<td><em>Agathosma mucronulata</em></td>
<td>1045.5±48.8</td>
</tr>
<tr>
<td><em>Agathosma ovata</em></td>
<td>1202.5±92.0</td>
</tr>
<tr>
<td><em>Calodendrum capense</em></td>
<td>301.7±19.1</td>
</tr>
<tr>
<td><em>Coleonema album</em></td>
<td>942.2 ± 34.9</td>
</tr>
<tr>
<td><em>Coleonema pulchellum</em></td>
<td>1126.7 ± 68.1</td>
</tr>
<tr>
<td><em>Murraya koenigii</em></td>
<td>339.8±27.7</td>
</tr>
<tr>
<td><em>Murraya paniculata</em></td>
<td>930.7±67.7</td>
</tr>
</tbody>
</table>
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-

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 PATTERNSON, 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/fruit 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 40°C for four days. Dried leaves were ground into powders and stored in air-tight containers at 23±1°C 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 30°C. 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 scalpel into McCartney bottles containing yeast malt broth (Sigma Y3752) and incubated at 37°C 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 1x10⁶ 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 1st 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 37°C 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

<table>
<thead>
<tr>
<th>Plants</th>
<th>Trichophyton rubrum</th>
<th>Trichophyton mentagrophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. E MIC mg/ml</td>
<td>Acetone MFC mg/ml</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Greater than 1.0</td>
<td></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>Greater than 1.0</td>
<td></td>
</tr>
</tbody>
</table>
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*

<table>
<thead>
<tr>
<th>Plants</th>
<th>Microsporum gypseum</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. E</td>
<td>Acetone</td>
<td>Methanol</td>
<td>Ethanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MIC mg/ml</td>
<td>MIC mg/ml</td>
<td>MIC mg/ml</td>
<td>MIC mg/ml</td>
<td></td>
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<tr>
<td></td>
<td>MFC mg/ml</td>
<td>MFC mg/ml</td>
<td>MFC mg/ml</td>
<td>MFC mg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. album</em></td>
<td>6.250</td>
<td>6.250</td>
<td><strong>0.391</strong></td>
<td>3.125</td>
<td><strong>0.195</strong></td>
<td>3.125</td>
<td><strong>0.391</strong></td>
<td>3.125</td>
</tr>
<tr>
<td><em>C. pulchellum</em></td>
<td>6.250</td>
<td>6.250</td>
<td><strong>0.781</strong></td>
<td>3.125</td>
<td><strong>0.049</strong></td>
<td>1.563</td>
<td><strong>0.781</strong></td>
<td>3.125</td>
</tr>
<tr>
<td><em>Murraya koenigii</em></td>
<td><strong>0.781</strong></td>
<td>6.250</td>
<td><strong>0.586</strong></td>
<td>6.250</td>
<td><strong>0.391</strong></td>
<td>6.250</td>
<td><strong>0.391</strong></td>
<td>6.250</td>
</tr>
<tr>
<td><em>C. capense</em></td>
<td>6.250</td>
<td>6.250</td>
<td><strong>0.781</strong></td>
<td>6.250</td>
<td><strong>0.781</strong></td>
<td>3.125</td>
<td><strong>0.781</strong></td>
<td>3.125</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Less than 1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td>Greater than 1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
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, spiroquinoxaline alkaloids, coumarins and isoflavonoids 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 6th 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.

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

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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>A. mucronulata oil</th>
<th>A. ovata oil</th>
<th>C. capense oil</th>
<th>Control</th>
<th>Starting material</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichophyton rubrum</em> diameter (cm)</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em> diameter (cm)</td>
<td>0.6</td>
<td>0.7</td>
<td>-</td>
<td>1.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>A. mucronulata oil FGI (%)</th>
<th>A. ovata oil FGI (%)</th>
<th>C. capense oil FGI (%)</th>
<th>Control FGI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichophyton rubrum</em> diameter (cm)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>566.67</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em> diameter (cm)</td>
<td>100</td>
<td>133.3</td>
<td>-</td>
<td>233.33</td>
</tr>
</tbody>
</table>
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 37°C

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>C. album oil</th>
<th>C. pulchellum oil</th>
<th>Control</th>
<th>Starting material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichophyton rubrum diameter (cm)</td>
<td>0.3</td>
<td>0.3</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes diameter (cm)</td>
<td>0.3</td>
<td>0.5</td>
<td>1.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>C. album oil FGI (%)</th>
<th>C. pulchellum oil FGI (%)</th>
<th>Control FGI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichophyton rubrum diameter (cm)</td>
<td>0</td>
<td>0</td>
<td>566.67</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes diameter (cm)</td>
<td>0</td>
<td>66.67</td>
<td>233.33</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>Murraya koenigii oil</th>
<th>M. paniculata oil</th>
<th>Control</th>
<th>Starting material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichophyton rubrum diameter (cm)</td>
<td>1.4</td>
<td>0.3</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes diameter (cm)</td>
<td>0.9</td>
<td>0.3</td>
<td>1.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>M. koenigii essential oil FGI (%)</th>
<th>M. paniculata essential oil FGI (%)</th>
<th>Control FGI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichophyton rubrum diameter (cm)</td>
<td>366</td>
<td>0</td>
<td>566.67</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes diameter (cm)</td>
<td>200</td>
<td>0</td>
<td>233.33</td>
</tr>
</tbody>
</table>
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* (SAJNI 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 thirty-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%), gammamyrurolene (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-methylethyl)-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-methylethyl)-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>
<thead>
<tr>
<th>Agathosma ovata</th>
<th>Agathosma mucronulata</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>β-Myrcene</td>
</tr>
<tr>
<td>22.03</td>
<td>34.05</td>
</tr>
<tr>
<td>1,6-Octadiene-3-ol, 3, 7-dimethyl-2-aminobenzoate</td>
<td>18.75</td>
</tr>
<tr>
<td></td>
<td>18.24</td>
</tr>
<tr>
<td>Trifluoroacetyl-lavandulol</td>
<td>Caryophyllene</td>
</tr>
<tr>
<td>12.97</td>
<td>7.4</td>
</tr>
<tr>
<td>Trans-β-Ocimene</td>
<td>(1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene</td>
</tr>
<tr>
<td>11.81</td>
<td>6.09</td>
</tr>
<tr>
<td>β- Ocimene</td>
<td>D-Limonene</td>
</tr>
<tr>
<td>5.65</td>
<td>6.06</td>
</tr>
<tr>
<td>Bicyclo germacrene</td>
<td>Eucalyptol</td>
</tr>
<tr>
<td>5.10</td>
<td>5.46</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>Bicyclo germacrene</td>
</tr>
<tr>
<td>3.47</td>
<td>5.38</td>
</tr>
<tr>
<td>D-Limonene</td>
<td>Trans-β-Ocimene</td>
</tr>
<tr>
<td>2.79</td>
<td>4.21</td>
</tr>
<tr>
<td>Thujone</td>
<td>2,4,4-trimethyl-2-vinyl-Guaia-1(10),11-diene</td>
</tr>
<tr>
<td>2.07</td>
<td>3.00</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>β- Ocimene</td>
</tr>
<tr>
<td>1.88</td>
<td>2.36</td>
</tr>
<tr>
<td>Caryophyllene</td>
<td>Others</td>
</tr>
<tr>
<td>1.74</td>
<td>7.75</td>
</tr>
<tr>
<td>Others</td>
<td>11.74</td>
</tr>
</tbody>
</table>
Table 6.2: List of major compounds present in the essential oils of *C. album* and *C. pulchellum*

<table>
<thead>
<tr>
<th>Coleonema album</th>
<th>%</th>
<th>Coleonema pulchellum</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td></td>
<td>Compound</td>
<td></td>
</tr>
<tr>
<td>Caryophyllene</td>
<td>24.91</td>
<td>Bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene</td>
<td>32.04</td>
</tr>
<tr>
<td>Trans-β-Ocimene</td>
<td>9.49</td>
<td>(+)-3-Carene</td>
<td>10.88</td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>7.96</td>
<td>β-Ocimene</td>
<td>9.46</td>
</tr>
<tr>
<td>Octahydro-7-methyl-3-methylene-4-(1-methylethyl)-β-copaene</td>
<td>7.53</td>
<td>Gamma-Elemene</td>
<td>8.95</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>7.00</td>
<td>α-Pinene</td>
<td>7.43</td>
</tr>
<tr>
<td>(+)-3-Carene</td>
<td>5.98</td>
<td>Caryophyllene</td>
<td>6.98</td>
</tr>
<tr>
<td>Bicyclo[3.1.0]hex-2-ene,4-methyl-1-(1-methyethyl)-β-phellandrene</td>
<td>5.97</td>
<td>β-Pinene</td>
<td>5.36</td>
</tr>
<tr>
<td>Bicyclogermacrene</td>
<td>5.11</td>
<td>β-Myrcene</td>
<td>4.47</td>
</tr>
<tr>
<td>A-Terpineol</td>
<td>3.69</td>
<td>(+)-4-Carene</td>
<td>3.76</td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>2.58</td>
<td>β-Phellandrene</td>
<td>3.73</td>
</tr>
<tr>
<td>1,3,6-Octatriene,3,7-dimethyl-, (Z) -β-Ocimene</td>
<td>2.25</td>
<td>α-Phellandrene</td>
<td>1.60</td>
</tr>
<tr>
<td>Gamma-Elemene</td>
<td>1.84</td>
<td>Others</td>
<td>5.34</td>
</tr>
<tr>
<td>Others</td>
<td>15.69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6.3: List of major compounds present in the essential oils of *M. koenigii* and *M. paniculata*

<table>
<thead>
<tr>
<th></th>
<th><em>M. koenigii</em></th>
<th></th>
<th><em>M. paniculata</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>%</td>
<td>Compound</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Caryophyllene</td>
<td>42.54</td>
<td>1, 6, 10-Dodecatriene, 7,11-dimethyl-3-methylene</td>
<td>38.62</td>
<td></td>
</tr>
<tr>
<td>(4aR-trans)-Guaia-9,11-diene</td>
<td>23.35</td>
<td>1,6-cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>Alpha-Guaiene</td>
<td>10.71</td>
<td>Caryophyllene</td>
<td>10.63</td>
<td></td>
</tr>
<tr>
<td>Humulene</td>
<td>6.93</td>
<td>Gamma-Elemene</td>
<td>8.55</td>
<td></td>
</tr>
<tr>
<td>Beta-phellandrene</td>
<td>2.66</td>
<td>Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)</td>
<td>5.42</td>
<td></td>
</tr>
<tr>
<td>Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)</td>
<td>2.22</td>
<td>Cis-beta-Farnesene</td>
<td>3.12</td>
<td></td>
</tr>
<tr>
<td>Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)</td>
<td>2.10</td>
<td>Beta-Bisabolene</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>Beta-Pinene</td>
<td>1.62</td>
<td>Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S-Cis)</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>7.87</td>
<td>Others</td>
<td>11.24</td>
<td></td>
</tr>
<tr>
<td>Compound</td>
<td>%</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cis-β-Farnesene</td>
<td>44.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphtalene,1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,(1S-cis)</td>
<td>4.88</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,6-Cyclodecadiene,1-methyl-5-methylene-8-(1-methylethyl)-,[S-(E,E)]</td>
<td>3.68</td>
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<td></td>
<td></td>
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<tr>
<td>Cis-β-Farnesene</td>
<td>3.29</td>
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<tr>
<td>β-Bourbonene</td>
<td>2.87</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Caryophyllene</td>
<td>2.86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>4.07</td>
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<td></td>
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<tr>
<td>Gamma-Muurolene</td>
<td>4.01</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bicyclo[5.2.0]nonane,4-methylene</td>
<td>2.03</td>
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<tr>
<td>Isolongifolene,9,10-dehydro</td>
<td>1.64</td>
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</tr>
<tr>
<td>Trans-α-Bergamotene</td>
<td>1.59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans-β-Ocimene</td>
<td>1.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>23.25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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

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% (*Table 7.2*).

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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>10 µl</th>
<th>20 µl</th>
<th>40 µl</th>
<th>Starting material</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col oil</td>
<td>Aga oil</td>
<td>Col oil</td>
<td>Aga oil</td>
<td>Col oil</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diameter (cm)</td>
<td>3.75</td>
<td>2.75</td>
<td>3.55</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td>4.05</td>
<td>2.9</td>
<td>3.68</td>
<td>2.55</td>
<td>3.28</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Fungus tested</th>
<th>10 µl FGI (%)</th>
<th>20 µl FGI (%)</th>
<th>40 µl FGI (%)</th>
<th>Control FGI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col oil</td>
<td>Aga oil</td>
<td>Col oil</td>
<td>Aga oil</td>
</tr>
<tr>
<td><em>Trichophyton rubrum</em></td>
<td>167</td>
<td>96</td>
<td>153</td>
<td>36</td>
</tr>
<tr>
<td><em>Trichophyton mentagrophytes</em></td>
<td>189</td>
<td>107</td>
<td>163</td>
<td>82</td>
</tr>
</tbody>
</table>
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

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

<table>
<thead>
<tr>
<th></th>
<th>Agathosma betulina</th>
<th>(%)</th>
<th>Coleonema album</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td></td>
<td></td>
<td>Compound</td>
<td></td>
</tr>
<tr>
<td>1-Mentone</td>
<td>36.35</td>
<td>β-Pinene</td>
<td>28.92</td>
<td></td>
</tr>
<tr>
<td>D-Limonene</td>
<td>29.84</td>
<td>(1R)-2,6,6-Trimethyl bicyclo[3.1.1]hept-2-ene</td>
<td>27.37</td>
<td></td>
</tr>
<tr>
<td>6-Methoxy-3(2H)-pyridazinone</td>
<td>8.35</td>
<td>β-Phellandrene</td>
<td>22.70</td>
<td></td>
</tr>
<tr>
<td>1,4,4-trimethyl-3-Ethoxy-4-methoxyphenol</td>
<td>7.25</td>
<td>Trans-β-Ocimene</td>
<td>7.14</td>
<td></td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>4.43</td>
<td>β-Ocimene</td>
<td>4.27</td>
<td></td>
</tr>
<tr>
<td>Cyclohexanone,2-(2-methylpropylidene</td>
<td>3.05</td>
<td>Gamma-Terpinene</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td>Pulegone</td>
<td>2.97</td>
<td>(+)-4-Carene</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>2.25</td>
<td>Eucalyptol</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>(1R)-2,6,6-Trimethyl bicyclo[3.1.1]hept-2-ene</td>
<td>1.33</td>
<td>p-Cymene</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>4.18</td>
<td>Others</td>
<td>4.35</td>
<td></td>
</tr>
</tbody>
</table>
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 bed sheets, 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.
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 alkoxy, 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 alkoxy 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 normalcy 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 (CD4) 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 inazole 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* peel 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.
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