An Investigation of Alternative Antifungals against *Phyllosticta citricarpa* Kiely and *Guignardia mangiferae*

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MASTER OF HEALTH SCIENCE: ENVIRONMENTAL HEALTH

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Bloemfontein, South Africa, 2016

DECLARATION OF INDEPENDENT WORK

I, Bheki Thapelo Magunga, do hereby declare that this research project submitted to the Central University of Technology, Free State for the degree MASTER OF HEALTH SCIENCE: ENVIRONMENTAL HEALTH is my own work and has not been submitted before to any institution by myself or any other person in fulfilment of the requirements for the attainment of any qualification.

Signature of student   Date

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SUMMARY

Citrus fruits have been cultivated for many years and consumed mostly as fresh produce or juice and considered as one of the most important crops in the agriculture sector (Abirami *et al.,* 2014: Paul, 2006). In South Africa, the citrus industry significantly contributes to the economy as the second largest earner of foreign exchange in terms of agricultural exports (Mabiletsa, 2003). However citrus production can be influenced negatively by pathogenic diseases resulting in significant economic decline such as Citrus Black Spot (CBS) caused by fungal pathogen *Phyllosticta citricarpa* (Timmer and Duncan, 1999; Davies and Albrigo, 1994). Recently infections due to *P. citricarpa* have resulted in lower citrus yield and huge economic loss impacting on the labour market due to job losses (Truter, 2010; Paul, 2006; Timmer and Duncan, 1999). (CBS) is a fungal disease of citrus leaves and fruit that causes superficial lesions on the rind of the fruit. However there are other strains associated with citrus, such as *Guignradia magniferae* (Meyer *et al.,* 2001). This strain is non-pathogenic and does not cause citrus black spot symptoms. This endophytic fungus is able to live within a plant without causing apparent disease unlike *P.citricarpa* known to result in CBS infection. In South Africa including other countries such as China and Australia *P. citricarpa* is considered to be phytosanitory important because of its role in international trade (Truter, 2010; Baayen *et al*., 2002). If the fruits contain CBS lesions, they can result in the rejection of the whole imported batch. When lesions are spotted on the fruits, repackaging is recommended and fruits could be sold at a lower market value, resulting in huge economic losses. Synthetic fungicides with antimicrobial activity are used to extend the storage-life of citrus fruits (Halueendo, 2008). However, over the past two decades, there has been great public concern about safety and side effects of synthetic agents in food preservation besides health implications (Ayoola *et al*., 2008). These agents are known to remain on the plant or within its tissues following treatment resulting in potentially toxic and carcinogenic effects on human and food systems (Sellamuthu, 2013). This indicates that growers and suppliers of citrus fruits are faced with the challenge of providing consumers with products that are attractive, free from disease, defects, toxic residues, and with longer shelf or storage-life (Sellamuthu, 2013). Synthetic fungicides often used to control

fungi are pesticides that inhibit fungal growth by targeting reproductive structures (ascospores and conidia) known to be important in the fungal life cycle. These structures play an important role in the growth and dispersal of fungi that habitually cause huge damages in agriculture resulting in critical losses of yield, quality and profit. Over the years farmers have used synthetic fungicides to control fungal growth; however synthetic fungicides use has increased concern due to their toxicity, polluting the environment and antimicrobial resistance. Moreover regular use of fungicides results in risk particularly if residues are retained in soil or transferred into water. This has a negative effect on soil organisms and carries a potential risk to long-term fertility of the soil. Furthermore, fungal resistance to currently available synthetic fungicides related to the overuse and abuse is more prevalent. In most cases resistance occurs due to modification of the target site (Halueendo, 2008). This is a concern because the products may become less effective or even inadequate for controlling fungal pathogens. This has influenced the search for rational approach to limit repeated use of synthetic fungicides. Kock *et al.,* 2007 showed that anti-inflammatory compounds such as acetylsalicylic acid (ASA), benzoic acid (BA) and salicylic acid (SA) possess antifungal properties. Although alternative methods are being researched to control postharvest decay during storage, natural plant products such as essential oils (EO's) and their hydrosol are gaining fame and the attention of researchers globally due to their biodegradable, eco-friendly, economical and safety properties. The EO's reported in various studies have shown to exhibit antifungal properties by targeting structures responsible for the life cycle of fungal organism as well such as ascospores and conidia for both in vitro and in vivo in different fresh produce (Prakash *et al.,* 2015). However there is limited information regarding the effect of essential oils against *P. citricarpa*. Therefore EO's can be ideal candidates for use as alternative fungicides as well against citrus black spot. In this current study, EO's and antifungal anti-mitochondrial compounds were tested for antifungal anti-mitochondrial properties. This is shown by a unique bioassay, with the mitochondrion-dependent sexual structure from *P. citricarpa* and *G. mangiferae* serving as indicators. It was revealed that the pathogenic fungi, *P. citricarpa* and *G. mangiferae* are also dependent on increased mitochondrion activity to effect spore-release and structure development. This was observed from the XTT assay (an indicator of metabolic activity) results where the activity of mitochondrion dehydrogenases were affected for both organism after the treatment with EO's. The study also showed that some cheaper alternative

methods such as using hydrosol can be used as antifungals against CBS, with the advantage over essential oils of being water soluble and consisting of EO's traces (Nazzaro *et al.,* 2013).

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$\Delta\psi$ m is signified by a yellow- green fluorescence (collected at 450 nm), while a low $\Delta\psi$ m is signified by a red fluorescence collected at 625 nm.

CHAPTER ONE

Background

An Investigation of Alternative Antifungals against *Phyllosticta citricarpa* Kiely and *Guignardia mangiferae*

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1.1 Citrus fruits

Citrus fruits has been cultivated for many years (Paul, 2006) and consumed mostly as fresh produce or juice and as one of the most important crops in the agriculture sector (Paul, 2006; Abirami *et al.,* 2014). It is a good source of numerous nutrients and natural occurring chemical compounds (Abirami *et al.,* 2014). They are an excellent source of Vitamin C (ascorbic acid) and folic acid, as well as a good source of fibre, thereby contributing to a healthy diet (Ihueze and Mgbemena, 2015; Abirami *et al.,* 2014). Additionally, they contain potassium, calcium, foliate, thiamin, niacin, vitamin B6, phosphorus, magnesium and copper. These may help to reduce the risk of heart diseases, some types of cancer and reduce the risk of pregnant women having children with birth defects (Ihueze and Mgbemena, 2015). Processed citrus products include citric acid, essential and distilled oils, jams, jellies, gel products and citrus alcohol, wines and brandies (Paul, 2006; Spiegel-Roy and Goldschmidt, 1996). By-products from juice extraction are important in soft drink, pectin and cattle feed production. Citrus fruits are also popular as ingredients in confectionary (Paul, 2006; Ray and Walheim, 1980) and several flavonoid compounds that are used by pharmaceutical and food industries (Paul, 2006; Spiegel-Roy and Goldschmidt, 1996). In South Africa, the citrus industry significantly contributes to the economy and it is the second largest earner of foreign exchange in terms of agricultural exports; however specific climate conditions are required to grow and yield fruits (Mabiletsa, 2003).

1.2 Climatic and geographic factors that influence cultivation

The growth of citrus requires a low frost incidence, enough moisture to sustain the trees, and suitable soils. These factors have a marked influence on the growth, development and productivity of trees (Davies and Albrigo, 1994; Timmer and Duncan, 1999). However, citrus fruits can be grown in a wide range of conditions. Temperature is the main factor that influences the global range of citrus production (Zekri, 2011; Paul, 2006; Spiegel-Roy and Goldschmidt, 1996; Davies and Albrigo, 1994). At a temperature of 13°C limited growth occurs in all citrus tree organs (Zekri, 2011). Extremely high temperatures above 50°C also influence growth and development of citrus trees (Zekri,

2011; Paul, 2006). Significant induction of flowering requires a period of drought of longer than 30 days and that temperature stay below 25°C for several weeks (Zekri, 2011; Paul, 2006). The degree of induction is proportional to the severity of and duration of stress. Flowering is not induced below 9.4°C (Zekri, 2011). Good quality irrigation water is a basic requirement for the successful cultivation of citrus (Paul, 2006; Srivastava and Singh, 2002). A lack of quality irrigation water limits citrus production in various regions of the world, including South Africa (Davies and Albrigo, 1994).

Citrus is grown in, and can adapt to, a wide range of soil conditions, but it grows best in sandy or clay loam soils. Soil properties may influence the growth habit of trees, especially root distribution. Adequate soil drainage is vital for growth as tree growth is reduced in poorly drained soils (Davies and Albrigo, 1994). Accumulation of free water in the root zone may also result in poor aeration and eventually lead to root injury, resulting in damage of the tree and/ or lower yield production, furthermore pathogenic microorganism also results in lower production costing the economy billions of Rands and job losses annually (Truter, 2010; Paul, 2006; Timmer and Duncan, 1999). Comparable to any other crop, citrus production can be inhibited by pathogenic diseases resulting in significant economic decline (Zekri, 2011; Davies and Albrigo, 1994). A disease of citrus may be defined as differences from the normal appearance, form or functioning of a citrus tree or its fruit. Diseases are classified into infectious (biotic) or non-infectious (abiotic) diseases (Paul, 2006; Timmer and Duncan, 1999). Abiotic diseases are caused by nutritional and genetic defects and incorrect cultural practices, such as the inappropriate application of chemicals (Timmer and Duncan, 1999). Biotic diseases are caused by bacteria, fungi and viruses. They may result in the death of citrus trees or seriously lower the production yield (Timmer and Duncan, 1999; Davies and Albrigo, 1994). Recently citrus biotic diseases have resulted in lower yield, huge economic loss and job losses. These biotic disease related to citrus fruits include fungal pathogen *Phyllosticta citricarpa* as a causal agent of citrus black spot.

1.3 Citrus black spot

Citrus Black Spot (CBS) is a fungal disease of citrus leaves and fruit that causes superficial lesions on the rind of the fruit. It is caused by the fungal pathogen *Phyllosticta citricarpa* Kiely (Hu *et al.,* 2014; Kotzé, 1981; Brodrick, 1969). However there are other strains associated with citrus, such as *Guirnradia magniferai*. This strain is non-pathogenic and does not cause citrus black spot symptoms (Meyer *et al.,* 2001). This endophytic fungus is able to live within a plant without causing apparent disease. It caused confusion in the past, since all isolates of *Guignardia* obtained from *citrus* were considered to be the citrus pathogen, *P. citricarpa.* The pathogenic strain is known to cause disease and symptoms associated with citrus black spot (Truter, 2010).The disease was initially described more than 100 years ago in New South Wales, Australia, and has subsequently been found in Africa, Asia, South America, and New Zealand (Hendricks *et al.*, 2013). Symptomatic fruits are unacceptable to the fresh and export markets due to the potential phytosanitary risk associated with the export of fruit from CBS affected production countries to CBS-free countries (Truter, 2010; Baayen *et al.*, 2002).

Phytosanitary risk is a measure of a plant health, with respect to the requirements of international trade with the aim of preventing the introduction of quarantine pests into other countries. This has resulted in other countries regulating the entry of plants and plant products and other materials capable of harbouring plant pests. Restrictive trade barriers due to CBS have been introduced particularly by the European Union (EU) and United State of America (USA) (Truter, 2010; Baayen *et al.*, 2002; European Union, 1998). Although CBS has been recorded in Florida, USA, trade restrictions regarding imports to the USA still apply (Lemon and McNally, 2010). In addition to the phytosanitary trade barriers, other economic losses attributed to CBS include premature fruit drop in heavy infected orchards, lower market value of symptomatic fruit and higher production costs due to extensive control programs.

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1.4 Phytosanitary barriers to trade and Pest Risk Assessments

As a result of international travel and trade, plant pathogens can be dispersed throughout the world. When these pathogens are introduced into a new area, and find a susceptible crop, they can have devastating consequences. Agricultural systems with an impoverished diversity are particularly vulnerable to new pathogens and such introductions have had significant economic impacts (Pimentel *et al.,* 2001; Baker *et al.,* 2000). Phytosanitary barriers to trade play a vital role in protecting a country from introduction of alien species by restricting the movement of plant material world-wide (Baayen *et al.,* 2002; European Union, 1998). However, countries may not impose unnecessary restrictions and these barriers are required to be based on scientifically justifiable principles (WTO, 1993). Ideally, the risk of pathogen introduction should be determined through a Pest Risk Assessment (PRA) that is supported by scientific research (International Plant Protection Convention, 1996).

Pest risk assessments evaluate the potential risks of introduction and establishment of a plant pest or pathogen into a new geographical location and assess the management options to reduce those potential risks (Rafoss, 2003). Pest Risk Assessments considers, amongst other things, the life-cycle, host specificity, including current and potential geographical distribution of the organism (McKenney *et al.,* 2003). If findings suggest that the risk of introduction is very low, phytosanitary measures may be removed in part or all together (McKenney *et al.,* 2003). In 2014, phytosanitary barriers to trade restricted the export of citrus fruit from CBS infected areas in South Africa, and several other citrus producing countries where the disease occurred, to the European Union (EU) and the United States of America (Commission on Phytosanitary Measures, 2016). However the restriction was later lifted with the condition of a more stringent criterion such as recording pre and post-harvest chemical treatments and mandatory registration of packing houses as well as on-site official inspections at citrus orchards (Commission on Phytosanitary Measures, 2016). Whole consignments of fruit may be rejected at packing houses or ports if, during inspection, they are found to contain

affected fruit (Bonants *et al.,* 2003). As a result, CBS has a great impact on the global citrus trade, and is of great concern to growers.

1.5 Production of Citrus fruits in South Africa and export market value.

Citrus is a common term and genus of flowering plants belonging to the *Rutaceae family*, originating and growing extensively in tropical and subtropical southern regions (Pham *et al.,* 2013). Citrus fruits were first planted in South Africa in the Eastern Cape around Bosheuvel in the year 1654 (Carstens *et al.,* 2012). The citrus trees planted were believed to be from St Helena Island, transported by a ship to the Cape of South Africa, however not all citrus fruits in South Africa are from St Helena Island (Carstens *et al.,* 2012). Previous studies indicates that some came from other parts of the world including India and Brazil (Carstens *et al.,* 2012). Due to the settlers some of the citrus trees were moved from the Cape of South Africa to the inland parts of the country (Carstens *et al.,* 2012).

Currently provinces that produce citrus fruit in South Africa include KwaZulu-Natal, Mpumalanga, Limpopo, North West and the Eastern Cape (Figure 1) (Department of Agriculture, Forestry and Fisheries 2013; Carstens *et al.,* 2012). In KwaZulu-Natal major citrus production areas are Pongola, Nkwalini and KwaZulu Natal Midlands (Figure 1) (Philp, 2006, Department of Agriculture, Forestry and Fisheries, 2013). In the Eastern Cape, major citrus production areas include Eastern Cape Midlands, Sundays River Valley and Patensie, the Boland region and Ceres region are the main citrus production areas in the Western Cape. Onderberg, Nelspruit (also known as Mbombela) and Senwes are the main citrus production areas in Mpumalanga while the major areas in Limpopo are Hoedspruit, Letsitele and Vhembe (Figure 1) (Philp, 2006; Department of Agriculture, Forestry and Fisheries, 2013).

The fruits grow in variety of soil types provided the soil is well drained, fertile, well-aerated with the pH around 6-6.5 (Department of Agriculture, Forestry and Fisheries, 2013). However there are important differences between productions regions in South

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Africa based mainly on climate. The Western Cape and Eastern Cape are considered 'cooler' citrus growing areas, the cooler climate allows farmers to respond to consumer demand for easy peelers including clementines and satsumas, and most of the country's easy peelers are produced in these two regions (Philp, 2006; Department of Agriculture, Forestry and Fisheries, 2013). Farm sizes are also smaller and most citrus fruits in the Western and Eastern Cape are packed by privatized cooperatives in huge facilities that are amongst the largest in the world (Philp, 2006). In Mpumalanga, Limpopo and KwaZulu-Natal, the climate is warmer and better suited to the cultivation of grapefruit and Valencia oranges. Farm sizes in these regions are larger and many farmers pack in smaller privately owned facilities (Department of Agriculture, Forestry and Fisheries, 2013 and Gianessi *et al.*, 2012). It is clear that citrus represents one of South Africa's most important fruit group by value and volume (Department of Agriculture, Forestry and Fisheries, 2013).

The South African citrus industry is currently the second largest exporter of fresh citrus fruit worldwide; exporting a total of 1.4 million tons annually to the most sophisticated world markets which demand high quality standards (Gianessi *et al.*, 2012; Carstens *et al.,* 2012 and Kotze., 1981). The export of citrus fruits to Europe dates far back from 1926 (Kotze, 1981). This industry plays a huge role in agriculture and the economic growth and development of the country. Its impact is more on job creation with the industry producing more than 20 million citrus trees that are planted on over 60 000 hectares by 1300 producers growing for export and 2200 smaller producers in South Africa. This provides employment to more than 100, 000 workers within the citrus sector (Truter, 2010; Halueendo, 2008). Citrus fruit produce that is exported yearly from South Africa is 65% of the total citrus production worldwide (Gianessi *et al.*, 2012). However within the Southern African Development Community (SADC), citrus fruits are not only grown in South Africa, they are also planted in Zimbabwe, Swaziland and Mozambique, although the production volume is much smaller when compared to South Africa (Department of Agriculture, Forestry and Fisheries, 2013).

Citrus fruits are mainly consumed for nutritional purposes, in particular for vitamin C and the peel can be used for feeding livestock and preparing compost (Halueendo, 2008). Moreover water that is extracted from the composted orange is used to control other

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plant diseases and the disease control rate is reportedly comparable with that of chemical treatment (Halueendo, 2008). Interestingly citrus fruits are available throughout the year because in Northern hemisphere they are harvested between October to June, while in Southern hemisphere they are harvested between April and November even though there are so many diseases associated with them, such as citrus black spot (Halueendo, 2008).

Citrus Producing Regions

Total Hectares	Total
Eastern Cape	12 508
KZN	3 405
Limpopo *	25 674
Mpumalanga *	6 817
Northern Cape	1 215
Swaziland	1 774
Western Cape	8 961
	60 355

ZIMBABWE

Limpopo

Letsitele

Limpopo Province
4 048 | 1 422 | 3 943 | 386 | 15 672

Hoedspruit

Nelspruit
Onderberg

Gauteng
Mpumalanga
Senwes Tvl

SWAZILAND
913 | 57 | 34 | 52 | 719

North West

2 371 | 209 | 929 | 451 | 2 857

Pongola

KwaZulu-Natal
1 650 | 335 | 571 | 49 | 801

NAMIBIA

Vaalharts

Free State

Northern Cape
324 | 94 | 337 | 64 | 397

LESOTHO

Nkwalini

KZN
Midlands

Eastern Cape
234 | 2 021 | 5 185 | 1 651 | 3 439

Eastern Cape Midlands

Key to Hectares	Total
Grapefruit & Pummelos	9 564
Lemon & Limes	4 726
Navel	14 832
Soft Citrus	5 200
Valencia & Midseasons	26 034
	60 355

Western Cape

Sundays River Valley

Western Cape
Boland   74 | 589 | 3 831 | 2 568 | 2 150

Patensie

* NOTE: The hectares reflected for Limpopo and Mpumalanga have changed from
previous reports due to the redefinition of the provincial boundaries.

Figure 1 Provinces of South Africa producing citrus. (Taken from Department of
Agriculture, Forestry and Fisheries official report, 2013).

1.6 Origin of Citrus black Spot and *Phyllosticta citricarpa*

1.6.1 Citrus black spot

Citrus black spot is found in various citrus producing continents throughout the world; however it is known not to occur in Europe, Central America and the Caribbean Region (Carstens, *et al.,* 2012; Truter, 2010; Agostini, *et al.,* 2006). It was reported first in Australia by Benson in 1895 then Kiely named and described it in 1948 (Everett and Rees George, 2006). Nevertheless in South Africa CBS was discovered first by Doige in 1929 along the coastal region of Kwa-Zulu Natal (former Natal) near Pietermaritzburg (Halueendo, 2008). The disease started spreading slowly in Pietermaritzburg where it fluctuated from seasonal and made considerable damages in the 1940 (Figure 2) (Halueendo, 2008). Additionally, the disease was also found present in Mpumalanga, Limpopo, North West, Gauteng and Eastern Cape (Figure 2) (Gianessi *et al.,* 2012). Even though history indicates that the first citrus propagating material introduced into South Africa was planted in the Western Cape Province from countries where CBS was known to be present (Figure 2) (Carstens, *et al.,* 2012). The presence or absence of CBS disease in the citrus producing provinces of the Western Cape and Northern Cape to date was never recorded (Carstens, *et al.,* 2012).
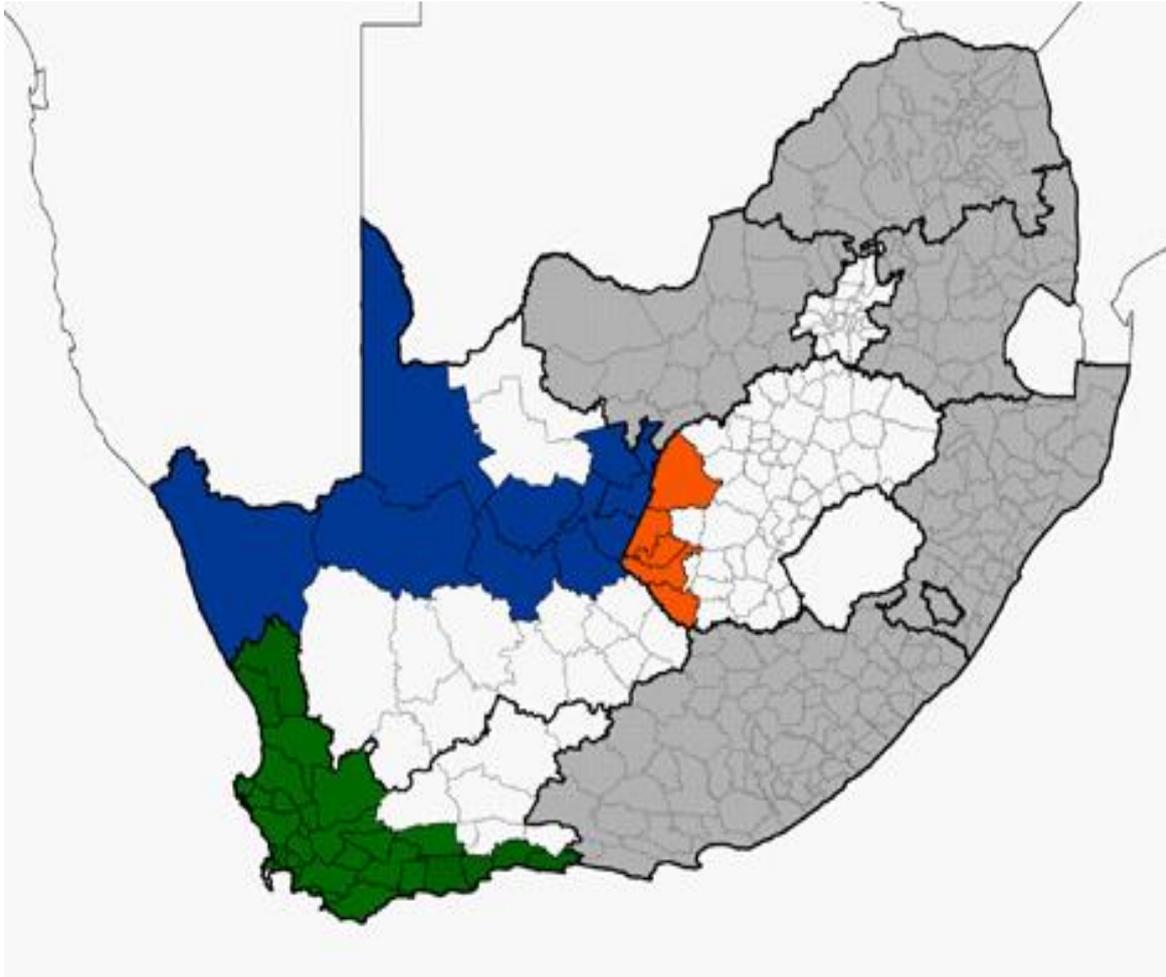
Figure 2: Provinces where Citrus black sport (CBS) is prevalent and CBS free areas in South Africa (Carstens, *et al.,* 2012).

Provinces in which CBS is present ☐
Northern Cape - CBS free ▇
Free State - CBS free ▇
Western Cape - CBS free ▇
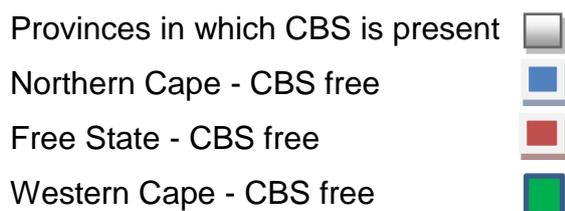
Ascomycetous fungus *Guignardia citricarpa* Kiely (anamorph or asexual stage is called *Phyllosticta citricarpa*) is the fungal organism responsible for causing CBS which occurs on citrus fruits (Bulanon *et al.,* 2013; Roberts *et al*., 2012 and Baayen *et al.,* 2002). However taxonomic classification indicates that the asexual stage was named *Phoma citricarpa* McAlpine, which was later changed to *Phyllostictina citricarpa* (McAlpine) around mid-nineteen sixties. Moreover the name was further changed to *P. citricarpa*

around 1973 (Halueendo, 2008). *P. citricarpa* incites lesions on citrus fruit but it does not result in internal decay. The fungus primarily infects the fruits although stems and leaves can also be infected (United State Department of Agriculture, 2011). Heavy infection near the pedicel of the developing fruit may lead to premature fruit drop and the losses may be substantial because affected fruits are no longer suited for the fresh fruit market (Agostini *et al.*, 2006; Baayen *et al.,* 2002). Almost all citrus cultivars are susceptible to CBS; this includes late maturing oranges, lemons, mandarins and grape fruit (Gomez, 2010; Halueendo, 2008). Due to the favourable conditions of summer rainfall, *P. citricarpa* tends to be more prevalent in summer than other seasons (Halueendo, 2008).

The species is frequently confused with *Guignardia mangiferae*, which is non-pathogenic, and is commonly isolated as an endophyte from citrus fruits and a wide range of other hosts (Wulandari *et al.*, 2009). Some studies have substantiated that the two species are not similar, for example on cherry decoction agar; the growth rate of *Phyllosticta citricarpa* isolates is lower than those of *G. mangiferae* (Baayen, *et al.,* 2002). However, conidia and ascospores dimensions are similar, but *P. citricarpa* form conidia with barely visible mucoid sheaths, whereas *G. mangiferae* form ascospores with thick sheaths (Baayen, *et al.,* 2002). *P. citricarpa* produce rare infertile perithecia, whereas fertile perithecia are formed by *G. mangiferae* (Baayen, *et al.,* 2002). Colonies of *P. citricarpa* are less dark than those of *G. mangiferae*, with a wider translucent outer zone and a lobate rather than entire margin (Baayen, *et al.,* 2002). On oatmeal agar, *P. citricarpa* forms a yellow pigment (Baayen, *et al.,* 2002). This fungus harbours strains from citrus fruits with classical black spot lesions usually containing pycnidia. *G. mangiferae* harbours endophytic strains from a wide range of host species, as well as strains from symptomless citrus fruits or fruits with minute spots (<2-mm diameter) without pycnidia (Baayen, *et al.,* 2002). These observations support the historic distinction between slowly growing pathogenic *P. citricarpa* and fast-growing, non-pathogenic *G. mangiferae* (Baayen, *et al.*, 2002).

1.6.2. Taxonomic classification of *Phyllosticta citricarpa*

Name: *Phyllosticta citricarpa* (McAlpine) Aa, 1973

Synonyms*: Phoma citricarpa* McAlpine, 1899

         Guignardia citricarpa Kiely, 1948

         Phyllostictina citricarpa (McAlpine) Petr., 1953

         Leptodothiorella sp. (spermatial state)

Taxonomic position: Eukaryota, Fungi, Ascomycota, Pezizomycotina,
               Dothideomycetes, Botryosphaeriales, Botryosphaeriaceae

Common names: Common names: Black spot, hard spot, shot-hole, freckle spot, virulent spot, and speckled blotch of citrus (English), Maladie des taches noires (French), Schwarzfleckenkrankheit (German)

(European and Mediterranean Plant Protection Organization Quarantine Pest, 2011).

1.7 Life cycle of *Phyllosticta citricarpa*

The life cycle of *Phyllosticta citricarpa* is shown in (Figure 3). These life cycle occurs in two stages (Figure 3): Stage 1; production of ascospores. Under favourable conditions, the ascospores originating from the fallen leaves deposited on the orchard's floor germinate from perithecia (Figure 3). As soon as the perithecia is fully mature, it swells and eject ascospores that are further dispersed by currents of air/ wind for long distance or water for short distance where they are deposited on leaf and fruit surfaces, and germinate to form quiescent infections (Figure 3) (Halueendo, 2008; Agostini, *et al.,* 2006).

Stage 2; is the production of conidia, the conidia structures are produced in conidiophores. Conidia can be found on dead twigs, where they are dispersed by water, raindrops splash and water during irrigation into the leaves and fruits (Figure 3) (Halueendo, 2008). Although in other countries conidia are not regarded as the main

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source of CBS, in Brazil, conidia are epidemiologically important just like ascospores because rainfall is not restricted to one season and flowering sometimes occurs more than twice yearly (Halueendo, 2008; Paula, *et al.,* 2005). Moreover if the South African conditions can be similar to those of Brazil, due to climatic change currently occurring, conidia can also be regarded as the main source of CBS just like the ascospores (Citrus black spot, 2009; Kotze, 1981).

Different symptoms of the fungal pathogen can be observed on the infected fruits. The fungus is most easily isolated from hard spot or virulent spot symptoms, but grows slowly and is often overrun in culture by faster growing saprophytes such as *Colletotrichum gloeosporioides* (Agostini, *et al.,* 2006). Rainfall has a positive correlation with disease development during susceptible periods and the development of the disease is negatively correlated with rainfall after petal fall when infection occurs (Wickert *at al.*, 2012). Rainfall allows the release of mature ascospores, however too much rainfall can disrupt discharge of the ascospores (United States Department of Agriculture, 2011). This leads to the decomposition of the dead leaves thus eradicating the CBS causal agent. Moreover too much rainfall prevents pseudothecia formation as the leaves become colonised by saprobes (United States Department of Agriculture, 2011).

Conducting Koch's postulates is difficult because of the extended latent period of the fungus (Everett and Rees George, 2006). It is uncertain whether the different symptom types are due to infection by different propagules, infection at various stages of fruit maturity, environmental factors, or some combination of these factors (Agostini, *et al.,* 2006). Symptomless fruit from regions where CBS is present may develop symptoms during transport (Everett and Rees George, 2006). Exposure of fruit to intense light and high temperatures speeds up the development of CBS symptoms (Agostini, *et al.,* 2006).
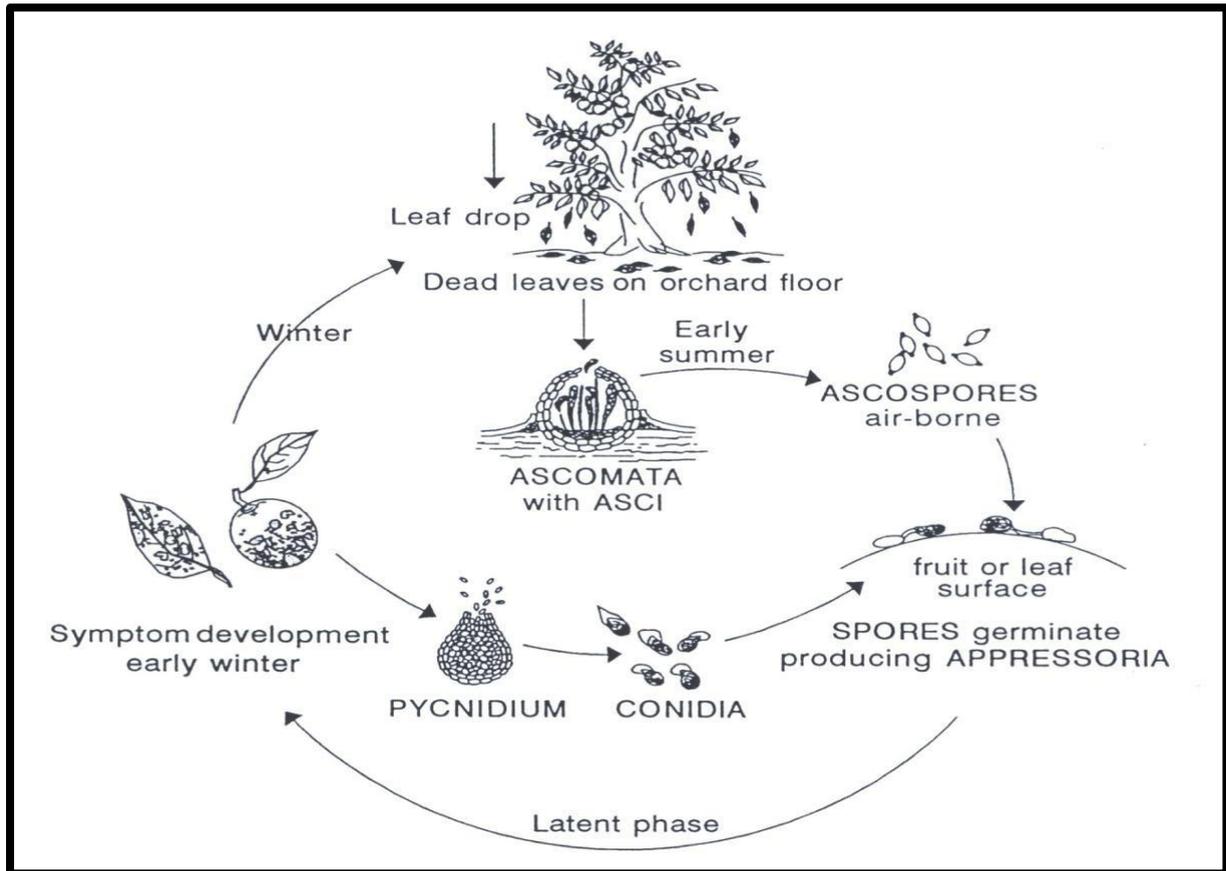
Figure 3: Life cycle and spreading of *Phyllosticta citricarpa* (Department of plant pathology, 2011).

1.8 Symptoms of *Phyllosticta citricarpa*

Benson who discovered citrus black spot (1895) in South Wales made drawings which were believed to be symptoms of the citrus black spot disease, whereas in South Africa the presence of CBS was first discovered by Doige in 1929 in Kwazulu Natal, later it was found in other provinces as well, such as Mpumalanga, Limpopo, North West, Gauteng and Eastern Cape (Halueendo, 2008). The epidemiology of citrus black spot is influenced by the presence of the organism, optimum climatic conditions for infection, tree growth cycle and the fruit age in relation to susceptibility to infection and symptom development (USDA, 2011). Symptoms on fruit normally develop late in the season; fruits that do not have symptoms at harvest may develop CBS in transport or storage (Bulanon *et al.,* 2013; DPP, 2011; Agostini, *et al.,* 2006). If symptoms develop before maturity, fruits often drop, resulting in loss of yield (Gianessi *et al.,* 2012). Leaf spots may occur as well (Figure 4 a), especially on the most susceptible citrus species such

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as lemon (Bulanon *et al.,* 2013). However, fruits may be severely infected with minor symptoms on foliage. Leaves symptoms first appear in mature leaves as tiny circular red to red-brown spots (Figure. 4a) that do not exceed 3 mm. Later they become sunken necrotic spots with a light centre and dark brown or black ring, often with yellow halo (Bulanon *et al.,* 2013).

Other symptoms of CBS can also occur on the fruits (Agostini, *et al.,* 2006). The most typical symptoms, known as hard spot, normally starts as small brick red spots with black margins which increase in size and develop necrotic tissue in their center and often bear conidia of the asexual stage of the *P. citricarpa* (Figure 4b) (Bulanon *et al.,* 2013; Agostini, *et al.,* 2006). The hard spot (figure 4b) normally appears at the beginning of fruit maturity and mostly pre-harvest, but on rare occasions they may appear just before the color of the fruits changes from green to yellow (Halueendo, 2008). Hard spot normally appears on the side that is exposed to sunlight (Halueendo, 2008). Hard spot can be replaced by freckle spots (Figure 4c) when the environmental conditions continue to be conducive. These freckle spots are similar to the hard spot except that their development occurs after the colour of the fruit change and this reduces the quality of the fruits (Halueendo, 2008, European and Mediterranean Plant Protection Organisation, 2011). The lesions render the fruit unmarketable for many fresh markets. After two to three weeks of freckle spot development, virulent spots start to develop with the onset of warmer conditions, they spread rapidly merging or running together, involving approximately two thirds of the fruit surface within four to five days and assuming irregular shapes (Figure 4c) (European and Mediterranean Plant Protection Organization, 2011).

Furthermore freckle spots may develop into virulent spot and these virulent spots may engulf freckle and hard spot lesions. Virulent spot is a spreading sunken necrotic lesion without defined borders and varies from brown to brick red which may extend deeper into the rind (Figure 4c) (Bulanon *et al.,* 2013; Agostini, *et al.,* 2006). False melanose consists of small black necrotic lesions and can occur in a tearstain pattern, normally occur months after the fruit develop resistance to the CBS (Figure 4d) (Gianessi *et al.,* 2012 and Halueendo, 2008). Other symptoms include cracked spot (Figure 4e) (Bulanon *et al.,* 2013). However there are various methods that are used to control the spread of this pathogenic organism, including the use of synthetic fungicides.

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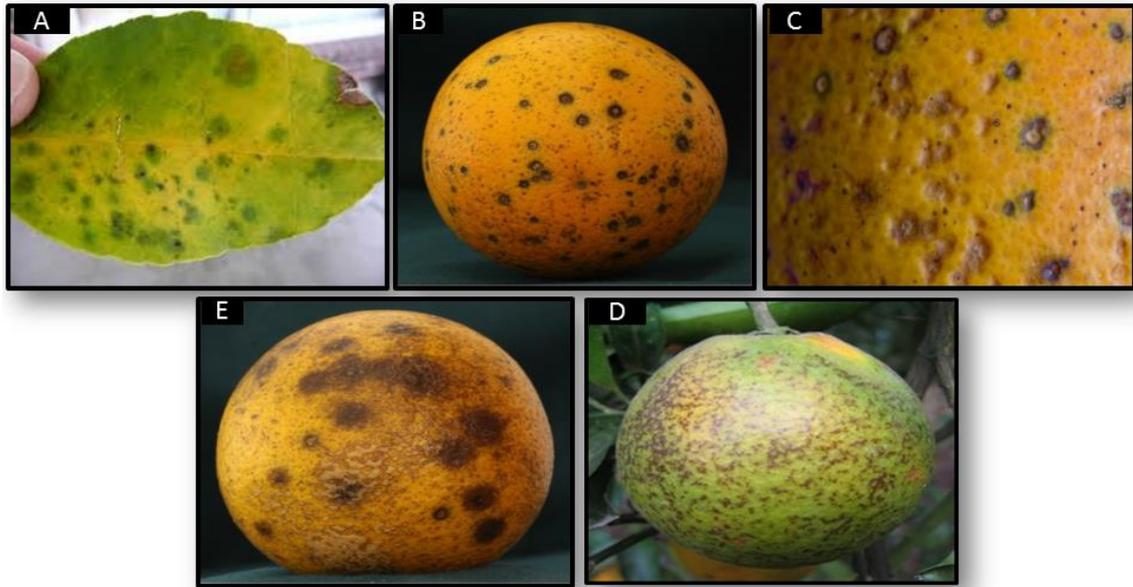
Figure 4. (A) Photograph depits leaves symptoms in mature leaves with tiny circular red to red-brown spots; (B) Photograph showing hard spot symptoms; (C) Photograph showing early virulent spot symptoms, (D) Photograph showing false melanose symptoms; (E) Photograph showing cracked spot symptoms (cited from Department of Plant Pathology, 2011).

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CHAPTER TWO

CITRUS BLACK SPOT CONTROL

SYNTHETIC FUNGICIDES AND ALTERNATIVE NATURAL FUNGICIDES FOR CITRUS BLACK SPOT CONTROL

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2.1 Abstract

Synthetic fungicides are currently used to eradicate fungal pathogens including Citrus Black Spot. However their excessive use has resulted in numerous drawbacks such as damage to the environment and negative effects on human health. Consequently demands to reduce the use of synthetic fungicides have increased and resulted in the search for alternative fungicides. The current study compared antifungal properties of essential oils with that of known anti-mitochondrial antifungal compounds against *Phyllosticta citricarpa* and *Guignardia mangiferae.* Essential oils (EO's) Thyme, Geranium, Citronella, Lavender and Eucalyptus were characterized using Gas Chromatography-Mass Spectrometry (GC-MS). Consequently their antifungal properties and that of known anti-mitochondrial antifungal compounds were evaluated and compared *in vitro*. Scanning Electron microscopy (SEM) was used to evaluate morphological changes that occurred due to EO's and anti-mitochondrial antifungal compounds. While the XTT colorimetric assay was used to measure the metabolic state of treated and untreated cells by determining the activity of mitochondrion dehydrogenases enzyme. In addition, Rhodamine (Rh) 123 was used to map mitochondrion function ($\Delta\psi_m$). Results from the bio-assay showed that EO's and anti-mitochondrial compounds inhibited fungal growth; this was indicated in a bio-assay demonstrating an inhibition zone (no growth), asexual zone and maximum growth zone. It is believed that the observed results occur through inhibition of structures with increased mitochondrial activity, indicated by the bio-assay results, SEM, XTT colorimetric assay and trans-membrane potential ($\Delta\psi_m$). The current study suggests essential oils can be used as alternative antifungals to minimize the spread of *P. citricarpa.*

Key words: Anti-mitochondrial antifungal compounds, bio-assay*,* conidia, citrus black spot, essential oils, *Phyllosticta citricarpa.*

2.2 Introduction

One of the major concerns currently facing citrus growers around the world is the spread of citrus black spot (CBS), a plant disease that threatens fruit marketability and citrus tree health (Bulanon *et al.,* 2013). The disease is caused by the fungus *Phyllosticta citricarpa*, which for many years has been confused with *Guignardia mangiferae* (anamorph: *Phyllosticta capitalensis*) (Wulandari *et al*., 2009). These species differ with respect to their ability to cause citrus disease, growth in different culture media and morphological features (Wulandari *et al*., 2009). *G. mangiferae* is a ubiquitous endophyte to citrus plants because no symptoms are known to be caused by this fungus on citrus hosts. Furthermore this fungus has been isolated from other plant hosts other than citrus plants (Baayen *et al.,* 2002). Besides the wider host range, the geographic distribution of *G. mangiferae* is also much wider than that of *P. citricarpa* and remarkably includes regions in which the disease caused by *P. citricarpa* has not been reported (Stringari *et al.,* 2009; Everett and Rees- George, 2006).

Furthermore growth difference in culture media has been reported in literature between the two species; *G. mangiferae* is known to produce ascospores in culture media, while, *P. citricarpa* produce conidia (Stringari *et al.,* 2009; Baayen, *et al.,* 2002). These reproductive structures (ascospores and conidia) are known to be important in the fungal life cycle because they play an important role in growth and dispersal, resulting in huge damage in agriculture such as critical losses of yield, quality and profit (Paul, 2014; Halueendo, 2008). *P. citricarpa* can spread readily in the natural environment with warm, wet or humid climates with summer rainfall, and adequate inoculums, resulting in an increased incidence of CBS (Carstens *et al.,* 2012; Pest Identification Database, 2007). Citrus black spot is important economically as it results in lesions that render the fruit not marketable even though it does not cause fruit decay (Bulanon *et al*., 2013; Halueendo, 2008). There are two direct impacts of CBS on the citrus industry: firstly, pre-harvest whereby the citrus tree that is affected by CBS bears less fruit, leading to yield decrease (Department of Agriculture Forestry and Fisheries, 2013). The second impact is visible lesions on the harvested fruit (Chung *et al.,* 2005). The post-harvest effects of CBS are not always hidden and there is likelihood that export citrus fruit may

develop CBS while being transferred to fruit ports (Department of Agriculture Forestry and Fisheries, 2013).

In South Africa including other countries such as China and Australia *P. citricarpa* is considered to be phytosanitory important because of its role in international trade (Halueendo, 2008). If the fruits contain CBS lesions, they can result in the rejection of the whole imported batch (Halueendo, 2008). When lesions are spotted on the fruits, repackaging is recommended and fruits could be sold at a lower market value, resulting in huge economic losses (Halueendo, 2008). Synthetic fungicides with antimicrobial activity are used to extend the storage-life of citrus fruits (Ayoola *et al*., 2008). However, over the past two decades, there has been great public concern about safety and side effects of synthetic agents in food preservation besides health implications (Ayoola *et al*., 2008). These agents are known to remain on the plant or within its tissues following treatment resulting in potentially toxic and carcinogenic effects on human and food systems. This indicates that growers and suppliers of citrus fruits are faced with the challenge of providing consumers with products that are attractive, free from disease, defects, toxic residues, and with longer shelf or storage-life (Sellamuthu, 2013).

Strict regulations enforced by the fresh produce-importing countries regarding the minimum pesticide residue levels on fresh produce; increasing resistance to synthetic fungicides by fungal pathogens and waste disposal of fungicides have necessitated the search for a natural novel fungicides to replace synthetic fungicide applications in the packing line as postharvest treatment (Sellamuthu, 2013). Plant extracts including essential oils (EO's) are recognized as potential sources of natural compounds to improve the shelf life and the safety of citrus fruits and other fruits (Xi Yap *et al.,* 2014). Various EO's are known to possess different biological properties such as anti-inflammatory and anti-fungal properties. Furthermore EO's represent a distinctive group of possible novel drug compounds, due to their complex chemical composition that make them functionally versatile (Xi Yap *et al.,* 2014). Moreover, it is proposed that essential oils may target structures with increased mitochondrial activity such as reproductive structures (ascospores and conidia) (Prakash *et al.,* 2015).

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2.3 Citrus black spot control

Plant pathogenic fungi are omnipresent in the environment; many require a plant host to continue their life cycle, releasing numerous spores with a purpose of withdrawing nutrients from an infected plant (Gianessi *et al.,* 2006). Synthetic fungicides often used to control fungi inhibit fungal growth by targeting reproductive structures (ascospores and conidia) known to be important in the fungal life cycle. These structures play an important role in the growth and dispersal of fungi that habitually cause huge damages in agriculture resulting in critical losses of yield, quality and profit (Halueendo, 2008; Paula, 2005). Synthetic fungicides are widely used in South Africa and other countries to counteract these invading fungi (Mahfouz *et al.,* 2013). Over the years farmers have used synthetic fungicides to control fungal growth; however synthetic fungicides use has increased concern due to their toxicity, polluting the environment and antimicrobial resistance (Romanazz *et al.*, 2013; Martinez, 2012). Moreover regular use of fungicides results in risk particularly if residues are retained in soil or transferred into water. This has a negative effect on soil organisms and carries a potential risk to long-term fertility of the soil (Martinez, 2012; Wightwick *et al.,* 2010).

Some of the chemicals penetrate the soil surface where they persist over a period of time and migrate off-site due to leaching and/or runoff (Wightwick *et al.,* 2010). In addition, some migrate off-site due to aerial drift. Once they have migrated off-site they enter nearby water sources, such as rivers, lakes and dams and groundwater resources where they cause adverse effects to aquatic organisms (Wightwick *et al.,* 2010). Furthermore, fungal resistance to currently available synthetic fungicides related to the overuse and abuse is more prevalent (Brooks and Roberts, 1999). Some fungicides are dangerous to human health, such as vinclozolin known to result in carcinogenic effects in human peripheral blood; these fungicides have since been banned from use (Hrelia, 1996). Flucycloxuron (FCX) and Diflubenzuron (DFB) are used to control fungal infections, however, they possess numerous side effects on organisms other than the target pest, they are poisonous when contacted and ingested (Rouabhi, 2010; Rouabhi *et al.,* 2009).

Moreover, the use of synthetic fungicides relies on many factors to mitigate the invading fungi including plant species; these include formulation methods and climatic factors such as temperature and rainfall (Yulia, 2009). Additionally, the use of most synthetic fungicides has been banned in many countries due to their toxic effects, long degradation, accumulation in the food chain and destroying useful microorganisms (Mahfouz *et al.,* 2013). For over 35 years the agricultural industry has faced problems arising from the development of resistance in fungal pathogens of crops against the fungicides used to control them (Brent and Hollomon, 2007). Phenylamides fungicides were first introduced in late nineteen seventies; they were developed to act specifically against oomycete pathogens, having no effect recorded on other classes of fungi (Brent and Hollomon, 2007).

However the first cases of resistance occurred in 1980, when it was applied to cucumbers for control of downy mildew (*Pseudoperonospora cubensis*) and to potatoes for control of late blight (*Phytophthora infestans*) (EPPO, 2011; Brent and Hollomon, 2007). Resistance appeared also in grape downy mildew (*Plasmopara viticola*) and in tobacco blue mould (*Peronospora tabacina*). The manufacturer was forced to immediately withdraw the product from use against foliar diseases due to unexpected occurrence of resistance (Brent and Hollomon, 2007). Dicarboximides (iprodione, vinclozolin and procymidone) have been used since the mid- nineteen seventies. They replaced benzimidazole fungicides, since it was no longer effective (Brent and Hollomon, 2007). After three years of intensive use, resistant strains were detected (Wightwick *at al.,* 2010). The proportion of resistant strains usually varies greatly with time of year; they reportedly decline after dicarboximide treatment ceases and increase again when it is resumed (EPPO, 2011; Wightwick *et al.,* 2010).

Furthermore, other fungicides such as copper sprays control plant pathogens by denaturing the proteins; thereby destroying enzymes that are critical for cell functioning. They also act by denaturing enzymes of the respiration pathway in the sporangia and zoospores (Rosenberger, 2012). The most used copper sprays are Bordeaux mixture, copper oxychloride and cupric oxide (Rosenberger, 2012; Brent and Hollomon, 2007). Nonetheless their disadvantage is that they are non-selective as a result they can kill plant cells as well, resulting in slowing down of the vegetative development of the plant (Rosenberger, 2012; Brent and Hollomon, 2007). Dithiocarbamates are organic sulphur

compounds derived from bidithiocarbamic acid, their salts are 1, 2- bidithiocarbamates and the common chemical names of these fungicides are based on the chemical groups (methyl, ethyl, etc.) and metals in them (Brent and Hollomon, 2007). They affect sporangial and zoospore germination in particular, but also mycelia development, disrupting important biochemical processes involving enzymes of the thiol group (Rosenberger, 2012). In comparison to the copper fungicides the dithiocarbamates are not phytotoxic and can therefore be used throughout growing period. However, they lack persistence because they are easily washed off in the rain (Russell, 2006).

Other fungicides that were used but not discussed above are mentioned in Table 1 together with the years when resistance was observed, resistant organisms and their related crop. Table 2 indicates the mechanism of resistance by microbes to some fungicides. Even though synthetic fungicides remain the most common method used to control fungal disease, their negative impact such as negative health implications to human, damage to environment and the risks of fungi developing resistance has become intolerable. In most cases resistance occurs due to modification of the target site of site (Table 2) (Paul, 2014). This is a concern because the products may become less effective or even inadequate for controlling fungal pathogens (Paul, 2014). This has influenced the search for rational approach to limit repeated use of synthetic fungicides. Alternative approaches such as using natural fungicides essential oils (EO's) and its hydrosol as well as antifungals, anti-inflammatory compounds can be a best alternative because they are safe, limit human and animals risk and environmental risk (Yulia, 2009).

Table 1 Classes of synthetic fungicides resistance in various crops and the year of discovery (Cited from Brent and Hollomon, 2007).

Date first observed (Approx.)	Fungicide class	Years of commercial use before resistance observed (Approx.)	Main crop diseases and pathogens affected
1960	Aromatic hydrocarbons	20	Citrus storage rots, *Penicillium* spp.
1964	Organo-mercurials	40	Cereal leaf spot and stripe, *Pyrenophora* spp.
1969	Dodine	10	Apple scab, *Venturia inaequalis*
1970	Benzimidazoles	2	Many target pathogens
1971	2-Amino-pyrimidines	2	Cucumber and barley, powdery mildews *Sphaerotheca fuliginea* and *Blumeria graminis*
1971	Kasugamycin	6	Rice blast, *Magnaporthe grisea*

1976	Phosphorothiolates	9	Rice blast, *Magnaporthe grisea*
1977	Triphenyltins	13	Sugar beet leaf spot, *Cercospora betae*
1980	Phenylamides	2	Potato blight and grape downy mildew, *Phytophthora infestans* and *Plasmopara viticola*
1982	Dicarboximides	5	Grape grey mould, *Botrytis cinerea*
1982	Sterol Demethylation inhibitors (DMIs)	7	Cucurbit and barley powdery mildews, *S. fuliginea* and *Blumeria graminis*
1985	Carboxanilides	15	Barley loose smut, *Ustilago nuda*
1998	Quinoneo utsideInhibitors (QoIs; Strobilurins)	2	Many target diseases and pathogens
2002	Melanin BiosynthesisInhibitors (Dehydratase) (MBI-D)	2	Rice blast, *Magnaporthe grisea*

Table 2: Mechanism of fungicides action in various crops (Brent and Hollomon, 2007).

Fungicide or fungicide class	Mechanism of resistant
Aromatic hydrocarbons	Unknown, but show cross-resistance with dicarboximides hydrocarbons and phenylpyrroles
Organo-mercurials	Detoxification by binding substances
Dodine	Unknown
Benzimidazoles	Altered target site (ß-tubulin)
2-Amino-pyrimidines	Unknown
Kasugamycin	Altered target site (ribosomes)
Phosphorothiolates	Metabolic detoxification
Phenylamides	Possibly altered target site (RNA polymerase)
Dicarboximides and Phenylpyrroles	Altered target site (protein kinase involved in osmoregulation)
DMIs	Increased efflux; altered target site; decreased demand for target-site product; target-site over-production
Carboxanilides	Altered target site (succinate-ubiquinone oxidoreductase)
QoIs (strobilurins)	Altered target site (ubiquinol-cytochrome c reductase)
Melanin Biosynthesis Inhibitors (Dehydratase) MBI-D	Altered target site (scytalone dehydratase)

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Kock *et al.,* 2007 showed that anti-inflammatory compounds such as acetylsalicylic acid (ASA), benzoic acid (BA) and salicylic acid (SA) possess antifungal properties. A yeast bio-assay was developed to expose antifungal properties of these compounds with *Eremothecium ashbyi* used as test organism (Kock *et al.,* 2007). Similar findings were also observed by Leeuw *et al.,* 2009 and Ncango *et al.,* 2010 in their research using *Mucor circinelloides* and *Aspergillus fumigatus* respectively. After exposing yeast and fungi to these drugs, asci formation and sporangia were affected (Ncango *et al.,* 2010; Leeuw *et al.;* 2009; Kock *et al.,* 2007). These authors further showed that asci and sporangia contain increased mitochondrion activity when compared to hyphae. It is believed that these drugs selectively target structures with increased mitochondrion activity (Ncango *et al.,* 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007).

Although alternative methods are being researched to control postharvest decay during storage, natural plant products such as essential oils (EO's) and hydrosol, a by-product of essential oil production are gaining fame and the attention of researchers globally due to their biodegradable, eco-friendly, economical and safety properties (Sivakumar and Bautista, 2014). The EO's reported in various studies have shown to exhibit antifungal properties by targeting structures responsible for the life cycle of fungal organism as well such as ascospores and conidia for both in vitro and in vivo in different fresh produce (Imelouane *et al.,* 2009; Meepagala *et al.,* 2002; Caccioni and Guizzardi, 1994; Vaughn and Spencer, 1991). However there is limited information regarding the effect of essential oils against *P. citricarpa*. Therefore EO's can be ideal candidates for use as alternative fungicides as well against citrus black spot (Macias *et al.,* 1997).

2.4 Alternatives natural fungicides (Essential Oils (EO's)

Essential oils are volatile oily liquids of the secondary metabolism of scented plants and are obtained from plant parts, such as flowers, leaves, seeds, bark, fruits and roots (Moussaoui *et al.*, 2013; Nazzaro *et al.,* 2013). Although reported to have low solubility in water, they are soluble in fats, alcohol, organic solvents and other hydrophobic substances and are generally liquid at room temperature (Baser and Demirci, 2007).

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They are stored in specialized plant cells, usually oil cells or ducts, resin ducts, glands or trichomes (glandular hairs) (Baser and Demirci, 2007; Pengelly, 2004). They are widely used as food flavours and preservatives to prevent growth of food-borne bacteria and moulds, and to extend the shelf life of processed foods (Moussaoui *et al.*, 2013). Essential oils play an important role in plant defence mechanism against pathogenic microorganisms and other pests (Nazzaro *et al.,* 2013; Liu *et al.*, 2009). Furthermore they attract some insects to favour the dispersion of pollen and seeds, or repel undesirable pests; they also work against herbivores by reducing their appetite for plants (Adebayo *et al.*, 2013; Bakali *et al.*, 2012).

In addition to the above mentioned properties they also have a number of other biological properties that can be used to prevent and treat human systemic diseases, including infectious diseases (Nazzaro *et al.,* 2013). Moreover they have been reported as exceptionally good therapeutic agents for chemo prevention, cancer suppression, anti-diabetic activity and lowering serum cholesterol and triglycerides (Bigos *et al.*, 2012). Many of them have high activity against Gram-positive and Gram-negative bacteria, as well as against viruses and fungi (Bigos *et al.*, 2012). Due to their antimicrobial activity, essential oils may be applied in fighting against drug-resistant bacteria, fungi and even viruses and for prevention of the resistance formation of pathogenic microbes (Bigos *et al.*, 2012).

The EO's mechanisms of action works according to three aspects: i) the presence of OH groups that are able to form hydrogen bonds with enzymes, modifying a variety of intracellular functions; ii) action on microbial morphology due to interactions with membrane enzymes, resulting in the loss of rigidity and integrity of the hypha cell wall; and iii) changes in permeability of cell membranes, granulation of the cytoplasm and cytoplasmic membrane rupture (Amiri, *et al.,* 2008). Modification of intracellular function occurs due to the hydrophobic nature of EOs which allows them to penetrate microbial cells and cause alterations in its structure and functionality (Tassou *et al.*, 2000). In some cases, essential oils also alter membrane permeability by destroying the electron transport system. The destruction of the electron transport prevents energy production and disrupts the proton motive force, protein translocation and synthesis of cellular

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components, resulting in cell lysis and death (Nazzaro *et al.,* 2013; Tassou *et al.*, 2000). Furthermore, the EO's can lead to a decrease in the intracellular concentration of ATP, an event that is linked to the destruction of the microbial membrane (Nazzaro *et al.,* 2013). Moreover, some compounds present in EOs are also responsible for interfering with proteins involved in the transport of essential molecules into the cell (Nazzaro *et al.,* 2013; Tassou *et al.*, 2000). This results in the destabilisation of the phospholipid bilayer, the destruction of the plasma membrane function and the loss of vital intracellular components and the inactivation of enzymatic mechanisms (De Oliveira *et al.,* 2011; Tassou *et al.*, 2000).

Lastly, in bacteria the permeability barrier provided by cell membranes is responsible for many cell functions, including maintaining the energy of the cell, membrane-coupled energy-transducing processes, solute transport and metabolic regulation (Nazzaro *et al.,* 2013; Gill *et al.,* 2006). The cell membrane is also essential for controlling the turgor pressure (Lambert *et al.,* 2001). Toxic effects of essential oils on membrane structure can be used to explain the antimicrobial activity of the oils. In fact, the mechanisms of action of the EO's include the degradation of the cell wall, damaging the cytoplasmic membrane, cytoplasm coagulation, damaging the membrane proteins and increased permeability leading to leakage of the cell contents. This results in a reduction of proton motive force including reduction in the intracellular ATP pool (Lambert *et al.,* 2001).

All three aspects are interrelated, the hydrophobicity of these compounds leads them to cross cell membrane and interact with cell compounds, and consequently they affect both membrane and intracellular enzymes (Avila-Sosa, *et al.,* 2012). Some hydrophobic compounds present in EO's change the permeability of the microbial membranes for cations such as H^+ and K^+ that cause a change in the flow of protons, modifying cell pH and affecting chemical composition of the cells and their activity (Amiri, *et al.,* 2008). The key for their activity is the ability of hydrophobic compounds to dissolve in the lipid phase of the cytoplasmic membrane, but higher solubility does not always mean greater antimicrobial action (Amiri, *et al.,* 2008).

The loss of differential permeability of the cytoplasmic membrane is the cause of cell death because this results in an imbalance in intracellular osmotic pressure, disruption of intracellular organelles, leakage of cytoplasmic contents and finally cell death (de

Oliveira *et al*., 2011). The interaction with cell membranes may also lead to the leakage of some cellular components, including ATP, the main energy-storing molecule. Phenolic compounds are known to alter microbial cell permeability, allowing the loss of macromolecules from the interior (de Oliveira *et al*., 2011). Other events that may lead to dysfunction of the membrane and subsequent disruption include: (i) dissipation of the two components of the proton motive force (pH gradient and electrical potential); interference with the system of generating energy (ATP) in the cell; (ii) inhibition of enzymes; and prevention of substrate utilization for energy production (El-Mogy and Alsanius, 2012; de Oliveira *et al*., 2011).

2.5 Rationale

The spread of citrus black spot (CBS) is a major concern in the citrus industry because the disease threatens fruit marketability and citrus tree health. In most countries the disease is considered to be phytosanitory important because of its role in the international trade. If the fruits contain CBS lesions, this can result in the rejection of the whole imported batch; moreover the disease can spread readily in the natural environment resulting in an increased incidence of CBS. It is therefore of paramount importance to control and manage the disease; however there is a great public concern about safety and side effects of synthetic fungicides currently used to control citrus black spot. Synthetic fungicides are known to have carcinogenic effects on humans and are also toxic to the environment. Furthermore, microorganisms tend to develop resistance to most synthetic fungicides. This problem has prompted research into the identification of new ways with broad activity in treatment of microbial disease in plants. Although alternative methods are being researched to control citrus fruits diseases, natural plant products such as essential oils and their hydrosol are gaining popularity and drawing the attention of researchers. In nature, essential oils play an important role in the protection of plants. They contain a wide variety of secondary metabolites that are capable of inhibiting or slowing the growth of bacteria, yeasts, fungi and even viruses. The oils and their components have activity against a variety of microbial targets, particularly the membrane and cytoplasm, and in some cases, they completely change the morphology of the cells, EO's can be ideal candidates for use as alternative antifungal compounds.

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2.6 Aim of the study

With the above information as background, the aim of the study was to assess the use of essential oils and hyrosol as alternative antifungals in the bio-control of the fungal plant pathogen *Phyllosticta citricarpa,* the causative agent of citrus black spot and *Guignardia mangiferae* (ubiquitous endophyte to citrus plants)*.*

2.7 Objective of the study

The specific objectives were:

- To determine structures with increased mitochondrial activity on *Phyllosticta citricarpa* and *Guignardia mangiferae* using Rhodamine 123.
- To characterize essential oils (EO's) using Gas Chromatography Mass Spectrometry (GC-MS).
- Perform bio-assays based on the agar diffusion method were activity of essential oils (EO's), hydrosol and known antifungal anti-mitochondrial compounds will be measured along a concentration gradient across the agar plate
- Perform bio-assays based on the agar dilution were the activity of the EO's and antifungal anti-mitochondrial compound will be measured based on the growth diameter of the tested organism.
- To map the mode of action of essential oils using:
    - o Scanning electron microscope to evaluate morphological changes that occur due to the effect of essential oils on the organism.
    - o Use XTT colorimetric assay to determine the effect of essential oils on activity of mitochondrion dehydrogenases.

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CHAPTER THREE

MATERIALS AND METHODS

MATERIALS AND METHODS

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3.1 Introduction

Previous studies show that essential oils (EO's) and antifungal anti-mitochondrial compounds with ant-inflammatory properties, affect fungal sexual structure development due to their anti-mitochondrial action. In this current study, EO's and antifungal anti-mitochondrial compounds were also tested for antifungal anti-mitochondrial properties. This is shown by a unique bioassay, with the mitochondrion-dependent sexual structure from *Phyllosticta citricarpa* and *Guignardia mangiferae* serving as indicators. The bio-assay is based on the agar diffusion method and agar dilution method. Using the agar diffusion method, activity of compounds are measured along a concentration gradient across the agar plate (i.e. from position of compound addition), done by observing the growth inhibition-zone and changes in fungi reproductive structures from the asexual-zone to the maximum growth zone. Three distinctive zones were observed on a plate; an inhibition-zone where growth is completely inhibited, asexual zone without sexual stages and maximum growth zone with both asexual and sexual zones. With regards to the agar dilution method on the other hand, the activity of the compound is measured based on the growth zone, with the small growth zone indicating high antifungal activity of the tested compound, while the highest growth zone indicate low antifungal activity of the tested compound. This bioassay can be used in the screening for various EO's from plant sources with Antifungal-anti-mitochondrial actions. In addition, EO's may serve as alternative to synthetic fungicides to combat the dispersal of *P. citricarpa* that is notorious for causing citrus black spot diseases on citrus fruits.

A bio-assay was constructed using *Phyllosticta citricarpa* and *G. mangiferae* to evaluate the ability of essential oils (EO's) and antifungal anti-mitochondrial compounds to selectively target fungal sexual structure development. The conidia and ascospores release including mitochondrion activity of *P. citricarpa* and *G. mangiferae* over decreasing concentration gradients of various essential oils (EO's) and antifungal anti-mitochondrial compounds were assessed. For the purpose of this study, any compound that inhibits mitochondrion activity in a direct or indirect manner will be referred to as an anti-mitochondrial *P. citricarpa* is a citrus pathogen widely responsible for causing Citrus

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black spot disease on citrus fruits as well as lesions on the leaves of citrus fruits. Uncovering novel effective anti-mitochondrial antifungals will therefore be of importance in combating this fungus.

Experimental section

3.2 Strains used and cultivation

Phyllosticta citricarpa and Guignardia mangiferae preserved at the national collection of fungi (ARC-PPRI) in Pretoria South Africa were used in the study. The fungus were cultivated on yeast-malt (YM) agar at 25 $^{\circ}$C in Petri dishes until spore-releasing-structures in *Phyllosticta citricarpa* and *Guignardia mangiferae* were observed respectively (Ncango *et al.,* 2010).

3.3 Mitochondrial mapping for *Phyllosticta citricarpa* and *Guignardia mangiferae*

In order to determine which structures possessed increased mitochondrial activity, mitochondrial mapping was carried out. This was performed according to Ncango *et al.* (2008). In short, fungal cells *Phyllosticta citricarpa* and *Guignardia mangiferae* were scraped from YM plates. To remove agar and debris, fungal cells were washed separately with PBS in 2 ml plastic tubes and then treated with 31 µl Rhodamine 123. Cells were treated for 1 h in the dark at room temperature after which cells were washed again with PBS to remove excess stain. These were fixed on microscope slides and viewed under the confocal laser scanning microscope. Rh123 is a cationic lipophilic mitochondrion stain used to map mitochondrion function (Δψm) selectively. This is attributed to the highly specific attraction of this cationic fluorescing dye to the relative high negative electric potential across the mitochondrion membrane in living cells. With

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this dye, a high Δψm is signified by a yellow-green fluorescence (collected at 450 nm); while a low Δψm is signified by a red fluorescence collected at 625 nm.

3.4 Essential oil characterization (GC-MS)

In order to characterize essential oils that were going to be used in the current study gas chromatography mass spectrometry (GC-MS) was carried out. Briefly, essential oils were dissolved in hexane (10% hexane) and injected in a Finnigan Focus Gas Chromatograph (GC) which was operated under the following conditions: the injector temperature was set at 230° C. The GC was equipped with an AB-1MS (30 M X0.25 mm id 0.25 μm) capillary column. Helium was used as carrier gas at a constant flow of 1 mL min^{-1} (at a split ratio of 50:1). The temperature programme was set at 40° C for four minutes and then raised at 5° C min^{-1} to 200° C and then held at 200° C for 1 minute and then raised at 5° C to 220° C where it was held for 10 min. Mass analysis of the oils was done using a Finnigan Focus DSQ mass spectrometer. The ion source temperature was set at 250° C with an ionization voltage of 70 eV and mass scan range of 50-650 amu. Individual GC peaks and mass spectra were identified by searching commercial libraries and this was followed by expert matching of MS data.

3.5 Bio-Assay Preparation and Application

3.5.1 Agar diffusion method for *Phyllosticta citricarpa*

The bio-assay was based on the agar diffusion method where activity of essential oils Citronella (*Cymbopogon nardus*), Eucalyptus (*Eucalyptus globulus*), Geranium (*Pelargonium graveolens*), Lavender *(Lavandula latifolia*) and Thyme (*Thymus vulgaris)*), hydrosol (Lengana (*Artemisia afra*), Lavender (*Lavandula latifolia*), Thyme (*Thymus vulgaris)* and rosemary (*Rosmarinus officinalis*) hydrosol) and known antifungal anti-mitochondrial compounds which were used a positive control

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(Acetylsalicylic acid [ASA]; Salicylic acid [SA]; Benzoic acid [BA]) and Ethanol (negative control) were measured along a concentration gradient across the agar plate (i.e. from position of compound addition) by observing the growth inhibition-zone. To ensure precise comparison of all tested compounds (EO's, hydrosol and anti-mitochondrial compounds) the bio-assays were carried out according to the method of Ncango *et al.* (2010). Briefly, each fungus (*Phyllosticta citricarpa* and *Guignardia mangiferae*) was suspended separately in sterilized distilled water (dH20) and 0.2 ml was streaked out on YM (0.5% m/v agar) to produce a homogenous lawn across the surface of the agar. Subsequently, a well (0.5 cm in diameter and depth) was constructed at the centre of the Petri dish and 46 µl of each essential oil was added separately to each plate containing the organism. The same procedure was carried out for known antifungal anti-mitochondrial compounds, and 96 % ethanol was added alone as a control. Similar amounts of essential oil hydrosol were tested for antifungal activity using the bio-assay only for *P. citricarpa*. All plates were incubated at 25°C until different textured growth zones were observed. Since the bio-assay has been evaluated as a qualitative screen for compounds with specialized antifungal activity, no attempts were made at this stage to determine MICs (minimum inhibitory concentration).

3.5.2 Agar dilution method *Guignardia mangiferae*

Activity of essential oils Citronella (*Cymbopogon nardus*), Eucalyptus (*Eucalyptus globulus*), Geranium (*Pelargonium graveolens*), Lavender *(Lavandula latifolia)* and Thyme (*Thymus vulgaris)*, and known antifungal anti-mitochondrial compounds which were used a positive control (Acetylsalicylic acid [ASA]; Salicylic acid [SA]; Benzoic acid [BA]) and Ethanol (negative control) was based on the agar dilution method. *Guignardia mangiferae* was employed in the anti-fungal investigation adopting the method utilized by (Erasto *et al.,* 2006; Aliero *et al.,* 2006; Afolayan and Meyer, 1997). Potato Dextrose Agar (PDA) was prepared and autoclaved before the addition of each essential oils and known antifungal anti-mitochondrial compounds. Each essential oils and known antifungal anti-mitochondrial compounds were mixed with molten agar at 45 °C to final concentrations of 46 ul, then poured into Petri dishes. Each plate was swirled carefully until the agar was evenly distributed, and left overnight for residual solvent to evaporate. Plates containing PDA with the respective solvents (ethanol) served as negative

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controls. The prepared plates were inoculated with plugs obtained from the actively growing margin of fungi plates and incubated at 25°C until growth was observed. The diameter of the fungal growth was measured and recorded per growth zone of each essential oils and known antifungal anti-mitochondrial compounds. Significant differences within the means of the treatments and negative controls were observed.

3.6 Scanning electron microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to evaluate morphological changes that occur due to activity of essential oils and other compounds on the fungus *Phyllosticta citricarpa* and *Guignardia mangiferae* in terms of shape and structure scanning electron microscopy (SEM) was used (AL-Bayaty *et al.,* 2011; AL-Bayaty *et al.,* 2010). Preparation of cells for analysis using SEM was carried out according to Ncango *et al*. (2010). Briefly, treated and untreated cells (from agar diffusion method) of *P. citricarpa* and *Guignardia mangiferae* (from agar dilution method) were fixed using 3% v/v of a sodium phosphate buffered glutaraldehyde solution at pH 7.0 and a similarly buffered solution (1% m/v) of osmium tetroxide for 1 h. Subsequently, the material was dehydrated in a graded series of ethanol solution (30%, 50%, 70%, 90%, and 100% for 30 min per solution). Next, the ethanol-dehydrated material was critical-point dried, mounted and coated with gold to make it electrically conductive. This preparation was then examined using a SEM. Micrographs were taken to investigate the general pattern of the fungi for specific morphological features.

3.7 Quantitative measurement of metabolic state

The XTT (a tetrazolium salt) colorimetric assay was used to determine the activity of mitochondrion dehydrogenases, an indicator of metabolic activity was done according to (Moss *et al.,* 2008; Strauss *et al.,* 2007; Bachmann *et al.,* 2002). Cells of *Phyllosticta citricarpa* and *Guignardia mangiferae* were scraped off from different textured zones (representing treated and untreated cells) on agar diffusion plates (bio-assay). Five millilitres of PBS was used to suspend 1g of cells from each respective zone. Following

this, 2.5 ml XTT [2.5 g XTT in 1L Ringer's lactate solution] and 400 µl menadione were then added. Cells were then incubated at 37° C for 3 h in the dark. A 96-well, flat bottom polystyrene microtiter plate was used and 150 µl of the formazan product was transferred to each well and the formazan product in the supernatant spectrophotometrically measured in terms of optical density at 492 nm using Spectramax ME2 (Molecular Devices). This was repeated for all the cells grown on agar diffusion plate (Bio assay) treated with different essential oils, hydrosol and different anti-mitochondrial compounds.

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CHAPTER FOUR

RESULTS AND DISCUSSION

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RESULTS AND DISCUSSION

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4.1 Mitochondrion mapping

Research suggests that sexual structures (ascospores and conidia) are characterized by elevated mitochondrial activity when compared to hypha (Ncango *et al.,* 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). *Phyllosticta citricarpa* the causal agent of citrus black spot is known to only produce conidia in culture media, while its close relative *Guignardia mangiferae* is known to produce ascospores in culture media (Stringari *et al.,* 2009; Baayen, *et al.,* 2002). *G. mangiferae* was used *in vitro* as a model organism because it is closely related to *P. citricarpa* in order to determine whether or not ascospores possess elevated mitochondrial activity. Even though *G. mangiferae* is known as endophytic and non-pathogenic on citrus fruits, it is believed that if the ascospores of *G. mangiferae* contain elevated mitochondrial activity, similar results will be observed for *P. citricarpa in vivo* because of the similarity of these organisms.

Previous studies have shown that spore-releasing-structures such as yeast asci, sporangia and phialides with increased mitochondrion activity are more sensitive to mitochondrial inhibitors when compared to vegetative cells and hyphae (Dieryckx *et al.,* 2015; Ncango *et al.,* 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). These authors also reported that increased mitochondrion activity is important to effect spore-release-structure development; commonly used anti-inflammatory drugs such as aspirin reportedly target the development of these structures probably by decreasing energy production necessary for normal development and spore release (Ncango *et al.,* 2010; Swart *et al.,* 2010).

In the current study mitochondrial activity in *P. citricarpa* and *G. mangiferae* structures was mapped using Rhodamine 123. This is a cationic lipophilic mitochondrion stain used to map mitochondrion function selectively. This is attributed to the highly specific attraction of this cationic fluorescing dye to the high negative electric potential across the mitochondrion membrane in living cells (Armstrong, 2006; Costantini *et al.,* 2000; Johnson *et al.,* 1980). With this dye, a high $\Delta\psi$ m is signified by a yellow-green

fluorescence (collected at 450 nm), while a low $\Delta\psi$ m is signified by a red fluorescence collected at 625 nm. Results in the current study showed that Rh 123 selectively stains the sexual structures i.e. conidia of *P. citricarpa* and ascospores for *G. mangiferae* respectively (Figure 4.1 and 4.2). Based on these results it is evident that conidia of *P. citricarpa* and ascospores *of G. mangiferae* possess increased mitochondrion activity when compared to hyphae. It would be of value to explore the use of antifungal anti-mitochondrial compounds including essential oils in combating *P. citricarpa* since it depends on these structures for development and dispersal. Furthermore if EO's can target ascospores development in *G. mangiferae*, it is believed that similar results can be expected against ascospores produced by *P. citricarpa in vivo* since this organism does not produce ascospores on agar media to enable *in vivo* screening. Literature indicates that the antimicrobial properties of essential oils depend mainly on their composition (Prakash *et al.,* 2015 and Bakkali *et al.,* 2008), it is thus essential to characterize the oils in order to elucidate their mode of action against fungi. EO's properties were analysed using the using Gas Chromatography Mass Spectrometry (GC-MS).
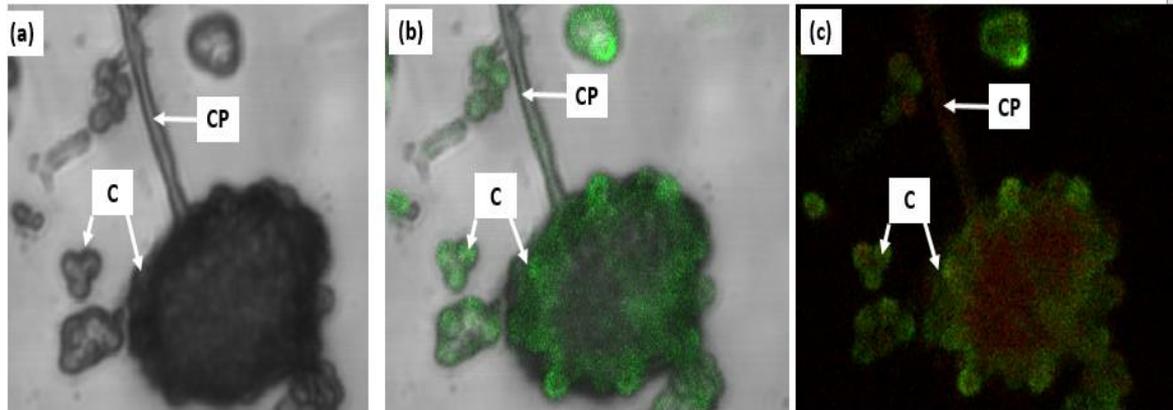
Figure 4.1. Confocal laser Scanning Micrographs of *Phyllosticta citricarpa* stained with Rhodamine (Rh) 123. (a) Light Micrographs showing cluster conidia. (b) Immunofluorescence superimposed on corresponding light micrograph of cluster conidia. (c) Only immunofluorescence micrograph of cluster conidia. C, conidia; CP, conidiophores; a high $\Delta\psi$ m is signified by a yellow-green fluorescence (collected at 450 nm), while a low $\Delta\psi$ m is signified by a red fluorescence collected at 625 nm.
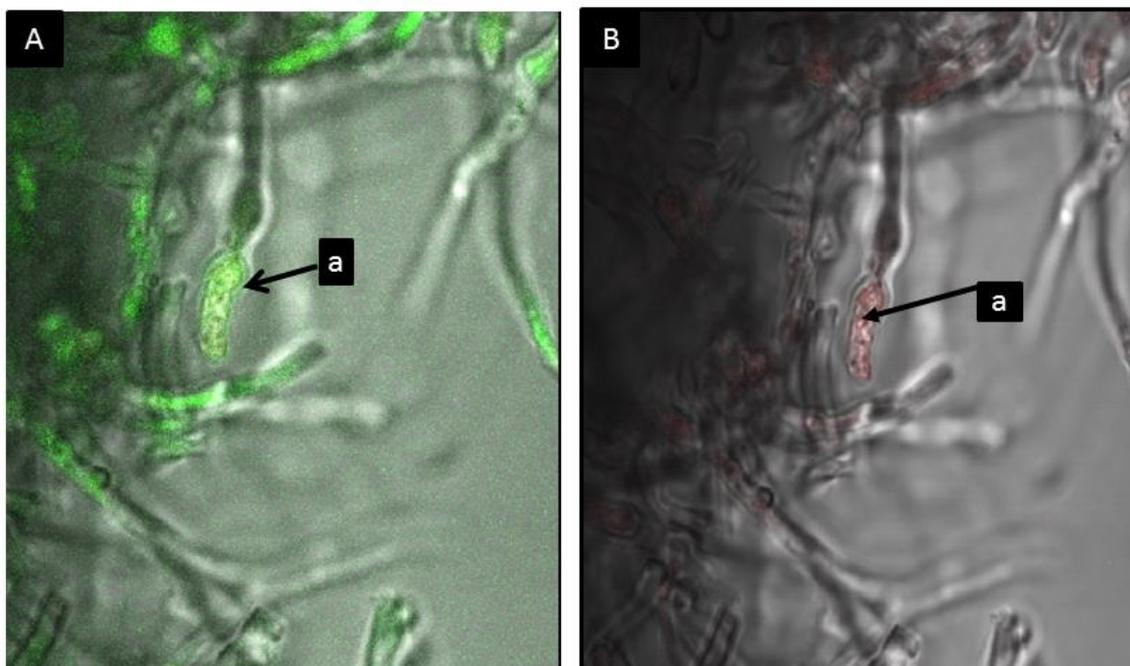
Figure 4.2. Confocal laser Scanning Micrographs of *Guignardia mangiferae* stained with Rhodamine (Rh) 123. (a) Light Micrographs showing stained ascospores. (b) Normal light micrograph of stained ascospores. a, ascospores; a high $\Delta\psi$ m is signified by a yellow-green fluorescence (collected at 450 nm).

4.2 Essential oil composition

To elucidate the mode of action of essential oils, it is important to characterize the oils in order to evaluate compounds that bequeath essential oils their antimicrobial properties. Table 4.1 shows the chemical composition of the six studied EO's analysed using Gas chromatography mass spectrometry (GC-MS). The analysis revealed the presence of terpenes and/or terpenes derivatives in almost all the essential oils, as the main compounds. Thyme oil (*Thymus vulgaris)* (i) and (ii), Geranium oil (*Pelargonium graveolens*), Citronella oil (*Cymbopogon nardus*), and Eucalyptus oil (*Eucalyptus globulus*) consisted mostly of terpenes as major compounds. Terpenes and their derivatives play a major role in the antimicrobial activities of essential oils (Lan-phin and Vy, 2015). On the other hand Lavender consisted of terpenes in trace amounts. Citronella essential oil consisted mostly of monoterpene hydrocarbons, mainly Limonene (14%), Citronellal (10.1%), ß-Pinene (13.1%), and α-Pinene (11.7%) as main

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compounds. The frequent occurrence of these as main components in Citronella have been previously recorded by Wany *et al.* (2013), they further indicated that the presence of these components bequeath this oil with the inhibitory effects against some fungus. Eucalyptus oil consisted of 1,8-Cineol (17.7%), Cyclopentane methyl (52.5%) and Hexane, 3-methyl (13.9%) as main constituents. Comparing these findings with those observed in a study conducted in Tunisia by (Marzoug *et al.,* 2011) important variations were noted, firstly the percentage of 1,8-cineole and Hexane, 3-methyl were lower than the current study. However the chemical composition can differ based on the origin of the plants, soil composition, plant organ, vegetative cycle phase and climate (Moussaoui *et al.*, 2013; Nazzaro *et al.,* 2013; Faleiro, 2011; Marzoug *et al.,* 2011). Though there were slight differences in the chemical composition, the antimicrobial effect of Eucalyptus was observed in both studies, this is due to the presence of oxygenated compounds such as 1,8-cineole (Marzoug, *et al.,* 2011).

Table 4.1: Chemical composition of the essential oils used in the study (Citronella, Eucalyptus, Rose Geranium, Lavender and Thyme oil).

Essential Oils plant (%)						
Compounds	Citronella	Eucalyptus	Lavender	Rose Geranium	Thyme i	Thyme ii
1,8-Cineol	-	17.7	-	-	-	-
2-Carene	1.7	-	-	-	-	-
Aristolene	-	-	--	2.7	-	-
Benzyl benzoate	9.9	-	-	-	-	-
Benzene, I methoxy-4methyl-2-(1methyl ethyl)	-	-	-	-	-	5.8
Borneol	-	-	-	-	0.5	-
Bisabolene	-	-	3.5		-	-
Bisabolol	-	-	1.6	-	-	-
Butanoic acid, 2 methyl, methyl ester	-	-		-	-	3.3

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			-		
1,3,8 p-Methatriene	-	-	-	-	-	5.0
Camphene	-	-	3.1	-	1.3	-
Caryophyllene	-	-	-	-	1.2	9.0
Caryophyllene oxide	-	-	-	-	1.4	2.3
Citronellal	10.1	-	-	-	-	
5-caranol, trans trans- (+)-	-	-	7.9	-	-	-
Citronellol	2.6	-	-	20.2	-	-
Citronellyl acetate	-	-	-	16.3	-	-
Citronyll tiglate	-	-	-	0.2	-	-
Cyclopentane 1,2 dimethyl	4.8	-	-	-	-	-
Cyclopentane methyl	-	52.5	3.5	-	-	-
Diphenyl ether	-	-	-	3.0	-	-
Geranoil	0.7	-	-	-	-	-
1.6 cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl) [S,(E,E)]	-	-	4.3	-	-	3.3
Farnesene	-	-	5.7	-	-	-
Geranyl tiglate	-	-	-	0.5	-	-
Heptane	-	1.8	-	-	-	-
Heptane-3-methyl	-	1	-	-	-	-
Hexane, 3-methyl	-	13.9	-	2	4.5	-
Hexane- 1- methoxy	-	-	0.5	-	-	-
4-Hexan-1-ol, 5 methyl-2-(1-methylethanyl) acetate	-	-	8.6	-	-	-
Isocryophillene	-	-	8.4	-	-	-
Limonene	14	-	-	-	-	-
Mesitlylene	-	1		-	-	
Naphthalene, 1.2.3.4.4a.5.6.7 octahydro-4a-methyl	-	-	-	-	-	8.5
Naphthalene 1.2.3.4.4a.5.6.7 octahydro-4a-methy	-	-	-	-	-	0.04
o-Xylene	-	1.4		-	-	

Pinocarvone	-	-		-	1.5	
Phenol-2methyl-5-(1-methylethyl)	-	-	-	-	-	7.6
1,6 Octadien-3-ol, 3.7 dimethyl	-	-	7.7	-	-	7.0
2,6-Octadin-1-ol, 3.7 dimethyl, acetate	-	-	3.0	-	-	-
ß-Pinene	13.1	-	-	1.9	-	
Pinene	-	-	-	-	-	4.4
Terpinene	-	-	-	-	3	
Thymol	-	-	-	-	32.1	
Toluene	2.2	1.7	-	1.3	-	-
(1R),2.6.6 Trimethylbicyclo[3.1.1]hept -2-ene	-	-	4.2	-	-	3.7
Tau cadinaol	-	-	-		-	0.5
α-Pinene	11.7	-	-	8.4	5.1	-
ρ-Cymene	-	-	-	-	20.4	-

Lavender oil *(Lavandula latifolia)* consisted of Isocryophillene (8.4%), 5-caranol, trans trans-(+)- (7.9%), 4-Hexan-1-ol, 5 methyl-2-(1-methylethanyl) acetate (8.6%), and 1,6 Octadien-3-ol, 3.7 dimethyl (7.7%) as the main components. These results are different from those reported in other studies (Jianu *et al.,* 2013), it is quite evident that the concentrations of 4-Hexan-1-ol, 5 methyl-2-(1-methylethanyl) acetate, Isocryophillene were slightly higher, in the present study. These variations could be due to differences in location, elevation, genetic makeup of the plant or due to an adaptive process to particular ecological conditions (Jianu *et al.,* 2013). Jianu *et al.,* 2013 also observed a wide variation in the quantitative composition of Lavender oil depending on plant genotype and cultivation area.

The chemical composition of Rose Geranium oil is presented in Table 4.1, Citronellol (20.2%), Citronellyl acetate (16.3%), and α-Pinene (8.4%) were the main constituents. Comparing the results observed in the present study with the study done by Dzamic *et al.* (2014), it can be seen in both studies that Rose Geranium consist of significantly high amounts of citronellol, though other constituents differ significantly. For example the current study has high chemical composition of α-Pinene and Citronellyl acetate when compared to the oil in the Džamić *et al.* (2014) study. Two types of Thyme oils obtained from different suppliers were used in the current study; they were referred to as Thyme (i) and Thyme (ii) respectively. Thyme oil (i) constituted of Thymol (32.1%), and ρ-Cymene (20.4%) as main compounds, there were other minor compounds detected in ranges between 0.5-5.1%. Thyme oil (ii) consisted of Caryophyllene (9.0%), Naphthalene, 1.2.3.4.4a.5.6.7 octahydro-4a-methyl (8.5%), Phenol-2methyl-5-(1-methylethyl) (7.6%) and 1,6 Octadien-3-ol, 3.7 dimethyl (7.0%) as main compounds. Other studies where Thyme was characterized (Elshafie *et al.,* 2015; Grigore *et al.,* 2010) indicated that Thyme oil consist of high amounts of p-cymene and its monoterpene phenol derivative thymol. Moreover, similar results were found in studies conducted in Brazil and India (Shabnum and Wagay, 2011; Porte and Godoy 2008). However, Thyme oil (ii) in this study differed from (Elshafie *et al.,* 2015; Grigore *et al.,* 2015; Shabnum and Wagay, 2011; Porte and Godoy 2008) findings, because neither Thymol, nor ρ-Cymene could be detected. These variations occur because of the difference in cultivation area (Jianu *et al.,* 2013).

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The difference in Thyme oil composition indicates that the two studied Thyme oils may possess different antifungal effects against the tests organism because antifungal properties of essential oil are a result of these components or the interplay between these compounds. Generally EO's have long been considered to possess anti-inflammatory properties and antimicrobial activity, with activity against fungi (Prakash *et al.,* 2015; Bakkali *et al.,* 2008). In fact other studies further reported that EO's can target structures with increased mitochondrial activity (Prakash *et al.,* 2015). Studies by (Ncango *et al.,* 2010; Leeuw *at al.,* 2009; Kock *et al.,* 2007) have indicated that structures with increased mitochondrial activity such as ascospores, conidia, asci, sporangia and phialides play an important role in the fungal life cycle and development. If these structures are inhibited they will probably limit the spread of the fungi since they can act as the prime source of infection (Kock *et al.,* 2007). Consequently, antifungal anti-mitochondrial activity of EO's oils, hydrosol and antifungal anti-mitochondrial compounds (positive controls) were tested using the agar diffusion bio-assay for *Phyllosticta citricarpa* and agar dilution bio-assay method for *Guignardia mangiferae*.

4.3 Antimicrobial activities of essential oils on *Phyllosticta citricarpa* and *Guignardia mangiferae*

The current study was done according to the findings of (Ncango *et al.,* 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007) which exposed that acetylsalicylic acid; benzoic acid and salicylic acid possess antifungal properties. However the current study not only proposed to assess antifungal properties of tested compounds but to also sought to elucidate the mode of antifungal action of all tested compounds. Studies have shown that compounds that inhibit fungal growth by first targeting development structures with increased mitochondrial activity could serve as an efficient strategy to reduce the spread of fungi (Ncango *et al.,* 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). The results of antifungal activity of the known antifungal anti-mitochondrial compounds, essential oils (EO's) and hydrosol, against *Phyllosticta citricarpa* obtained from the agar diffusion bio-assay are shown in Table 4.2 and Figures 4.3, 4.4 and 4.5. When antifungal properties of known antifungal anti-mitochondrial compounds, essential oils (EO's) and hydrosol were assessed in a bio-assay using *Phyllosticta citricarpa* as test

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organism, three distinctive zones were observed on a plate, an inhibition-zone (i) where growth was completely inhibited, asexual zone (a) without sexual stages and maximum growth zone with both asexual and sexual zones (m).

The antifungal activity of the known antifungal anti-mitochondrial compounds and essential oils (EO's) against *Guignardia mangiferae* analysed using agar dilution method resulted in different growth zone diameter (Table 4.3 and Figures 4.6 and 4.7). A small growth zone diameter indicates high inhibition of the fungus *G. mangiferae*. In *Phyllosticta citricarpa* antifungal anti-mitochondrial compounds showed antifungal activity, here Benzoic acid [BA] appear to have produced the largest inhibition zone (20mm), while Salicylic acid [SA], produced second largest inhibition zone (10mm), with Acetylsalicylic acid [ASA] producing a smaller inhibition zone (3 mm) in comparison to other anti-mitochondrial compounds assessed (Table 4.2 and figure 4.3). These observations are in line with the findings by Kock *et al.* (2007) that acetylsalicylic acid; benzoic acid and salicylic acid possess antifungal properties. Results from other studies have indicated similar findings that known antifungal anti-mitochondrial compounds have potential antifungal activities against pathogenic fungi; these potential antifungal activities are believed to be associated with conidia and ascospores (Ncango *et al.,* 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). Furthermore, (Yong *et al.,* 2016; Colombo *et al.,* 2011; Di Bonaventura *et al.*, 2006) believe that this interaction results in changes in prostaglandin production, membrane potential, and reduction of extracellular polysaccharide leading to the cell death. In good agreement with previous studies showing that known antifungal anti-mitochondrial compounds (Panahirad *et al.,* 2014; Qi *et al.,* 2012; Trofa *et al.,* 2009; Leeuw *et al.,* 2009, 2007; Wu *et al.,* 2008; Sebolai *et al.,* 2008; Meyer *et al.,* 2006; Cory and Cory, 2005; Amborabé *et al.,* 2002) can directly impede growth in several fungal species, the present study documents that antifungal anti-mitochondrial compounds have potential inhibitory effects against *Phyllosticta citricarpa.*

Antifungal activity of essential oils is shown on Table 4.2 and Figure 4.4 determined using the agar diffusion Bio-Assay. Growth inhibition zone diameters were also measured; three distinctive zones similar to those observed using antifungal anti-mitochondrial compounds were detected, the results showed that essential oils

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influenced growth of *Phyllosticta citricarpa* negatively. Among all oils tested, Thyme oil (*Thymus vulgaris)* (i) produced the highest inhibition zone moreover; essential oils resulted in significant inhibition of the fungus *P. citricarpa* when compared to other tested compounds. Thyme oil (i) exhibited excellent antimicrobial activity against this fungal pathogen with the inhibition zone 55mm. In addition, Geranium and Citronella produced the second largest inhibition zones with a diameter of 17 mm and 14 mm respectively.

However, Lavender oil *(Lavandula latifolia*) and Eucalyptus oil (*Eucalyptus globulus*) when compared with the other essential oils did not display inhibition zones (Figure 4.4). The different varieties of Thyme oil (i and ii) produced different diameter size inhibition zones, the two varieties of the Thyme oils possessed different antifungal effects against the test organism probably due to the differences in chemical composition (Table 4.2 and Figure 4.4). Several authors (Moussaoui *et al*., 2013; Nazzaro *et al.,* 2013; Faleiro, 2011) indicated that factors such as soil composition, plant organ, vegetative cycle phase and climate influence the quality and quantity of the extracted EO's, which was probably the case with the two EO's.

Furthermore hydrosol used in the current study showed antifungal effects against the test organism with Rosemary hydrosol (*Rosmarinus officinalis*) and Thyme oil hydrosol indicating the highest inhibition zone of 15 mm and 10 mm respectively (Table 4.2 and Figure 4.5). However Lavender hydrosol and Lengana (*Artemisia afra)* hydrosol showed no antifungal effect against the test organism (Table 4.2 and Figure 4.5). These results indicate that hydrosol can be used as antifungals against CBS, with the advantage over essential oils of being water soluble, cheaper and consisting of EO's traces (Nazzaro *et al.,* 2013). The ethanol used as negative control indicated no significant inhibition zone in diameter; it is therefore concluded that ethanol alone had no effect against *P. citricarpa* the causative agent of CBS. Subsequently antifungal activity of the known antifungal anti-mitochondrial compounds and essential oils (EO's), against *Guignardia mangiferae* was assessed using the agar dilution method.

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Agar dilution bio-assay analysis of the antifungal activity of the known antifungal anti-mitochondrial compounds revealed that all the known antifungal anti-mitochondrial compounds possess inhibitory effects against the test organism varying in growth diameter from 2 mm up to 15 mm (Table 4.3 and Figure 4.6). This was done by mixing Potato Dextrose Agar (PDA) with EO's and antifungal anti-mitochondrial compounds respectively and inoculate *G. mangiferae* to observe the growth in the presence of each EO's and known antifungal anti-mitochondrial compounds respectively after incubation. These results are in good agreement with previous studies done by (Dieryckx *et al.,* 2015; Ncango *et al.*, 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007) that known antifungal anti-mitochondrial compounds can directly inhibit fungal growth. Similar results were also obtained from a study done by Dieryckx *et al.* (2015) were known antifungal anti-mitochondrial compounds were shown to inhibit the growth of fungal pathogen *Botrytis cinerea* through penetration into the fungal cells. Interestingly, several studies further reported that in addition to *in vitro* inhibition of microorganisms, known antifungal anti-mitochondrial compounds can also inhibit *in vivo* the growth of various microorganism in interaction with the plants, resulting in alteration of mitochondrial respiration (Carviel *et al.,* 2009; Cameron and Zaton, 2004). Based on these results it was also concluded that known antifungal anti-mitochondrial compounds used in the study possess antifungal activity against *G. mangiferae.*

Antifungal activity of essential oils determined using the agar dilution method is shown on Table 4.3 and Figure 4.7. Growth zone diameters were measured; with growth zones similar to those observed using antifungal anti-mitochondrial compounds were also detected, the results showed that essential oils influenced growth of *G. mangiferae* negatively. Among all oils tested, Thyme oil (*Thymus vulgaris)* (i and ii) presented the smallest growth zone, which resulted in significant inhibition of the fungus *G. mangiferae*. Thyme oil (i and ii) exhibited excellent antimicrobial activity against this fungus with smallest growth zone of about 2 mm in diameter. In addition, Citronella produced the second smallest growth zones with a diameter of 20 mm. In addition Geranium oil produced the third smallest growth zone of about 70 mm. Results from the current study are in agreement with a study done by Naidoo, (2007) that EO's can act as chemical defence against plant pathogenic microorganism, this was proven in the current study through bio-assay analysis. The existence, of

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antimicrobial activity in the EO's, would be of considerable benefit to the plant considering the large number of chemical composition of the oils (Svoboda and Hampson, 1999). It is therefore most likely that the antifungal properties are attributed to different mechanisms; therefore fungal resistance might not be a problem (Carson *et al.*, 2002; Skandamis *et al.*, 2001).

However, Eucalyptus oil (*Eucalyptus globulus*) and Lavender oil *(Lavandula latifolia)* when compared with the other essential oils have shown to possess largest growth zones of 85 mm and 90 mm respectively (Fig 4.7). This is an indication that not all EO's can inhibit fungal growth, a study conducted by Naidoo, (2007) also showed that some EO's are not capable of inhibiting fungal pathogens, where EO's from *Cymbopogon validus* failed to inhibit *Candida albicans* and *Aspergillus flavus.* The different varieties of Thyme oil (i and ii) produced similar growth diameter, thereby indicating similar antifungal properties against the test organism even though their chemical properties were different (Table 4.3 and Figure 4.7). Although inferences about oil composition can be made from the plant species name, oil composition can be influenced by environmental factors, meaning that two identical plants grown under different conditions are unlikely to produce identical oils which might have been the case in this study regarding the Thyme oils used.

Ethanol (negative control) indicated significant highest growth zone of 90 mm in diameter; it was concluded that ethanol alone had no effect against *G. mangiferae.* These results are in agreement with studies done by (Ncango *et al.*, 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007) respectively where ethanol alone did not show any inhibitory effect against the different tested fungal organism. Detailed microscopic analysis of *P. citricarpa a*nd *G. mangiferae* treated with EO's was then conducted using the scanning electron microscope in order to determine the effects of the EO's on the conidia and ascospores respectively and thereby elucidate the mode of action of the tested compounds.

Table 4.2 Bio-assays of *Phyllosticta citricarpa* showing effects of different known antifungal anti-mitochondrial, Essential oils and hydrosol compounds measured in mm.

| Compounds tested, EO's and Hydrosol	Inhibition zone(mm)
Benzoic acid (BA)	20
Salicylic acid (SA)	10
Acetylsalicylic acid (ASA),	3
Thyme (*Thymus vulgaris)* oil (i)	55
Geranium (*Pelargonium graveolens*)	17
Citronella(*Cymbopogon nardus*)	14
Thyme oil (ii) (*Thymus vulgaris*)	15
Eucalyptus oil (*Eucalyptus globulus*)	0
Lavender (*Lavandula latifolia*) oil	0
Rosemary (*Rosmarinus officinalis*) hydrosol	15
Thyme (*Thymus vulgaris)* oil Hydrosol	10
Lengana (*Artemisia afra*) Hydrosol	0
Lavender (*Lavandula latifolia*) Hydrosol	0
Ethanol (Control)	0
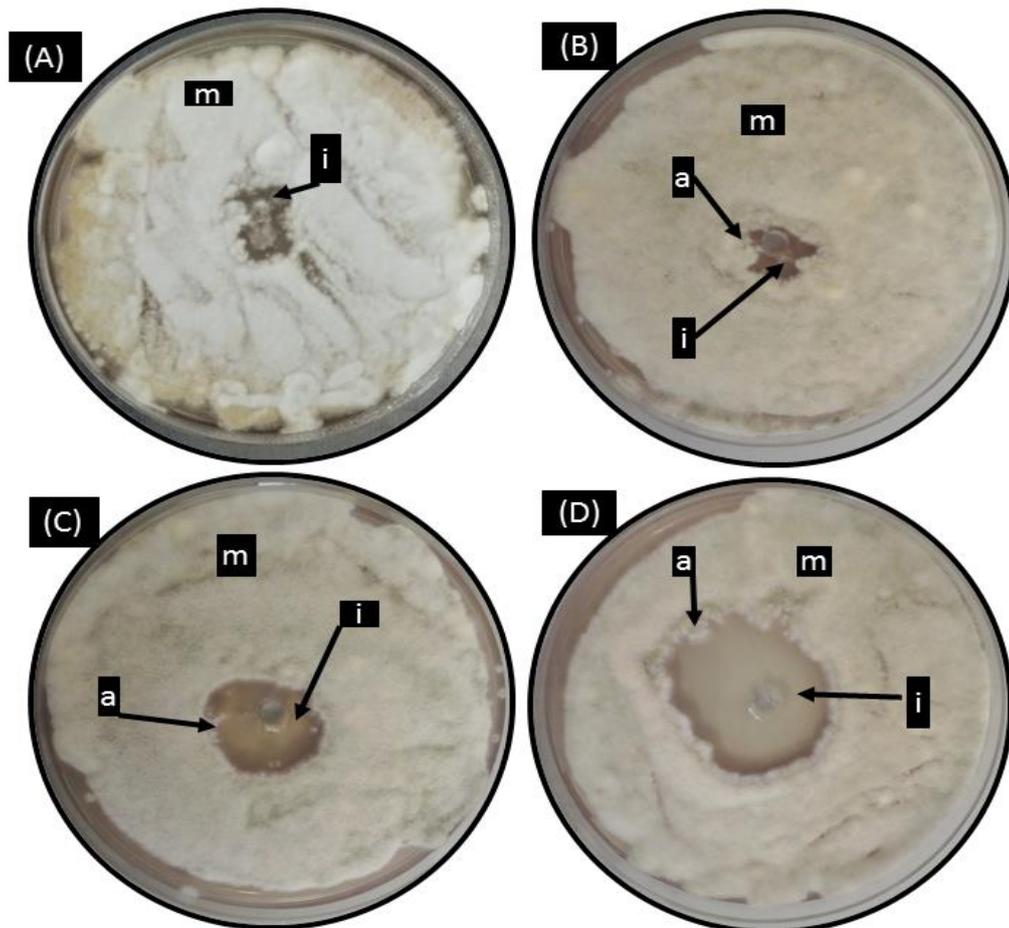
Figure 4.3. Bio-assays of *Phyllosticta citricarpa* showing effects of different known antifungal anti-mitochondrial compound. (A) Ethanol (control), (B) Acetylsalicylic acid [ASA], (C) Salicylic acid [SA] and (D) Benzoic acid [BA]. i,- Inhibition zone; a - asexual zone; m- maximum growth zone.
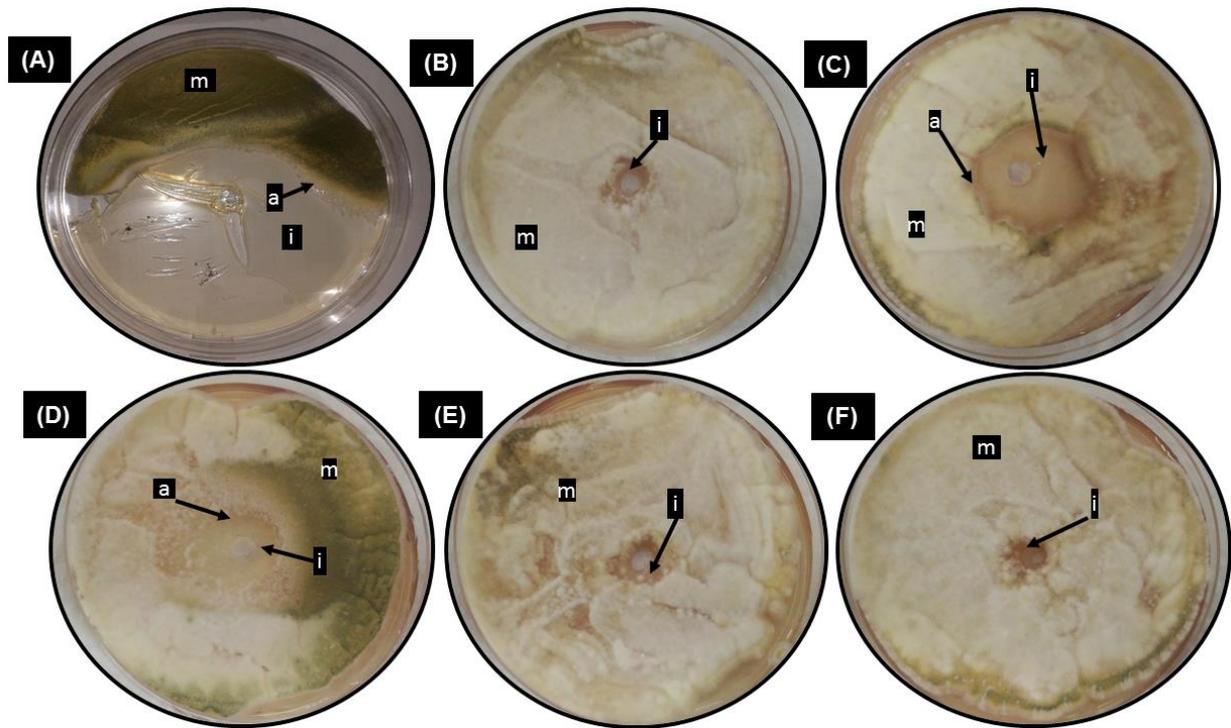
**Figure 4.4.** Bio-assays of *Phyllosticta citricarpa* indicating effects of different essential oils. (A) Thyme oil (*Thymus vulgaris)* i, (B) Geranium oil (*Pelargonium graveolens*), (C) Citronella oil (*Cymbopogon nardus*), (D) Thyme oil (*Thymus vulgaris)* ii, (E) Lavender oil *(Lavandula latifolia*) and (F) Eucalyptus oil (*Eucalyptus globulus*). i,-Inhibition zone; a - asexual zone; m- maximum growth zone.
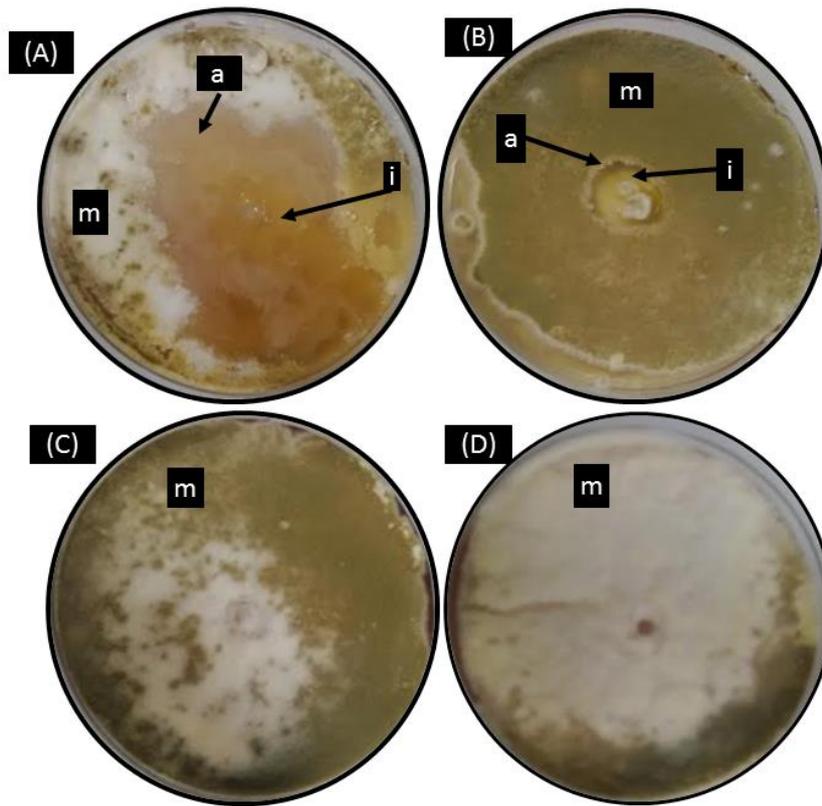
Figure 4.5. Bio-assays of *Phyllosticta citricarpa* indicating effects of different Hydrosol. (A) Rosemary (*Rosmarinus officinalis*) hydrosol, (B) Thyme oil (*Thymus vulgaris)* hydrosol, (C) Lengana (*Artemisia afra*) Hydrosol and (D) Lavender *(Lavandula latifolia*) Hydrosol. Growth inhibition-zone (i), to asexual zone (a) to maximum growth zone (m).

Table 4.3 Bio-assays (agar dilution method) of *Guignardia mangiferae* showing effects of different known antifungal anti-mitochondrial and Essential oils measured in mm**.**

| Compounds tested and EO's	Inhibition zone(mm)
Benzoic acid (BA)	2
Acetylsalicylic acid (ASA),	2
Salicylic acid (SA)	15
Thyme (*Thymus vulgaris)* oil (i)	2
Thyme oil (ii) (*Thymus vulgaris*)	2
Citronella(*Cymbopogon nardus*)	20
Geranium (*Pelargonium graveolens*)	70
Eucalyptus oil (*Eucalyptus globulus*)	85
Lavender (*Lavandula latifolia*) oil	90
Ethanol (Control)	90
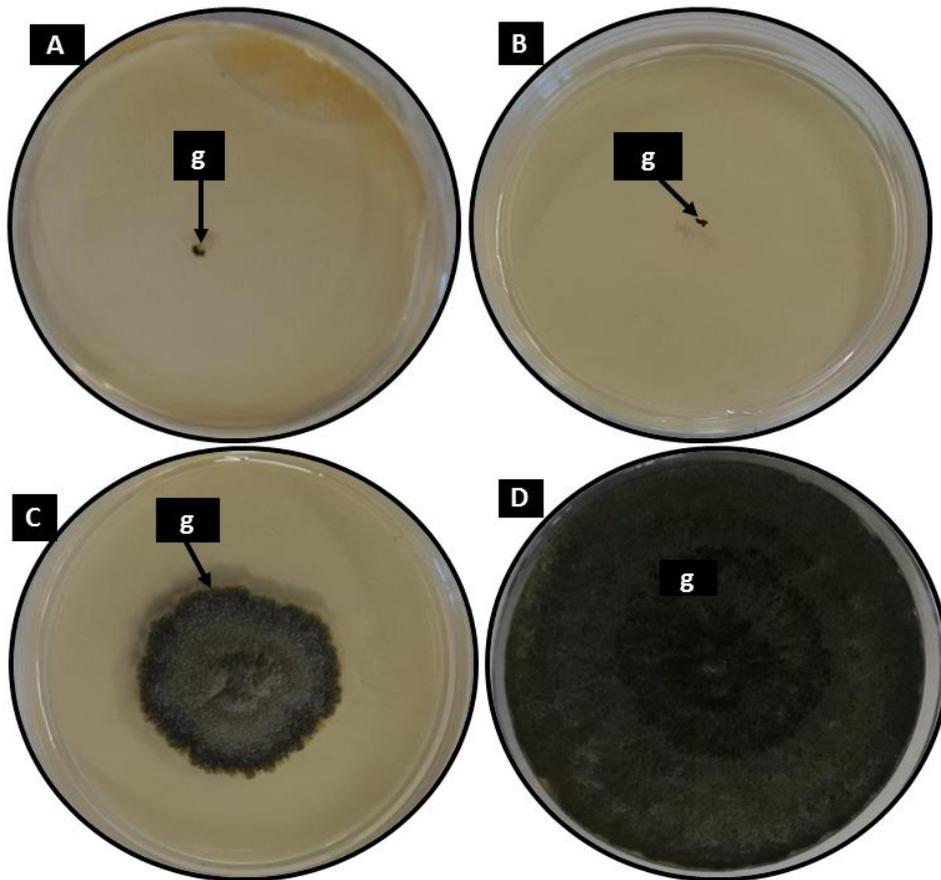
Figure 4.6. Bio-assays of *Guignardia mangiferae* showing effects of different known antifungal anti-mitochondrial compound. (A) Acetylsalicylic acid [ASA], (B) Benzoic acid [BA], (C) Salicylic acid [SA] and (D) Ethanol (control). (g) Growth zone.
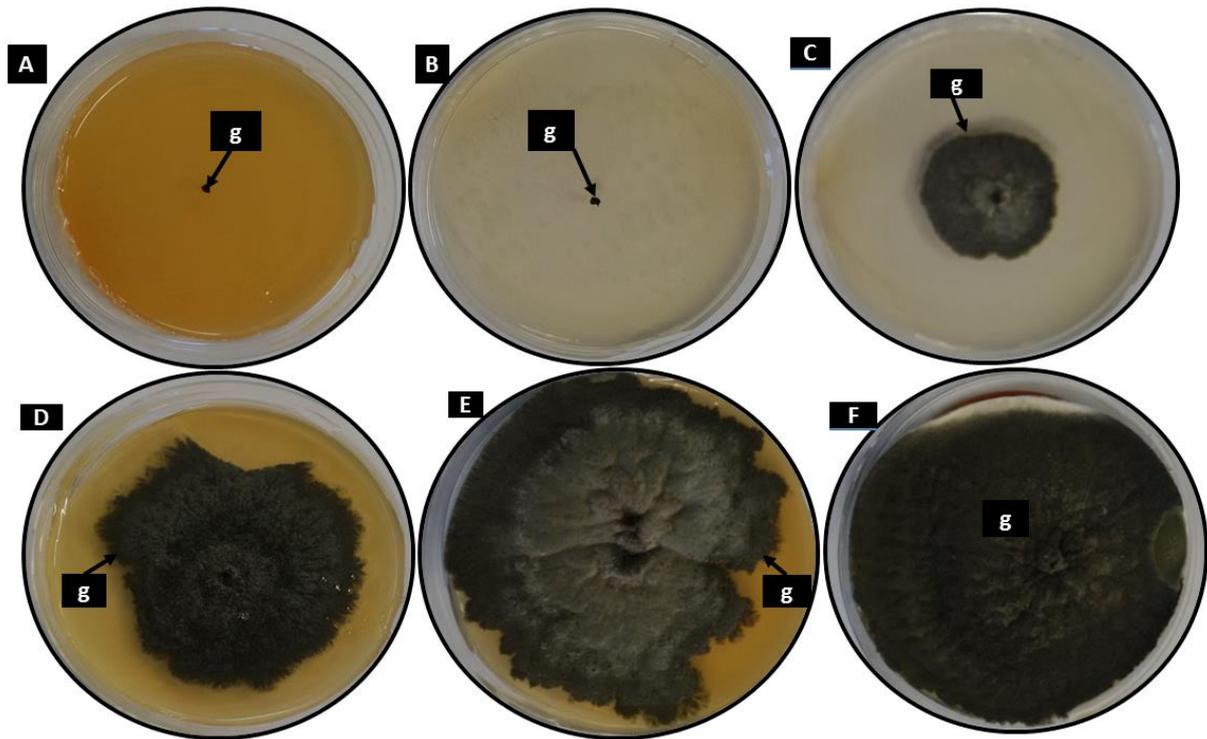
Figure 4.7. Bio-assays of *Guignardia mangiferae* indicating effects of different essential oils. (A) Thyme oil (*Thymus vulgaris)* i, (B) Thyme oil (*Thymus vulgaris)* ii, (C) Citronella oil (*Cymbopogon nardus*), (D) Geranium oil (*Pelargonium graveolens*), (E) Eucalyptus oil (*Eucalyptus globulus*) and (F Lavender oil *(Lavandula latifolia*). (g) Growth zone.

4.4 Scanning Electron Microscopy (SEM) of *Phyllosticta citricarpa* a**nd** *Guignardia mangiferae*

Previous studies have shown that *Phyllosticta citricarpa* does not form perithecia with ascospores on the agar media, although it is able to produce them on the field from fallen leaves. However its counterpart *Guignardia mangiferae* is able to produce ascospores on the agar media (Baayen *et al.,* 2002). As expected, this was also observed in the current study. *P. citricarpa* produced conidia from structures called conidiophores, while *G. mangiferae* produced ascospores (Haleeondo, 2008; Baayen *et al.,* 2002). This section investigated whether essential oils (EO's) can target conidia of *P. citricarpa* using scanning electron microscopy (SEM). When essential oils (EO's)

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were applied to the agar diffusion bio-assays, three distinctive zones were observed across the concentration gradient (Figures 4.4); an inhibition-zone (i) where growth was completely inhibited, asexual zone (a) without sexual stages and maximum growth zone with both asexual and sexual zones (m). Morphology of treated and untreated cells was assessed using scanning electron microscopy (Figure 4.8 A-F). Analysis of the area towards inhibition zone using scanning electron microscopy indicated that conidia structures were completely inhibited by some essential oil at a high concentration gradient, whereas some EO's did not inhibit conidia structures at a high concentration gradient. In Figure 4.8 A and B (Thyme oil (i) and Thyme oil (ii) inhibited conidia structures with increased mitochondrial activity completely, indicating the antifungal anti-mitochondrial properties of these oils. Additionally, the oils affected the morphology of hyphae (Fig 4.8 A-B), hyphae appeared granular and wrinkled. Based on these findings it can be said that Thyme oil (i) and (ii) can inhibit part of *P. citricarpa* life cycle by targeting conidia structures. Furthermore it is believed that this occurs because Thyme oil (i) and Thyme oil (ii) consist of high amount of terpenes which play an important role in plant defence mechanism against pathogenic fungi (Nazzaro *et al.,* 2013; Martínez, 2012; Liu *et al.*, 2009). Interestingly, Thyme oil (ii) did not have large inhibition zone on the bio-assay findings but it showed a negative effect against conidia. Figure 4.8 C, D and E (Citronella, Geranium and Eucalyptus oil) indicated no effect on conidia structures, shown by the high amount of conidia and conidiophores, unlike Thyme (i) and (ii) which completely inhibited these structures.

These findings indicate that Geranium, Citronella and Eucalyptus do not possess anti-mitochondrial properties against the test organism, although they possess antifungal properties. Figures 4.8 F (Lavender oil) also showed to possess no inhibitory effect against the test organism, because of numerous conidiophores structures present. This indicates that Lavender oil does not possess anti-mitochondrial effects against the test organism; moreover, Lavender did not show any antifungal effects through observation of the bio-assay results. However these results were not surprising as Lavender oil does not have terpenes as major constituent. Ethanol (Figure not shown) used as the control did not have any antifungal anti-mitochondrial properties against the test organism, indicated by a high amount of conidia thereby concluding that ethanol does not have any effect on conidia structure of *Phyllosticta citricarpa*. Similar

tests were also done on *Guignardia mangiferae* however these time ascospores were targeted because of the properties of this organism when grown on agar media.

Presence of conidia is known not to occur on the agar media for *Guignardia mangiferae* (Haleeondo, 2008; Baayen *et al.,* 2002)*.* This was also the case in the current study, since only ascospores could be discerned by microscopic analysis. After essential oils (EO's) were applied to the agar dilution bio-assays of *Guignardia mangiferae*, detailed microscopic analysis was done using scanning electron microscopy (Figures 4.9). In Figure 4.9 A and B (Thyme oil (i) and Thyme oil (ii)) inhibited ascospores structures with increased mitochondrial activity completely indicating the antifungal anti-mitochondrial properties of these oils. Based on these findings it can be also said that Thyme oil i and ii can inhibit *G. mangiferae* by targeting ascospores structures. Figure 4.9 C and D (Citronella and Geranium indicated similar results as Thyme oil (i) and (ii) on the effect of ascospores structures, which is shown by the high amount of hyphae cell with no ascospores present, thereby completely inhibited ascospores structures. This is an indication of anti-mitochondrial effect of these EO's.

However, (Figures 4.9 E and F) Eucalyptus and Lavender oil possess no inhibitory effect against the test organism, because of numerous amounts of ascospores structures that were present. This indicates that Eucalyptus and Lavender do not possess anti-mitochondrial effects against the test organism; moreover, Lavender did not show any antifungal effect from the bio-assay results. Ethanol (Figure not shown) used as the control did not have any antifungal anti-mitochondrial properties against the test organism, and this is indicated by a high amount of ascospores. It is therefore concluded that ethanol does not have any effect on ascospores structure *G. mangiferae*.

Based on the results from scanning electron microscopy for *Phyllosticta citricarpa* and *Guignardia mangiferae* it is evident that both the conidia and ascospores can be targeted by some essential oil (EO's), thereby indicating the antifungal anti-mitochondrial properties of some EO's. These occur through the inhibition of enzyme

systems including those involved in energy production in a cell and synthesis of structural components such as mitochondrion dehydrogenases enzyme thereby decreasing metabolic state of the cells, leading to cell death (Nazzaro *et al.,* 2013; Martínez, 2012; Liu *et al.*, 2009). The current study has shown that essential oils (EO's) can be used as alternative antifungal anti-mitochondrial agents against *Phyllosticta citricarpa* and *Guignardia mangiferae* determined using Bio-Assay, (agar dilution and agar diffusion) and scanning electron microscopy. Results in the current study also showed that spore-release-structures such as conidia and ascospores with increased mitochondrion activity are more sensitive to mitochondrial inhibitors including EO's compared to vegetative cells and hyphae analysed using scanning electron microscopy (SEM) (Ncango *et al.*, 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). This may be of value in combating fungi that depend mainly on these structures for dispersal.
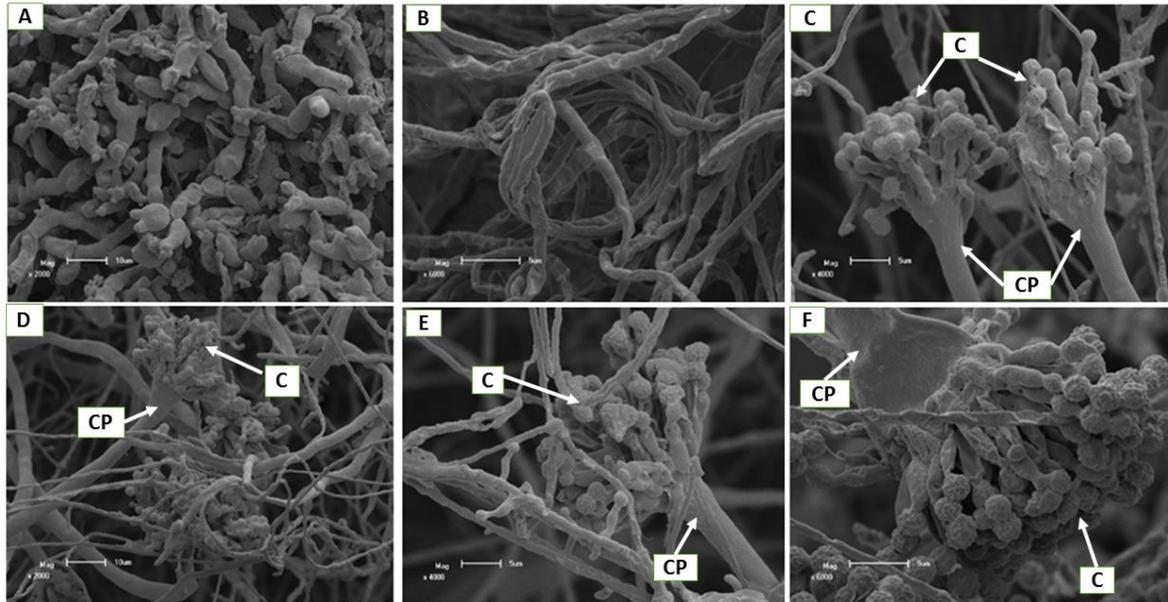
Figure 4.8. Detailed Scanning Electron microscopy analysis. *(A Phyllosticta citricarpa* Cell treated with Thyme oil (*Thymus vulgaris)* i. *(B P. citricarpa* cell treated with Thyme oil (*Thymus vulgaris)* ii. *(C P. citricarpa* Cell treated with Citronella oil (*Cymbopogon nardus*). *(D P. citricarpa* Cell treated with Geranium oil (*Pelargonium graveolens*). *(E P. citricarpa* Cell treated with Eucalyptus oil (*Eucalyptus globulus*). *(F P. citricarpa* Cell treated Lavender *(Lavandula latifolia*). *C, conidia; CP, conidiophores.* All treated cells were scraped from minimal growth zone in bio-assay (agar diffusion method) plate.
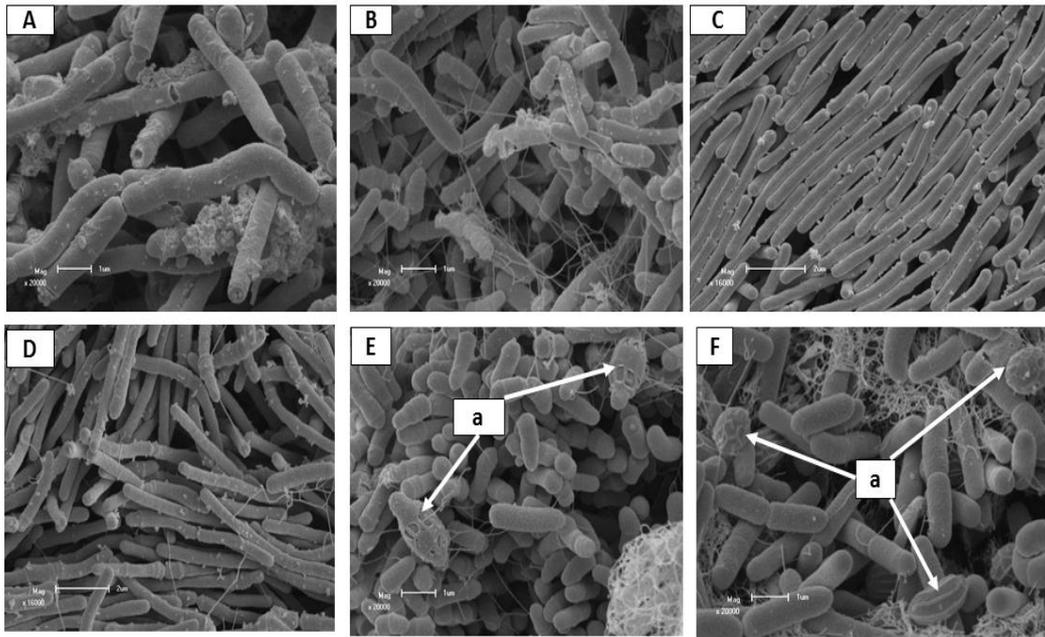
Figure 4.9. Detailed Scanning Electron microscopy analysis. *(A Guignardia mangiferae* Cell treated with Thyme oil (*Thymus vulgaris)* i. *(B G. mangiferae* cell treated with Thyme oil (*Thymus vulgaris)* ii. *(C G. mangiferae* Cell treated with Citronella oil (*Cymbopogon nardus*). *(D G. mangiferae* Cell treated with Geranium oil (*Pelargonium graveolens*). *(E G. mangiferae* Cell treated with Eucalyptus oil (*Eucalyptus globulus*). *(F G. mangiferae* Cell treated Lavender *(Lavandula latifolia*). *a, ascospore*; h, hyphae).

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4.5 Quantitative measurement of metabolic state

Electron scanning microscopy indicated that conidia of *Phyllosticta citricarpa* and the ascospores of *Guignardia mangiferae* also shown to possess increased mitochondrial activity are affected by the essential oils. It was therefore important to determine the effect of the antimicrobial compounds on the activity of mitochondrion dehydrogenases, an indicator of metabolic activity. This was done using XTT (a tetrazolium salt) colorimetric assay. The results of *Phyllosticta citricarpa*, mitochondrial dehydrogenase activity at different growth zones (asexual and maximum growth zone) (Figure 4.10) and *Guignardia mangiferae*, mitochondrial dehydrogenase activity on cells treated with Thyme oil (i) and untreated (Figure 4.11) were observed. Here, XTT (tetrazolium salt) was cleaved by various mitochondrial dehydrogenase enzymes to produce a coloured formazan product, which indicates fungal metabolic activity. For *P. citricarpa* as expected, the maximum growth zone contained increased mitochondrial dehydrogenase activity compared to asexual zone. *P. citricarpa* cells treated with Thyme oil (i) showed significantly higher ($p < 0.001$) mitochondrion activity in the maximum growth zone (0.96 ± 0.01 measured at 492 nm) compared to the asexual zone (0.46 ± 0.01 measured at 492 nm) (Figure 4.10).Similarly, the untreated *G. mangiferae* contained increased mitochondrial dehydrogenase activity compared to treated cells. *G. mangiferae* cells treated with Thyme oil (i) ($p < 0.001$) mitochondrion activity (0.71 ± 0.01 measured at 492 nm) compared to untreated (0.82 ± 0.01 measured at 492 nm) (Figure 4.11).This is an indication of the effect of essentials oils on structures with elevated mitochondrial activities when compared to vegetative cells and hyphae. This study further supports previous studies which have shown that spore-releasing-structures such as yeast asci, sporangia and phialides with increased mitochondrion activity are more sensitive to mitochondrial inhibitors when compared to vegetative cells and hyphae (Ncango *et al.*, 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007). Structures with elevated mitochondrial activity play an important role in the life cycle of *P. citricarpa* especially for the dispersal of the organism*.*
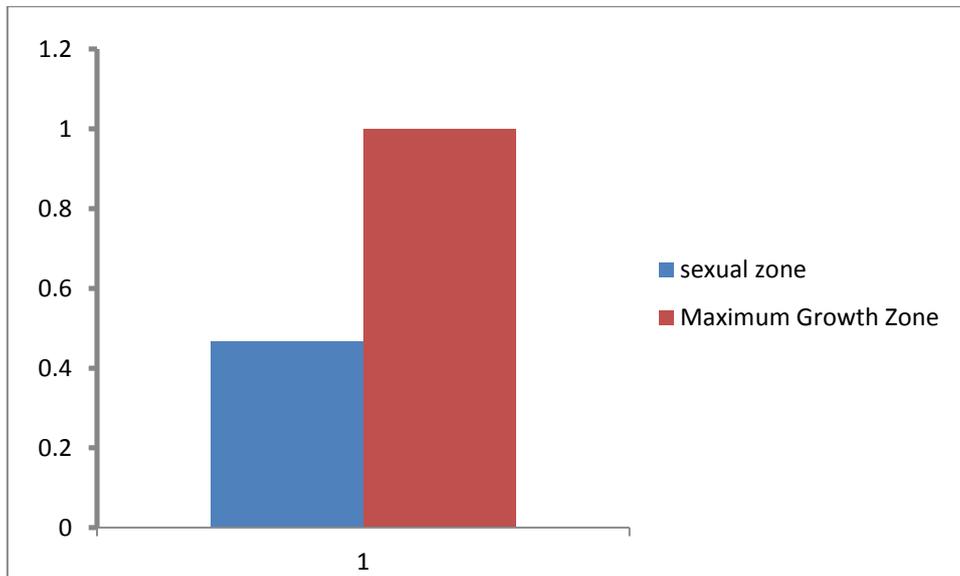
Figure 4.10. Results of the XTT assay studies performed on different growth zone (asexual and maximum growth zone) of *Phyllosticta citricarpa* treated with (Thyme oil i).
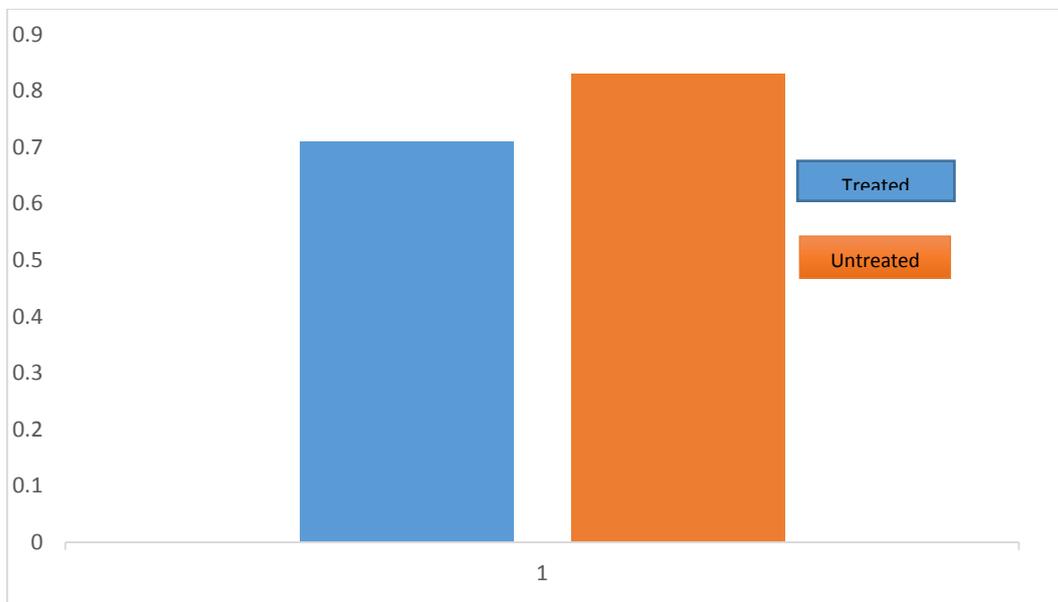

Figure 4.11. Results of the XTT assay studies performed on Treated (Thyme oil i) and untreated cells of *Guignardia mangiferae*.

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CHAPTER FIVE

CONCLUSION AND FUTURE

STUDIES

CONCLUSIONS AND FUTURE STUDIES

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5.1 Conclusions and Future Studies

Phyllosticta citricarpa is the causal agent of Citrus Black Spot (CBS) and is capable of infecting economically important citrus fruits (Hendricks *et al.,* 2013; McMillan, 1986). The disease was initially described over 100 years ago in Australia and has subsequently been found in Africa, Asia, South America, and New Zealand. *P. citricarpa* does not affect the quality of the fruit, meaning that the fruits are still suitable and safe for consumption; however in areas where the disease is endemic untreated citrus groves have been reported to experience up to 80% yield loss due to fruit drop. The economic impact due to the disease either through fruit drop or rejection for the fresh market because of the fruit lesions or potential quarantine of fruit sales to other citrus production countries could be estimated in the hundreds billions of Rands. The South African citrus industry alone plays an important role in job creation employing about 100 000 workers, marking South Africa the second largest exporter of citrus in the world.

However the incidence of citrus black spot (CBS) has continued to increase in citrus production areas, with severe infections resulting in premature fruit drop. In addition, asymptomatic fruit at harvest may still develop symptoms during transport or in storage. This has resulted in imposition and implementation of phytosanitary restrictions on citrus imported to the EU countries from countries in which CBS is known to occur. This is a major problem, especially in Southern Africa. As a result many in South Africa relying on this traded industry are seriously compromised because CBS infections result in huge job losses and drop in profits and a negative effect on the economy of the country.

Synthetic fungicides have long been used to control invading fungi, for example copper base fungicides such as Bordeaux mixture. Bordeaux mixture is a colloidal suspension created by mixing a solution of copper sulphate ($CuSO_4.5H_2O$) with a suspension of calcium hydroxide (hydrated lime; $Ca(OH)_2$) (Schwinn and Margot, 1991). Bordeaux mixture is an outstanding fungicide that has been used for 150 years to control some fungal diseases of tree fruits and nuts, vine fruits, and ornamentals. The ability of Bordeaux mixture to weather the fall, winter, and spring rains and to adhere to plants

makes it an excellent choice. Furthermore they have the advantages of good persistence and acting by targeting and denaturing enzymes of respiratory pathway in the conidia and ascospores (Schwinn and Margot, 1991). Other synthetic fungicides such as Dithiocarbamates are also commonly used. Dithiocarbamates an organic sulphur compounds which act by also targeting conidia and ascospores germination with the advantage of non-phytotoxic and low cost over Bordeaux mixture (Fernández, 2002). However both Bordeaux mixture and Dithiocarbamates are said to be toxic to plants and other organisms at increased levels. Furthermore, Bordeaux mixture has been found to be harmful to fish, livestock and result in a potential buildup of copper in the soil resulting in non-targeted soil organisms.

Literature also reported that Bordeaux mixture is toxic to both birds and mammals (including humans) particularly when misused (Martínez, 2012). For many years synthetic fungicides played a very important role in plant protection against fungal disease. However, the chemical residues in synthetic fungicides are known to remain on the plant or within its tissues following fungicidal treatment. Some chemical residues are able to enter nearby water sources and cause detrimental effect in the aquatic environment during run-off. These fungicide residues pose a great health risk to the consumer, environment and to the aquatic life, resulting in carcinogenic effects to human. Moreover resistance to currently available synthetic fungicides is also more prevalent resulting in the search for safe alternatives to synthetic fungicides (Ncango *et al.*, 2010; Mircus *et al.,* 2009).

The detrimental effect of synthetic fungicides on the environment, human and development of fungal resistance have intensified the search and development for alternative strategies. To overcome the disadvantages associated with synthetic fungicides, new ideal alternative methods to control Citrus black spot should be implemented. These should improve on the current available methods, meaning it should be easy to implement and should not have negative impact on the fruits, the environment and human health (Romanazzi *et al.,* 2012). This means that alternative fungicides should be biodegradable, eco-friendly, economical and possess safety properties as opposed to synthetic fungicides (Ncango *et al.*, 2010; Mircus *et al.,* 2009; Trofa *et al.,* 2009; Kock *et al.,* 2007). Antifungal anti-mitochondrial compounds with

anti-inflammatory properties have been reported in various studies and shown to exhibit antifungal properties by targeting ascospores and conidia structures (Caccioni and Guizzardi, 1994; Vaughn and Spencer, 1991). Studies by (Ncango *et al.,* 2010; Leeuw *et al.,* 2009 and Kock *et al.,* 2007) have indicated that structures with increased mitochondrial activity such as ascospores, conidia, asci, sporangia and phialides play an important role in the fungal life cycle and development. However, there has been particular interest in the activity of essential oils and their components against food-spoilage fungi. These essential oils with anti-inflammatory properties and their components have been shown to inhibit the growth of many fungi. Essential oils (EO's) have long been considered to possess antimicrobial activity, with activity against fungi (Prakash *et al.,* 2015; Bakkali *et al.,* 2008). The antimicrobial activity of essential oils can be attributed largely to the major groups of compounds found in them: monoterpenes, sesquiterpenes and nonterpenaceous components such as phenylpropanoids present in significant high proportions. Essential oils and its antifungal activity are becoming increasingly well described, with many studies reporting that EO's also target structures with increased mitochondrial activity such as ascospores and conidia (Prakash *et al.,* 2015; Bakkali *et al.,* 2008). Moreover, in the current study conidia of *P. citricarpa* and ascospores of *Guignardia mangiferae* have shown to contain elevated mitochondrial activity when compared to hypha. This was observed through the use of Rhodamine 123, a cationic lipophilic mitochondrion stain used to map mitochondrion function selectively. Other human, animal and agricultural fungal pathogens shown *in vitro* to be inhibited and/or killed by essential oils by targeting these structures, heightening interest in their agriculture application.

Essential oils are natural, volatile, complex plant compounds, oily or lipid-like in nature and frequently characterized by a strong fragrance (Bakkali *et al.,* 2008; Burt, 2004). They have a low solubility in water but are soluble in fats, alcohol, organic solvents and other hydrophobic substances and are generally liquid at room temperature. They are stored in specialized plant cells, usually oil cells or ducts, resin ducts, glands or trichomes (glandular hairs) and may be extracted from the leaves, flowers, buds, seeds, fruits, roots, wood or bark of plants by a variety of methods (Baser and Demirci, 2007; Pengelly, 2004). Essential oils are often described as secondary plant metabolites. Traditionally, secondary plant metabolites have been all those

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compounds synthesized by the plant which do not appear to be essential for plant growth and development or compounds that do not have an obvious function (Croteau *et al.,* 2000). Greater interest in the investigation of secondary metabolites in recent years has led to the discovery of roles they have in defence, signalling and as intermediates in secondary metabolism (Gershenzon and Dudareva 2007; McCaskill and Croteau, 1998).

Essential oils are not simple compounds or even simple mixtures of several individual compounds. They may contain up to approximately 100 components, although many contain about 20 to 60 (Dung *et al.,* 2008; Iscan *et al.,* 2005; Pengelly, 2004; Langenheim 1994). The compounds found in essential oils are from a variety of chemical classes, predominantly terpenes, but phenylpropanoids and other compounds also occur although at lesser frequency and often, but not always, in smaller proportions (Zuzarte and Salgueiro, 2015; Friedrich, 1976). Despite their history of being regarded as secondary, non-essential plant metabolites, it is becoming clear that essential oils and their components have specific biological functions (Vickers *et al.,* 2009; Gershenzon and Dudareva, 2007; Pichersky *et al.,* 2006), many of which lend themselves to commercial exploitation. Given the range and complexity of the compounds present in essential oils it is hardly surprising that they have the capacity to affect many biological systems.

Essential oils chemical composition was analysed in the current study using Gas chromatography mass spectrometry (GC-MS). The GC-MS provides a chromatographic profile of the chemical composition of essential oils. Every individual component of the essential oil is identified by the time at which the peak elutes on the trace. The data produced was then compared to an established 'profile' for that particular essential oil to finally determine the chemical composition of the oil. The GC-MS analysis revealed the presence of terpenes and terpenes derivatives in almost all the essential oils used in the study, as the main compounds, however the occurrence varied from all the essential oils tested. These compounds play a major role in the antimicrobial activities of essential oils. Furthermore these compounds are known to have many properties other than antimicrobial activities, such as anti-

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inflammatory properties, antiseptic and anti-cancer properties (Naidoo, 2007). Two types of Thyme oils obtained from different suppliers used in the current study (referred to as Thyme (i) and Thyme (ii) respectively) showed different chemical profiles, it is believed that the chemical composition of essential oils from a particular plant species can vary according to the geographical origin, harvesting period and cultivation area. It is therefore possible that variation in composition between these essential oils of the same plant species is sufficient to cause variability in the degree of susceptibility and this was also the case in the current study (Jianu *et al.,* 2013).

The current study was done in reference to the findings of (Ncango *et al.*, 2010; Leeuw *et al.,* 2009; Kock *et al.,* 2007) that acetylsalicylic acid; benzoic acid and salicylic acid possess antifungal properties which was also proven to be true against *P.citricarpa* and *G. mangiferae* in the current study. It was revealed that the pathogenic fungi, *Phyllosticta citricarpa* and *G. mangiferae* are also dependent on increased mitochondrion activity to effect spore-release structure development. This was observed from the XTT assay (an indicator of metabolic activity) results where the activities of mitochondrion dehydrogenases were affected for both organisms after the treatment with EO's. The study also showed that some cheaper alternative methods such as using hydrosol can be used as antifungals against CBS, with the advantage over essential oils of being water soluble and consisting of EO's traces (Weidenhamer *et al.,* 1993). It is concluded that EO's also target the development of these structures probably by decreasing energy production necessary for normal development and spore dispersal. This provides a dual function to these compounds, that is, anti-mitochondrial as well as antifungal. Moreover, EO's consist of complex composition, therefore, fungal resistance will not be a problem.

From the current study it can be concluded that many essential oils possess antifungal activity against *P. citricarpa* and *G. mangiferae*. With Thyme oil (i and ii) having the most potential antifungal anti-mitochondrial properties. It is believed that the present investigation together with previous studies provide support to the antifungal properties of essential oils. More over the current study has also reported for the first time the anti-mitochondrial properties of essential oils. The effective essential oils can

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therefore be used as alternative antifungals against citrus black spot towards the development of new antifungal agents. Additional in future, corresponding minimum inhibitory concentrations (MICs) should be determined for successful screening of EO's and evaluate *in vivo* to justify and further evaluate the potential of this oil as an antifungal agent in orchard. Future studies should also focus on the impact assessment of the actual fruits to identify possible changes such as taste, size and appearance after treatment with respective successful EO's.

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