Postharvest control of citrus pathogens using free and encapsulated essential oils.

POSTHARVEST CONTROL OF CITRUS PATHOGENS USING FREE AND ENCAPSULATED ESSENTIAL OILS

by

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DECLARATION

I hereby declare that the dissertation submitted for the degree M Tech: Biotechnology, at Tshwane University of Technology, is my own original work and has not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.

S.N. BOPIMA

Signature:……………………………

October 2018

DEDICATION

The completion of this study is firstly to the honour of **JEHOVAH BORE** (Lord Creator of all the earth), the renewer of my strength (Isaiah 40:31), our Father through the Lord Jesus Christ, in whom are hidden all the treasures of wisdom and knowledge (Colossians 2: 3), for putting everything together so that I can move that far in my academic achievement. Secondly, to my parents (**Constant Bob and Rita Bopima**), for all the sacrifices, belief and absolute love; and lastly in memory of the unforgettable unconditional and overwhelming support granted by **Liza Civava**. I am so grateful for the impact you made in this specific season of my life.

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ABSTRACT

South Africa is one of the major exporters of citrus fruit worldwide. During the season 2015/2016, the country was ranked the second largest exporter of oranges, and exported about 180 000 cartons of kumquat (*Citrus japonica*) to the European Union and the Middle East as major destinations. However, postharvest decay caused by green mould (*Penicillium digitatum*) and sour rot (*Galactomyces citri-aurantii*) is a threat to the industry. Kumquat in particular, is susceptible to postharvest rot caused by green mould. Health and environmental concerns with regard to synthetic fungicides have provided opportunities for research into green alternatives. The application of essential oils (EOs) as low risk mycobiocides for the postharvest management of decay on fruit, has gained momentum. However, the volatility of EO compounds and the possible phytochemical effects associated with EO application at high concentration have resulted in novel approaches for better application. The applications of EO as reducing and stabilizing agent in the synthesis of silver nanoparticles (SNPs), as well as the use of coating substances such as chitosan (CHI) and wax used as encapsulating matrices of EOs, have been investigated. In this study, the *in vitro* and *in vivo* inhibitory potential of selected EOs in the free and encapsulated form, and of oil-mediated SNPs, were investigated for the control of green mould and sour rot.

Both toxic medium and microtitre assays were used to compare the inhibitory effects of the oils, individually and in combination. At 500 µL/L, cinnamon leaf oil was the most effective against green mould, while lemongrass was the most effective against sour rot in the toxic medium assay. Spearmint oil was the least effective, because of the high concentration required for inhibition. Cinnamon performed best in the microtitre assay at 2000 µL/L. Oil-mediated SNPs were synthesised, characterised and their activities were subsequently assessed against the pathogens. The SNPs retarded the growth, suppressed sporulation and prevented spore germination of green mould, but were ineffective against sour rot. Chitosan alone demonstrated antifungal activity against green mould and sour rot at 7.5 mg/mL. The combination of CHI and EOs, both at their sub-inhibitory concentration, inhibited the growth of green mould and sour rot; and provided

100% inhibition of the mycelial growth after seven days of incubation. Essential oils alone and the mixture of EO with wax were tested in a semi-commercial trial on artificially wound-inoculated (green mould and sour rot) Valencia oranges. The outcome indicated that the inoculation conditions were too harsh, thereby limiting the ability of the EOs applied alone and/or with wax to control decay. The application of CHI alone and encapsulation of EO in CHI coating matrix at high concentrations resulted in poor performance of the substances to control sour rot infection on kumquat.

In vivo trials under commercial conditions indicated that both cinnamon leaf and spearmint oils at 3000 µL/L were able to control decay compared to the conventional treatment. In addition, the SNPs were also effective compared to the control treatments. Applying the EOs at higher concentration (8000 µL/L) and the use of a mixture of EO and wax resulted in a higher incidence of decay under all storage conditions tested. The *in vitro* and *in vivo* results did not correspond when using EOs. The use of high concentrations of EO possible affected fruit metabolism on kumquat since the fruit has a large surface to volume ratio. Spearmint and cinnamon leaf oils outperformed lemongrass oil when applied to commercial fruit, despite displaying less potential for pathogen control *in vitro*.

Potential hot-spots for contamination were sampled in the packhouse, but proved to have low spore counts, suggesting that the orchard may be the main source of kumquat contamination. Although there was no difference in the degree of ripening of kumquat at all tested warm water dip temperatures, treatment at 35 °C yielded the most promising results. A lower percentage of fruit rot and firmer rind were recorded at this temperature than for the others.

This study has contributed to the search for greener alternative applications of fungicides to fruit using natural substances. It has revealed that EOs in the free form hold promise for pathogen control and that encapsulated forms do not necessarily perform better. However, due to the volatile nature of EOs and the variable conditions in packhouse settings, it is recommended that future trials using EOs be carried out only in the packhouse, to mimic realistic environmental conditions and thereby guarantee the expected outcome.

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LIST OF ABBREVIATIONS

BC	Before Christ
BPW	Buffered peptone water
CFU	Colony forming units
CGA SA	Citrus Growers' Association of South Africa
CHI	Chitosan
CIN	Cinnamon leaf oil
CN-SNPs	Cinnamon leaf-mediated silver nanoparticles
CRI	Citrus Research International
DAFF	Department of Agriculture, Forestry and Fishery
DMI	Demethylation-inhibiting
DNA	Deoxyribonucleic acid
DW	Dry weight
EDS	Energy-dispersive spectroscopy
EC_{50}	Half maximum effective concentration
EO	Essential oil
EPA	Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FID	Flame ionization detector
FTIR	Fourier Transform infrared
GC	Gas chromatography
GRAS	Generally Regarded as Safe
GZT	Guazatine
HMW	High molecular weight
HWD	Hot water dip
HWRB	Hot water rinse brushing
HWT	Hot water treatment
IMZ	Imazalil
INT	*p*-Iodonitrotetrazolium violet
LG	Lemongrass
LG-SNPs	Lemongrass-mediated silver nanoparticles

LMW	Low molecular weight
MIC	Minimum inhibitory concentration
MMW	Medium molecular weight
MS	Mass spectrometer
NMR	Nuclear magnetic resonance
OD	Optical density
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PRO	Prochloraz
PYR	Pyrimethanil
RNA	Ribonucleic acid
RRI	Relative retention indices
SEM	Scanning electron microscope
SM	Spearmint oil
SM-SNPs	Spearmint-mediated silver nanoparticles
SNPs	Silver nanoparticles
SOPP	Sodium ortho-phenylphenate
TBZ	Thiabendazole
UK	United Kingdom
UNCTAD	United Nations Conference on Trade and Development
USA	United State of America
USDA	United State Department of Agriculture
UV	Ultraviolet

CHAPTER 1

BACKGROUND

1.1 INTRODUCTION

South Africa is one of the major exporters of citrus fruits around the globe. The country produces and exports a variety of citrus such as oranges, lemons, limes, soft citrus, grapefruits and kumquats. In the season 2015/2016, South Africa was ranked the second largest exporter of oranges in the world after Egypt (National Agricultural Marketing Council, 2016). During the same season, South Africa exported approximately 180 000 cartons of kumquats to the European Union (EU), Middle East and Indian Ocean Islands as major destinations with a share of 42.42%, 30.88% and 16.34%, respectively (DAFF, 2017). The citrus industry is the third largest horticultural industry in the country. The industry contributed about R9.69 billion to total gross value of South African agricultural production in the production season 2013/2014 (DAFF, 2015). The South African citrus industry is estimated to provide employment for more than 100 000 people. Besides large numbers of workers in the orchards and packhouses, there are many people employed throughout the supply chain services, which include transport, port handing and allied services, whose number is unspecified (DAFF, 2016). Moreover, it is estimated that approximately one million households depend on the citrus industry of the country as their sole source of revenue (DAFF, 2016). However, postharvest citrus decay is a threat to the industry since it causes wastage and quality deterioration due to decay, with consequent heavy economic losses. While decay losses can reach about 30% of the total production, it is estimated to attain as much as 50% in less developed countries (Strano *et al*., 2017).

Postharvest decay results from spores or mycelia infiltrating fruit injuries during handling throughout the fruit supply chain (harvesting, packing operations, transportation and storage). Green rot (*Penicillium digitatum*), blue rot

(*Penicillium italicum*) and sour rot (*Galactomyces citri-aurantii*) are the major fungal pathogens responsible for postharvest losses (Smilanick *et al.*, 2008; Hao *et al.*, 2010; Strano *et al*., 2017). All three are wound pathogens (Palou *et al*., 2008) that display similar decay symptoms; commencing as small watery spots, which progress to white fungal growth and eventually manifesting as blue, green or soft patches on the rind. Green mould is the most damaging postharvest disease of citrus fruit (OuYang *et al*., 2016). In a packhouse near Levubu, a farmer (S. Whitworth personal communication, 2017) indicated concern regarding green mould infection, which caused about 11% of kumquats decay after 6 to 7 days during exportation. Pathogen spores are ubiquitous in orchards where citrus is harvested (Boubaker *et al*., 2009). Factors that contribute to the prevalence of infection include tree age and condition, weather conditions and area of production (Palou *et al*., 2008). Although less common than green and blue moulds, sour rot is a potentially devastating postharvest citrus disease (Hao *et al.*, 2010; Karim *et al*., 2016). According to several researchers (Butler *et al.*, 1988; Feng *et al.*, 2011; El-Mohamedy *et al*., 2015), the high incidence of sour rot in all citrus-producing countries presents a continuous risk to the industry. For several years, the disease was regarded as one of the most economically important postharvest diseases of citrus in arid growing regions of the world (Smilanick & Sorenson, 2001; El-Mougy *et al.*, 2008).

Industries have, for many years, relied on the use of synthetic fungicides such as imazalil (IMZ), thiabendazole (TBZ), pyrimethanil (PYR), sodium *ortho*-phenylphenate (SOPP) and guazatine (GZT) for the prevention of postharvest pathogens (Kanetis *et al.*, 2007; Strano *et al*., 2017, Kellerman *et al*., 2018). However, serious environmental problems can result from irresponsible disposal of this hazardous waste. In addition, there is increasingly a build-up of fungal resistance to synthetic crop protection products (Bus *et al.*, 1991; Kinay *et al.*, 2007; Kitchen *et al*., 2016). Various fungicides including imazalil, pyrimethanil, thiabendazole are used for the control of green mould (Kellerman *et al*., 2014; Kellerman *et al*., 2016; Kellerman *et al*., 2018). However, their use is regulated depending on the legislation of countries (Strano *et al*., 2017). In contrast, guazatine was the only commercial fungicide applied for the control of sour rot since the 1980's (Rippon & Morris, 1981; Brown, 1988). However, this fungicide was not registered by the European Union (European Food Safety Authority, 2013), and its use was not authorized in several countries (European Food Safety Authority, 2010). However, it was recently banned in the European Union (EU) due to safety concerns (CGA SA, 2016). The European Union's measure led to the termination of the use of guazatine by the South Africa citrus industry, since the EU remains an export destination. Sodium *o*-phenylphenate can partially control sour rot, but its use is limited owing to the risk of fruit damage (Liu *et al.*, 2009; Feng *et al.*, 2011). Research has been conducted to find an alternative to replace guazatine for the control of sour rot. McKay *et al.* (2012) reported that propiconazole is an excellent fungicide for the integrated postharvest management of citrus sour rot as well as to control decay resulting from demethylation-inhibiting (DMI) sensitive isolates of *Penicillium* spp. However, the necessity for a safer and biodegradable natural product to replace synthetic fungicides remains the focus of researchers. The application of synthetic fungicides cannot be tolerated for citrus fruit such as kumquat. The fruit is not only eaten together with the peel and added to beverages (Peng *et al*., 2013), but also candied or preserved in sugar syrup (Lou & Ho, 2017).

Aromatic and medicinal plants, which contain a broad spectrum of secondary metabolites, some with antimicrobial properties, have received attention in the field of plant disease control (Sales *et al*., 2016). Many researchers have focussed on the application of essential oils (EOs) for postharvest disease control in fruit (Burt, 2004; Tripathi & Dubey, 2004; Combrinck *et al*., 2010) and in citrus specifically (Klieber *et al.*, 2002; Plaza *et al.*, 2004; Du Plooy *et al.*, 2009; Talibi *et al.*, 2012; Regnier *et al*., 2014). The ability of pathogens to induce resistance to EOs is minimised, since EOs contain a variety of compounds with different modes of action against microbes (Da Cruz Cabral *et al*., 2013; Swamy *et al*., 2016). Plaza *et al*. (2004) reported that thyme and cinnamon EOs significantly reduced the incidence of green and blue moulds on citrus. In addition, a study conducted by

Wu *et al*. (2017) proposed the inclusion of cinnamaldehyde (one of the major components of cinnamon leaf oil) in integrated management programmes for the control of sour rot. The *in vitro* and *in vivo* experiments involving mixtures of spearmint and lemongrass EOs indicated that these oils could control *P. digitatum, P. italicum* and *G. citri-aurantii* (Regnier *et al*., 2014). The antifungal activity of the oil mixture was attributed to the presence of high concentrations of *(R)*-carvone, geranial and neral. The individual application of cinnamon leaf, lemongrass and spearmint oils has been previously reported to completely inhibit the *in vitro* mycelial growth of green mould (Combrinck *et al*., 2010), while lemongrass was proved effective against sour rot (Regnier *et al*., 2014).

Nanotechnology is an emerging scientific approach that permits the manipulation of physical, as well as chemical properties, of a substance at the molecular level (Fakruddin *et al*., 2012). The use of nanometals as antimicrobial agents has also been investigated. Metal nanoparticles such as silver and zinc oxide have gained considerable attention due to their unique antibacterial, antifungal, and UV-filtering properties (Jasim, 2014). Nanosilver is the most studied and utilised nanoparticle for bio-systems. It has long been known to have strong inhibitory and bactericidal effects, as well as a broad spectrum of antimicrobial activities (Prasad & Swamy, 2013). Nano-sized silver colloidal solution has been applied as an antifungal treatment to inhibit various plant pathogens (Kim *et al*., 2012). Vilas *et al.* (2014) prepared silver nanoparticles (SNPs) using EO (*Myristica fragrans*) as reducing and stabilising agent. The generated SNPs were successfully tested against the bacteria *Escherichia coli* and *Staphylococcus aureus*. More importantly, the phytosynthesis of metal nanoparticles has been considered more advantageous over other biological methods due to their availability, and the rapid formation of stable nanoparticles with diverse morphology (Makarov *et al*., 2014). In addition, the natural approaches to synthesising nanoparticles offers the advantages of being non-toxic, less expensive and also enhance the production of nanomaterials when compared to physical and chemical methods (Vilas *et al*., 2016).

Owing to the volatility of the EOs compounds, a coating system to provide a slow release of these compounds is needed. Several studies have been conducted recently with chitosan (CHI) as a model for edible coating to improve the safety of food and to protect it from external environment factors (Cerqueira *et al*., 2011; El Guilli *et al*., 2016). Chitosan (1,4-linked 2-amino-2-deoxy-β-*D*-glucan) is a linear polysaccharide derived from chitin deacetylation (Vieira *et al*., 2016). Chitosan has become popular in food protection due to its unique biocompatibility, biodegradability, antimicrobial activity, and non-toxic characteristics (Palou *et al*., 2015). It has been reported to have excellent carrier properties for functional substances acting as antimicrobials and antioxidants agents (Yang *et al*., 2014). Furthermore, the incorporation of EOs in chitosan coatings reduces water permeability and can improve the antimicrobial efficacy, because the EOs constituents are released onto the fruit surface over time (Sánchez-González *et al*., 2011). Chitosan has been used as a coating matrix with the potential to inhibit green mould growing on Mandarin fruit cv. “Ortanique” (*Citrus reticulata* Blanco) (El Guilli *et al*., 2016). In addition, low molecular weight chitosan (LMWC) has been reported to maintain the quality and extend the shelf life of mature yellow citrus Murcott tangor (Chien *et al*., 2007). A study on grapes revealed the efficacy of chitosan alone, and as coating matrix for EOs, to inhibit fungal pathogens (Dos Santos *et al*., 2012).

1.2 PROBLEM STATEMENT

Green mould and sour rot remain the largest threat to citrus. There are several strategies available for the control of sour rot, but these are not highly effective. Fungicides that are effective against both pathogens, and are sufficiently complex to prevent the development of pathogen resistance, are urgently needed to use alone, or in combination regimes with existing fungicides. However, the use of synthetic fungicides is regarded as problematic from a safety perspective and their residue levels on the rind are restricted. In addition, it is undesirable to apply synthetic fungicides to kumquat, which is consumed with the peel. Essential oils have a proven track record of pathogen control *in vitro*, but application techniques for these oils remain largely unexplored. Studies are needed that focus on the development of techniques that prolong the inhibitory action of these volatile substances, while providing efficient delivery. Since EOs are highly volatile, researchers have investigated mechanisms that slow down the evaporation rate and ensure the controlled release of the active compounds (Sánchez-Navarro *et al*., 2013). A practical application of this knowledge would allow full fungal control during prolonged transport of fruit to destinations far from the production point. Essential oils have been applied in the wax coating applied to the rind (Du Plooy *et al*., 2009). Addition of SNPs and the coating of EOs into chitosan matrix may provide protection with lower concentrations of the oils, resulting in a prolonged shelf life of the fruits. It is also possible to dip or spray the fruit with aqueous solutions containing EOs, rather than wash them with synthetic fungicides. However, the efficacy of control has not been investigated. There is no information available on the use of EOs on kumquat. Due to their small size, the ratio of volume to surface area is very different from that of other citrus fruit and physiological conditions may result from the application of crop protection products.

Previous studies have indicated the multi-target pathogenic effect of EOs. A mixture composed of spearmint and lemongrass oil were able to control all three citrus pathogens mentioned (Regnier *et al*., 2014). The use of EO-mediated synthesis of SNPs and the encapsulation of EO in chitosan matrix to apply as citrus coating has been emphasised and evaluated. Although impossible to achieve, the ideal packhouse should provide a spore-free environment to fruit. The application of natural fungicides will be more effective when confronted with low spore loads. Different sections of the packline may be a serious source of infection. Kumquat producers in South Africa have adopted a warm water dip at 37 °C, but it is unclear whether this practice is substantiated by scientific evidence.

1.3 HYPOTHESIS

Essential oils, applied in the free form, alone and in combination, as well as in the form of EO-mediated matrices, will provide efficient antifungal control of citrus postharvest pathogens both *in vitro* and *in vivo*.

1.4 AIM AND OBJECTIVES

This study was aimed at determining the efficacy of various applications of EOs (individually and in mixtures, EO-mediated synthesised SNPs, encapsulated EO within a chitosan matrix, and EOs in commercial waxes), to apply on citrus for the control of challenging citrus postharvest decay pathogens *Penicillium digitatum* and *Galactomyces citri-aurantii*.

The specific objectives were to:

- identify EOs for further *in* vitro studies;
- optimise various ratios of EOs for the most effective control of both pathogens;
- evaluate *in vitro* the ability of silver nitrate alone, and in combination with EOs, to control both pathogens;
- evaluate *in vitro* the ability of EO-mediated SNPs, prepared according to published methods, to control both pathogens;
- evaluate *in vitro* the ability of EO-coated chitosan to control both pathogens;
- determine the antifungal ability of aqueous EOs applied by dipping the fruit or brushing with a mixture of EO and wax in the absence of synthetic fungicides;
- establish the potential antifungal efficacy of EOs, EO-mediated SNP suspension and EO-encapsulated into chitosan and waxes *in vivo* using kumquat;
- evaluate possible sources of spore contamination on the packline; and finally to
- determine the effect of a warm water dip at different temperatures on the decay and physiology of kumquat.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

In this chapter, an overview is provided of citrus fruit, their health benefits, the major postharvest diseases affecting citrus, conventional methods currently used to control these diseases, and the current state of research with regard to alternative antifungal agents from natural sources. More attention is paid to the use of essential oils (EOs), silver nanoparticles (SNPs) and EO-mediated SNPs, and chitosan (CHI) as an EO encapsulating matrix to apply to fruit as a natural coating.

Worldwide, South Africa is recognised as a producer and exporter of citrus, deciduous and subtropical fruits**.** Although South Africa was not on the top rank of soft citrus production in the world (ninth largest producer) for the season 2015/2016 (DAFF, 2016), the country still produces large quantities of soft citrus for export (DAFF, 2017). However, South Africa was ranked the second largest exporter of oranges in the season 2016/17 (DAFF, 2017). The South Africa citrus is not spared from the postharvest challenges that the sector faces. An ever-increasing world population has placed huge demands on agricultural and food products in terms of food security during the last decades (McCarthy *et al*., 2018). It has been estimated that 110% increase in food production will be required by 2050. Spoilage of fresh produce, resulting from fungal and bacterial infections remains one of the biggest risks to food security (Sekhon, 2014). Seven years ago, postharvest diseases were identified as responsible for up to 40% of the fresh produce losses in developing countries (Gustavsson *et al*., 2011). The citrus industry is not exempted from decay.

2.2 THE GENUS *CITRUS*

2.2.1 *Citrus* species

Citrus fruits belong to the genus *Citrus* L. (family Rutaceae) and are thought to originate from the tropical and subtropical regions of India, southern China, northern Australia and/or New Caledonia (Manner *et al*., 2006). In general, citrus performs best in subtropical climates, but the cultivars selected must be tolerant to cold spells (Rieger, 2002). Citrus is cultivated in more than 140 countries worldwide (Liu *et al.*, 2012), with summer, winter, bimodal or uniform rainfall (Manner *et al*., 2006). Citrus is considered as one of the world's most popular fruit crops. Although the number of natural species within the genus is uncertain, the most well-known and commercially important examples of citrus fruits include oranges, lemons, limes, grapefruit and tangerines (Liu *et al.*, 2012). A list of the most common citrus species is provided in Table 2.1.

Table 2.1: *Citrus* species and their commonly used names

Species	**Common name**
Citrus aurantifolia	lime
C. aurantium	sour orange
C. grandis	pomelo
C. hystrix	kaffir lime
C. limon	lemon
C. macroptera	wild orange
C. medica	citron
C. mitis	calamondin
C. paradise	grapefruit
C. reticulate	mandarin
C. sinensis	sweet orange
C. japonica (Fortunella spp)	kumquat

Source: Manner *et al*. (2006)

2.2.2 Nutritional value of citrus

Citrus fruit are available globally and make a valuable contribution to the human diet (Liu *et al.*, 2012). Although citrus fruits are well-known for their content of Vitamin C, they are also rich in nutrients such as organic acids (e.g. citric, malic, quinic and tartaric acids) and simple sugars (e.g. sucrose, glucose and fructose) (Lado *et al*., 2018), dietary fibre (cellulose, hemicelluloses and pectins) (Matheyambath *et al.*, 2016) and minerals (Zou *et al*., 2016). Several health benefits have been associated with the consumption of citrus (Table 2.2). For example, citrus has been described as a low energy density and low fat content food, since they provide fewer calories per gram of fruit. Citrus is therefore valuable in combating the increasing rate of obesity in both adults and children through the maintenance of a healthy body weight (Al-Snafi, 2016).

Table 2.2: Health benefits associated with the consumption of citrus fruit

Protective against	Reference
cardiovascular diseases	Testai & Calderone (2017)
cancer	Rawson *et al*. (2014); Cirmi *et al*. (2017)
neurological conditions	Cirmi *et al*. (2016)
cataracts	Kaur *et al*. (2017)
age-related macular degeneration	Elliot & Williams (2012)
osteoporosis	Shalaby *et al*. (2011)

The components involved mainly in the diseases prevention mechanisms of citrus include substances such as Vitamin C, dietary fibres, flavonoids, limonoids, carotenoids and folate. Flavonoids are well known anti-oxidants and have been implicated in preventing all the conditions referred to in Table 2.2.

2.2.3 Geographic distribution of citrus

Citrus fruits are cultivated predominantly in tropical and subtropical climates. A report of the United Nations Conference on Trade and Development (UNCTAD, 2004) indicated that most of the citrus species are cultivated between the 35ºN and 35ºS latitudes, with cultivation and production (about 70% of the global marketable citrus) mostly located in the Northern Hemisphere (Blasco *et al*., 2016). In a report of the Food and Agriculture Organisation of the United Nations (FAO, 2016), the production of citrus in the Northern Hemisphere accounted for 77.2% of the global production in the 2013/14 seasons. Countries with large production volumes in the Northern Hemisphere include China, United State of America (USA), Mexico, Egypt, Turkey, Morocco and countries of the Mediterranean region (FAO, 2016). Of the Mediterranean countries, Spain is the largest producer, with more than 55% of the European citrus output (Blasco *et al*., 2016). In the USA, California and Florida are acknowledged as major producers of particularly grapefruit (USDA, 2017). The production of citrus in the Southern Hemisphere is dominated by Brazil, South Africa and Argentina (FAO, 2016), which have a seasonal advantage for export to the Northern Hemisphere.

In the season 2016/2017, the global bulk production of citrus was dominated by oranges, soft citrus and lemons (DAFF, 2017). During the same season, Brazil produced a total of 19.2 million metric tons of oranges (38.2% of global share) and ranked as the world's top producer of oranges, followed by China with a share of 12.4%, the European Union (EU) (12%) and the USA (9.1%). In contrast, the production of soft citrus was dominated by China, the EU, Morocco and Turkey (DAFF, 2017). South Africa was ranked eighth for the production of oranges and tenth for soft citrus (DAFF, 2017).

Although China (USDA, 2018) and the USA (USDA, 2017) produce large volumes of citrus, they consume the largest proportion domestically. During the 2016/17 season, Egypt was ranked the first largest exporter of oranges, with a commanding share of 31%, followed by South Africa with 28% market share (DAFF, 2017). During the same season, South Africa exported 210 000 tons of soft citrus.

2.2.4 The South African citrus industry

The diverse weather and climatic conditions of South Africa enable the country to cultivate and produce a wide range of fruits. Worldwide, the country is well known for the production and exportation of citrus, deciduous and subtropical fruits. Citrus is produced in various regions of South Africa as depicted in Figure 2.2. The major production areas are situated in the Limpopo, Mpumalanga, KwaZulu-Natal provinces, as well as in the Northern, Western and Eastern Cape provinces; covering 70 055 hectares in the 2015/16 season (USDA, 2017).

Figure 2.1: Areas of citrus production in South Africa[1]

The citrus industry plays a major role in the economy of South Africa. It contributed R8.3 billion to the gross value of agriculture during the 2012/13 production season (DAFF, 2014). In addition, fifteen years ago, the industry employed over 100 000 people, who support about 600 000 dependants (DAFF, 2003), which represented

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[1]http://caesarfruit.com/Pages/Citrus.html

15% of the agricultural labour force (Potelwa, 2015). A report of the Citrus Growers' Association of South Africa (CGA SA, 2017) revealed that the main destination of citrus during the 2016/17 season was to the Middle and Far East, Northern Europe, Russia, North America, the United Kingdom (UK) and Asia. The percentage of distribution was dependent on the type of citrus. Oranges were exported mainly to Europe (37%), Middle East (23%), North America (7%), Russia (6%) and the UK (5%), and soft citrus mainly to the UK (35%), followed by Europe (30%), North America (10%), Asia (9%), Russia (7%) and the Middle East (6%).

2.2.5 Postharvest diseases affecting citrus

Green and blue moulds are two of the most devastating diseases of citrus world-wide and are caused by *Penicillium digitatum* Sacc. and *Penicillium italicum* Wehmer, respectively (Boubaker *et al*., 2016). Sour rot also poses a serious threat to the South African citrus industry. This disease results from infection of *Galactomyces citri-aurantii* E.E. Butler [anamorph: *Geotrichum citri-aurantii* (Ferraris) E.E. Butler] (McKay *et al*., 2012). These three pathogens are wound-obligate pathogens that require nutrients from a fresh fruit injury site for infection and disease initiation (Brown, 1979; Palou *et al*., 2008). Other postharvest pathogens affecting citrus include black rot caused by *Alternaria alternat*a pv. *citri*, brown rot caused by *Phytophthora citrophthora* and anthracnose caused by *Colletotrichum gloeosporioides* (Talibi *et al*., 2014).

Green mould *(Penicillium digitatum)*

For several decades, green mould has been considered the most economically devastating infection of citrus (Eckert & Eaks, 1989; Smilanick *et al*., 2006; Ouyang *et al*., 2016). Eleven years ago, the disease caused about 90% of citrus loss during postharvest handling (Macarisin *et al*., 2007). Green mould is highly destructive, even in the arid environments of the world (Smilanick *et al*., 2006).

The spores of *P. digitatum* are ubiquitous in the atmosphere and on the surface of fruits (Palou *et al*., 2008). The dispersion of conidia on the surface of fruit occurs in multiple ways, such as by water splashing from rain or irrigation sprinklers, through airborne soil dust particles, or by insects and other animals (Brown, 1979; Baudoin & Eckert, 1982). The orchard remains an important source of inoculum throughout (Palou *et al*., 2008).

Injured fruits are the most susceptible to infection. Wounds in the fruit rind are inflicted during harvesting, improper handling, or as a result of insect activities in the orchard (Brown, 2003). Susceptible wounds vary from deep injuries that extend into the susceptible mesocarp, to minor injuries involving individual oil glands of the flavedo (Brown & Ismail, 1978; Brown *et al*., 2000). Minor punctures result from rough handling, twigs within the canopy and the accumulation of sand grains in picking bags or on conveyor belts on the packline (Brown *et al*., 2000). Arimoto *et al*. (1995) reported that moisture and epicarp extracts from wounds increase infection and facilitate green mould development.

The conidia situated at the sites of injury penetrate the albedo of the peel; usually resulting in irreversible infection within 48 h at 20-25 ºC (Eckert & Eaks, 1989). Free water, nutrients and specific temperature, as well as pH conditions, are required for the germination of conidia within the rind wounds (Lahlali *et al*., 2006; Palou, 2014). Germination is stimulated by volatiles emitted from the host tissue (Stange *et al*., 2002). The development of the disease is mediated by complex interactions between pathogen virulence mechanisms and host defense responses relative to intrinsic and extrinsic factors (Palou, 2014). The intrinsic factors are mostly related to the fruit maturity. Infected immature fruit rarely results in the development of the disease, because of a range of complex mechanisms generated by the host that provide natural resistance to the disease. Mature ones are more susceptible to the development of decay. Extrinsic factors, also known as harvest or postharvest factors, encompass temperature, inoculum density, rind maturity and condition (Palou, 2014). Green mould infection is active in the temperature range 4-30 °C.

Palou (2014) reported that the pathogen was unable to grow at 37 °C, but flourished at 25 °C, the optimum temperature for germination and growth (Palou, 2014).

The symptoms of green mould involve primarily the formation of a water-soaked lesion, detectable within 24-36 h after infection (Barmore & Brown, 1982), followed by the development of an airborne white mycelium in the centre of the lesion, which expands radially (Palou, 2014). Thereafter, olive green conidiophores (non-sporulating white mycelium) form in the centre of the lesion (Benhamou, 2004). Finally, with time (after 7-8 days), the spores cover the whole surface of the fruit, accompanied by shrinking and a hollow mummified shell of the fruit if exposed to air (Palou, 2014).

Sour rot *(Galactomyces citri-aurantii)*

Sour rot is a common pathogen of citrus fruit rot across the globe (Suprapta *et al.*, 1997; Smilanick & Sorenson, 2001; Boubaker *et al*., 2016). It is soilborne and is ranked as the second biggest contributor to postharvest decay after *Penicillium* spp. (McKay *et al*., 2012). The disease is most frequent in arid and semi-arid regions of citrus production (El-Mougy *et al.*, 2008). As for green mould infection, sour rot infection also requires injury of the rind. The pathogen easily infects healthy fruits through injuries that extend into the albedo (El-otmani & Ait-oubahou, 2011). Although sour rot infection occurs by water splash (rain or irrigation sprinklers) and by airborne (soil dust particles) to the surfaces of fruit in the tree canopy, the organism is also transported to the fruit by fruit flies, which carry mycelium and spores from spoiled and macerated tissue to healthy stored fruits (Nazerian & Alian, 2013).

The susceptibility of the fruit to the pathogen depends on the maturity of the fruit, as well as the season. Mature fruits are more highly predisposed to the decay than immature ones (El-otmani & Ait-oubahou, 2011). High rainfall also contributes to an increase in the occurrence of sour rot infection (Liu *et al.*, 2010). Furthermore, humid environmental conditions and improper harvesting and packing methods, result in the spread of the arthroconidia (Nazerian & Alian, 2013).

The optimal growth temperature of sour rot is between 25 and 30 °C, while the growth of the pathogen is slow at temperatures ranging from 4 to 10 °C (Plaza *et al*., 2003). However, rapid development of decay occurs within five days of incubation at 25 °C, in the case of an active rot, otherwise a dry lesion (2-3 mm in diameter) results if infection is arrested (Baudoin & Eckert, 1982).

Initial symptoms of sour rot infection are closely related to those of green and blue moulds, with the visible formation of water-soaked areas that are light-brown. Infected fruit exposed to high relative humidity, leads to the development of a cream-coloured mycelium covering the lesion. Fruit are degraded into a slimy and watery mass at their final stage of decay (El-otmani & Ait-oubahou, 2011).

2.2.6 Industrial control of green mould and sour rot

Synthetic fungicides, introduced into the market between 1960 and 1970 (Ivic, 2010), are still extensively applied for the management of postharvest diseases in fruits commodities (e.g. mango, citrus). When applied to plants or crops, the fungitoxic active substances penetrate the infected wound and eliminate the established pathogen upon interaction (Ivic, 2010). Synthetic fungicides have been applied successfully for the control of citrus green mould and sour rot. Green mould is controlled by limited number of registered conventional synthetic fungicides including imazalil (IMZ), thiabendazole (TBZ), pyrimethanil (PYR) and prochloraz (PCZ). In addition, some other active ingredients such as azoxystrobin and fludioxonil have also been investigated (Kanetis *et al*., 2007; Horuz, 2010). Different mixtures of these compounds have also been commercially marketed (Palou, 2013).

Among several studies that have been carried out to investigate the effects of these fungicides against green mould, Kellerman *et al.* (2016) reported that dip tank curative control of green mould exposed to IMZ (500 mg/L) was successful after adjusting the solution pH to 6. The results indicated effective control on lemons (96.9-98.7%) and Clementine mandarins (39.3-57.9%) regardless of exposure time and temperature. However, longer exposure time was needed to reach 84.7-92.5% of control on navel oranges. Thiabendazole was found to be very effective as an *in vivo* curative dip treatment on Valencia oranges (Kellerman *et al*., 2014). The substance (TBZ) provided more than 96% of control over green mould at 1000 µg/mL. Although IMZ and TBZ demonstrated their effectiveness against green mould-sensitive isolates, they need to be supplemented by PYR for their prolonged use (Smilanick *et al*., 2006). The use of PYR in the control of postharvest citrus green mould is necessary, because the chemical provides good curative control (90.6%) in dip application on navel oranges (Kellerman *et al*., 2018). In the experiment, fruit were dipped for 90 s in 1000 mg/L of PYR solution. In addition, the effective application of PYR alone resulted in the control of IMZ- and TBZ-resistant isolates (Smilanick *et al*., 2006). Pyrimethanil was proven effective against green mould IMZ- and TBZ-resistant isolates when applied by immersion (500-600 mg/L) on Salustiana and Delta seedless oranges and Clementine mandarins in the northeast of Argentina (Vázquez *et al*., 2014). The antifungal effect of PCZ was demonstrated *in vitro* by Hao *et al*. (2010). The substance (PCZ) alone provided the lowest effective concentrations (EC_{50}) that caused 50% inhibition of mycelial growth and spore germination of *Geotrichum candidum*, *P. italicum* and *P. digitatum*. In the experiment, mandarins artificially inoculated with *G. candidum*, *P. italicum* and *P. digitatum* (spore suspensions: 10^6 conidia mL^{-1}) were immersed in a solution of tea saponin, PCZ and IMZ (200 µg/mL) for 60 s at 25 ˚C. After 10 days of storage at 20 ˚C, PCZ provided a slightly better performance than IMZ against both *P. italicum* and *P. digitatum* with about 20% of the fruit infected. However, IMZ presented a slight lower disease incidence against *G. candidum* than PCZ.

Although several commercial fungicides are active against *Penicillium* species to some extent, fewer can control *G. citri-aurantii*. For this reason, Liu *et al.* (2009) stated that sour rot is often considered as the most problematic pathogen. Thirty years ago, guazatine (GZT) was the only commercial fungicide able to control sour rot (Brown, 1988). A report of the Citrus Growers' Association of South Africa (CGA SA, 2017) revealed that GZT was applied in local packhouses until being banned by the EU in early 2016. Sodium ortho-phenylphenate (SOPP), a broad-spectrum phenolic-based fungicide is a possible alternative for sour rot control, but is known to cause phytotoxicity to citrus at concentrations required to effectively manage the disease (Brown, 1979; Kitagawa & Kawada, 1984).

Over the past decade, some synthetic fungicides have been investigated and applied for the control of sour rot. These fungicides included the demethylation-inhibiting (DMI) triazole fungicides such as metconazole, tebuconazole, propiconazole and cyproconazole (McKay *et al*., 2012). Of all the DMI fungicides, propiconazole was found highly effective for the control of sour rot in a study conducted by McKay *et al*. (2012). The results revealed that the control (water without supplementation) and treatment with propiconazole, cyproconazole, metconazole and tebuconazole resulted in 93.7% decay (control) and 11.4, 13.0, 31.3, and 35.1%, respectively. The lemons were infected 14 hours prior to the fungicide treatment and decay was recorded after six days storage at 20 °C.

A few other synthetic chemical fungicides have also been studied for their potential ability to control sour rot and green mould infections. In a study conducted by El-Mougy *et al*. (2012), members of the azole class (itraconazole, ketoconazole, fluconazole and stevia) were tested *in vitro* against green and blue moulds, as well as against sour rot. The four antifungal agents completely inhibited the mycelial growth of all three pathogens at a concentration of 400 µg/mL.

2.2.7 Constraints to the application of synthetic fungicides

Chemical fungicides offer advantages when applied in both curative and preventative scenarios. They are not only highly effective against postharvest pathogens, but also provide a long-term preventative action. In addition, they are easily handled, simple to apply and are available at low cost (Martínez, 2012). Nevertheless, their excessive and indiscriminate use has resulted in some negative effects (Sharma & Pongener, 2010; Mahmood *et al*., 2016). Large volumes of fungicides used in dip tanks as part of the packline are disposed, and may have serious repercussions for the environment (Du Plooy *et al*., 2009). The presence and persistence of synthetic chemical fungicides in the environment might lead to various harmful effects, including the destruction of organisms that promote fertility and health of agricultural soils, while polluting aquatic ecosystems *via* migration through surface and ground water (Wightwick *et al*., 2010; Mahmood *et al*., 2016). Exposure of mammalians, including humans, to synthetic fungicides may result in carcinogenicity and teratogenicity (Castro *et al*., 1999; Damalas & Eleftherohorinos, 2011). In addition, studies have demonstrated that continuous use of fungicides result in the development of fungal strains that are resistant to synthetic fungicides (Boubaker *et al*., 2009; Erasmus *et al*., 2015).

2.3 ALTERNATIVES TO CONVENTIONAL FUNGICIDES

2.3.1 Physical treatment

The use of physical treatments has gained momentum in recent years in the domain of the postharvest control of diseases because of their irrefutable benefits such as the total absence of residues in the treated product and insignificant environmental impact (Usall *et al*., 2016). Physical treatment methods include heat treatment, the application of microwave energy, hypobaric and hyperbaric treatment and ultraviolet radiation (Usall *et al*., 2016). However, there are no literature reports on the use of physical treatments other than heat treatment and UV-radiation for the postharvest control of green mould and sour rot in citrus. Some studies have been conducted on the application of ultraviolet irradiation using UV-C on Navel oranges, inoculated with *P. digitatum* and *P. italicum* (Gündüz *et al*., 2015), and on lemon using UV-B irradiation against green mould infection (Riuz *et al*., 2017). Treatment with UV-C, applied at dose of 3.17 kJ/m^2 caused maximum reductions of 2.75 and 3.33 log CFU/orange of *P. digitatum* for spot and wound inoculation methods, respectively (Gündüz *et al*., 2015). Although, *P. italicum* (blue mould) exhibited resistance at the dosage effective against green mould, the team suggested that the dose at which blue mould might be susceptible would lead to higher inhibitory activity against green mould. Riuz *et al*. (2017) found that exposure of lemon flavedo to 15 mg dry weight (DW)/mL of UV-B inhibited the growth of green mould infection.

Heat treatment, also referred to as thermal treatment, remains the most commonly studied physical treatment on citrus (Palou, 2013; García *et al*., 2016). Such treatment is mostly applied as a hot water dip (Palou, 2013) that may contain a chlorine sanitiser. All heat treatments induce anti-pathogenic effects by acting on the host-pathogen interaction (Usall *et al*., 2016). Heat treatment acts directly on the pathogen by changing the structure of nuclei and cell walls, thereby inducing the denaturation of proteins. This results in the destruction of mitochondria or outer membranes, thereby damaging the vacuolar membranes, and eventually depleting the food reserves of the fungus (Palou, 2013). Indirect action on the fruit involves the induction of several resistance mechanisms against the infection process and the development of the pathogen (Palou, 2013).

Hot water treatment is an approach that involves the use of water at elevated temperatures for the control of postharvest decay (Usall *et al*., 2016). The method is safe to both humans and the environment, and is free of regulation (Usall *et al*., 2016). The two ways through which HWT can be applied include a complete immersion of the fruit commodity (hot water dip, HWD) or in the form of hot water rinse brushing (HWRB) (Palou, 2013).

Thermal treatment is usually applied by immersing fruits for 2-5 min in water heated between 40 and 55 °C (Palou, 2013). The effectiveness of hot water dips is species, cultivar and fruit maturity dependent (Palou, 2013). In most cases, the treatment did not trigger physiological disorders such as surface damage and colour change (Strano *et al*., 2014). It has also been reported to have no negative effects on physico-chemical parameters such as fruit firmness, weight loss, juice content, soluble solids, pH, titratable acidity and sensory of the fruit (García *et al*., 2016).

Sivakumar and Fallik (2013) described a thermal treatment, whereby the fruit are rinsed with water sprayed above the roller brushes on the sorting line, followed by a rinse using pressurised recycled hot water, and finally, the fruit are dried using forced air. The set temperature is relatively high (55-65 °C) and commodities are treated for 10-60 s (Palou, 2013). Although studies have elaborated on the potential effects of heat treatment as an alternative to conventional chemical fungicides, it has some limitations. High energy costs, large volumes of water required and the generation of waste water are some of the disadvantages (Usall *et al*., 2016).

Curing, also known as hot air treatments, is a treatment approach whereby fruit are exposed for several hours or days, depending on the type, to an air atmosphere at temperatures above 30 °C at relatively high humidity (RH > 90%) (Usall *et al*., 2016). Palou (2013) achieved the best results when fruit was cured for two to three days (Palou, 2013). He reported 90% control of green mould using artificially inoculated citrus exposed to hot air at 35 °C for 72 h and incubated for 7-20 days at 20 °C. The application of curing for postharvest disease control on a commercial scale is unpopular due to the expense of heating, the immobilisation of large amounts of fruit and the possibility of some fruit cultivars developing physiological disorders (García *et al*., 2016; Usall *et al*., 2016).

Other physical tools that are applied for the reduction or delay of the growth of postharvest pathogens include cold storage, controlled and modified atmospheres (Usall *et al*., 2016). These tools are mostly used following the application of fungicides.

2.3.2 Biological control

The use of biological control agents as alternatives to conventional synthetic chemical fungicides have been intensively investigated over the last 30 years (Droby *et al*., 2016). Biological control has been used as a preharvest control measure in the orchard, to supplement synthetic chemical treatments, as part of an integrated disease management strategy over the past years (Ewekeye *et al*., 2013). Biological control, also known as biocontrol, is the purposeful usage of non-pathogenic living organisms (introduced or indigenous) as a means to naturally suppress the activity or population of one or more pathogens causing diseases in host plant (Cook, 1993; Babychan *et al*., 2017).

Certain traits have been identified for microorganisms to be ideal antagonists (Nune, 2012; Babychan *et al*., 2017; Dukare, 2017). These include:

- genetic stability,
- effectiveness at low concentration,
- ability to limit the growth of a wide range of pathogens,
- usefulness on several commodities,
- survival under stressful or undesirable environmental conditions,
- easiness in dispensing,
- resistant to most chemicals used for postharvest pathogen control,
- non-pathogenic to the host commodity,
- non-detrimental to human health and non-targeted life-forms,
- require simple and inexpensive nutritional substrate for growth and multiplication, and
- the formulation must provide a long shelf-life, as well as be economical.

The primary mode of action of effective biocontrol agents has been mainly the competition for nutrients and space (Spadaro & Droby, 2016; Babychan *et al*., 2017). However, additional modes of actions have also been suggested, such as antibiosis, induced resistance and parasitism (Babychan *et al*., 2017). Biocontrol agents consisting of bacteria (such as *Bacillus subtilis* and *Agrobacterium radiobacter*) and yeasts (such as *Cryptococcus laurentii* and *Rhodosporidium paludigenum*) exhibited remarkable antifungal activity potential against green mould (Mohammadi *et al*., 2017) and sour rot (Liu *et al*., 2010), when applied to lemons and mandarins, respectively.

Despite the many advantages that biocontrol offers, its application has some restrictions. The host-specificity, lower efficacy and slow action against plant diseases when compared to conventional fungicides, as well as the limited number of organisms suitable for application, pose problems (Chandrashekara *et al*., 2012; Droby *et al*., 2016). In addition, biocontrol is mostly used as a preventive measure and not in a curative manner (Chandrashekara *et al*., 2012). Furthermore, the organisms are active only under specific environmental conditions (Nega, 2014).

Biological control requires well-trained personnel to multiply and supply microorganisms and to regularly monitor the presence of the biocontrol population in the orchard to ensure sufficient levels (Chandrashekara *et al*., 2012). A major problem is that many potential biocontrol organisms display good performance in the laboratory and under greenhouse conditions, but fail to provide appropriate control in the field (Wang *et al*., 2003; Babyshan *et al*., 2017). The commercial use and application of biocontrol agents has been relatively slow. Further development would require a better understanding of the environmental parameters that affect biocontrol agents (Fravel, 2005). Moreover, economic and regulatory constraints constitute limiting factors for the successful introduction of the biocontrol product to the market. These constraints are related to the high costs associated with biocontrol product development, registration and marketing (Usall *et al*., 2016).

2.3.3 Plant essential oils as a natural means for food preservation

2.3.3.1 Brief background on the history of essential oils

Prior to the identification of volatile compounds as responsible for the fragrance of plants, essential oils were initially referred to as *Quinta Essentia* (Haagen-Smit, 1961; Sadgrove & Jones, 2015). Since the 1960s, EOs have been studied by researchers from various fields, including chemistry, plant physiology, pharmacology, cosmetics, and even historians have been interested in these materials because of their cultural roles (Haagen-Smit, 1961).

Aromatic plants have been long used (since about 2000 BC) as incense, perfume, cosmetics, and for culinary and medical applications, in ancient civilisations of Egypt, China, Arabia, Greece and by the Romans (Lawless, 2013). However, systematic studies on EOs occurred in the 19th century and were focussed primarily on the determination of the chemical compositions of EOs obtained from different plants. The French chemist, Jean Baptiste Andre Dumas (1800-1884) (Kubeczka, 2010), made a valuable contribution in this regard. The introduction of gas chromatography after the Second World War revolutionised the study of EOs and together with nuclear magnetic resonance (NMR) spectroscopy (Kubeczka, 2010), inroads were made towards the establishment of a database of EO constituents.

The first recorded investigations on the medicinal and antimicrobial properties of EOs took place in the era of the French chemist Rene Maurice Gattefosse in 1910. He applied pure and undiluted lavender oil to a severe burn on his hand and arm and as a result, the burning section healed without infection or scarring. Later, Dr Jean Valet was inspired by the work of Gattefosse to use EOs to cure injured soldiers during World War II (Lawless, 2013). Since then, many researches have been conducted into the use of EOs in the fields of medicine and food microbiology.

2.3.3.2 Application and chemical characteristics of plant essential oils

Essential oils are a mixture of natural complex volatile organic compounds synthesized as secondary metabolites by aromatic plants (Bakkali *et al*., 2008; Neiro *et al*., 2009) with a general molecular weight below 300 kD (Dhifi *et al*., 2016). They are isolated from different parts of aromatic plants such as flowers, leaves, tree underground organs (e.g. roots), rhizomes, seeds, fruits, wood and bark (Dhifi *et al*., 2016). The known methods used their isolation are steam or hydrodistillation methods (Gavahian *et al*., 2015). They are characterised by a strong odor (Bakkali *et al*., 2008).

Similar to other plant metabolites, volatile organic compounds are synthesised by plants to play a specific role within the plant, including the repulsion of detrimental insects acting as plague vectors, reduction of appetite to some herbivores through the induction of an unpleasant taste to the plant, protection against pathogenic microorganisms and attraction of pollinators to support the dispersion of pollens and seeds (Nazzaro *et al.*, 2017). These compounds can be isolated as EOs. The antimicrobial or other biological activities of EOs are linked to the occurrence of their bioactive volatile components (Calo *et al.*, 2015).

Although EOs might incorporate hundreds of bioactive compounds (Bleasel *et al*., 2002), many EOs are characterized by two or three main constituents that are present in high concentration (20-60%) (Swamy *et al*., 2016). Essential oils are mainly composed of terpenes, which may be oxygenated (terpenoids) (Nazzaro *et al*., 2013) and have alcohol, aldehyde, ester, ether, or ketone functional groups (Bakkali *et al*., 2008). Terpenes and terpenoids are synthesized through the Mevalonic Acid pathway within the cytoplasm of the cell (Nazzaro *et al*., 2013). Terpenes are composed of linked isoprene units, which are represented by the chemical formula $(C_5H_8)n$. However, the enormous variety in their chemical structures has led to several groups of classification, which include monoterpenes ($C_{10}H_{16}$) (Figure 2.2), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), and triterpenes ($C_{30}H_{40}$) (Swamy *et al*., 2016).

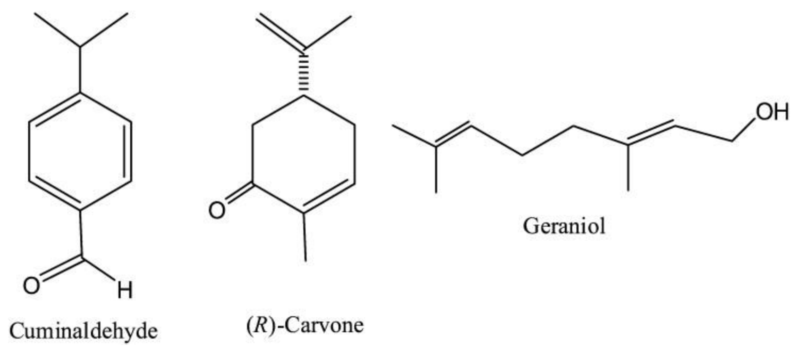

Figure 2.2: Chemical structures of the monoterpenes cuminaldehyde, R-carvone and geraniol[2]

Although EOs contain many sesquiterpenes (Chamorro *et al*., 2012), the most abundant constituents of EOs are the highly volatile monoterpenes (Russo *et al*., 2015). For example, monoterpenes present in lemongrass include geranial, neral and limonene. Spearmint oil contains carvone and limonene as the major constituents, while cinnamon leaf oil is composed mainly of eugenol and cinnamaldehyde. Eugenol is a volatile phenylpropanoid and not a terpene. Diterpenes

[2] https://www.sciencedirect.com/science/article/pii/S1226861510001263

are, due to their higher boiling point, absent from EOs. They can be extracted through the use of solvents into the non-volatile fractions (Turek & Stintzing, 2013).

2.3.3.3 Antimicrobial mode of action of EOs

The antimicrobial activity of EOs has been demonstrated against a wide range of bacteria, fungi and virusses (Swamy *et al*., 2016). Regnier *et al.* (2014) reported on the complete *in vitro* inhibition of the radial mycelial growth of the fungal fruit pathogen *Geotrichum citri-aurantii* by six EOs (*Citrus aurantium* var. *amara, Cymbopogon citratus, Cymbopogon martinii, Pelargonium graveolens roseum* Bourbon*, Organum vulgare and Thymus vulgaris*) out of fifty-eight EOs screened at a concentration of 1000 µL/L. The conidial germination of *Cladosporium cladosporioides* was significantly inhibited upon exposure to the EO isolated from the aerial parts of *Nepeta rtanjensis* (Grbić *et al*., 2011). They reported that the conidia of the organism were susceptible to the lowest concentration tested (0.1 µg/mL), causing maximum inhibition of the conidial germination (ID_{95}). Other studies have reported on the *in vivo* antifungal activities of EOs against fungi by inhibiting the growth of pathogens (Jhalegar *et al*., 2015; Liu *et al*., 2016; Souri & Bakhtiarizade, 2018).

The mode of action of a particular EO may differ depending on its chemical composition and the microorganism exposed to it (Dorman & Deans, 2000). Essential oils from a specific plant species may present chemotypic variations because of differences in the ecology, geography, age of the plant, as well as the time of harvest (Petretto *et al*., 2016). These variations in the chemotype contribute to the alteration in the biological activity of EOs (Bettaieb *et al*., 2014). For example, Petretto *et al*. (2018) observed that EOs from eight samples of *Cuminum cyminum* collected from three different geographical areas of Morocco had similar chemical composition; however, the proportion of the various components differed significantly depending on the origin of the plants. Therefore, the minimum inhibitory concentration (MIC) of the EOs against the tested bacteria differed based on their origin.

The principal mode of action of EOs involves the induction of cell death through the activation of two processes, namely apoptosis and/or necrosis (Sharifi-Rad *et al*., 2017). Apoptosis is a controlled, proactive, independent process that inflicts microbial cell damage upon application. In contrast, necrosis has been described as cell death resulting from exposure to environmental factors such as gases, temperature, pH fluctuations and water availability (Fink & Cookson, 2005). Both processes are results of cytotoxic effects of the EOs, arising from the complex interaction between different classes of compounds. In some cases, the main components are responsible for the activity, but this must be proven through individual testing of the pure compounds (Sharifi-Rad *et al*., 2017). The antimicrobial effect of EOs is facilitated by the lipophilic nature and low molecular weight of the chemical constituents of EOs (Machado *et al*., 2014; Russo *et al*., 2015). These characteristics permit lipophilic compounds in EOs to penetrate cell membranes, thereby causing changes to the membrane composition and increasing the fluid retention of the membrane, which ultimately results in leakage of ionic and cytoplasmic molecules (Solgi & Ghorbanpour, 2014). Changes in the membrane composition induce a reduction in ATP production, alteration of the pH gradient and hinder the proper functioning of the mitochondria, leading to cell death (Swamy *et al*., 2016). Some EOs display pro-oxidant element properties, leading to a change in the cellular redox potential, and subsequently compromise the survival of the cell (Sharifi-Rad *et al*., 2017). The cellular redox potential is the condition through which an organism balances the availability of electron (donors and acceptors) that play a critical role in metabolism and cell growth (Okegbe *et al*., 2014).

Relatively few investigations have scrutinised the mechanism of action of EOs against pathogenic fungi. It was demonstrated that contact of the EOs with fungi induces a loss of cell wall integrity, making it permeable to the plasma membrane whereby serious morphological alterations are caused in the hyphae (Sharifi-Rad *et al*., 2017). Dias Ferreira *et al.* (2013) observed significant damage to hyphal membranes and conidiophores of *Aspergilus flavus* using scanning electron microscopy, following exposure of the organism to EO of *Curcuma longa*. Disruption of the cytoplasmic membranes and intercellular organelles was observed by means of transmission electron microscopy when *Aspergillus niger* was exposed to EOs of *Thymus eriocalyx* (Ronniger) Jalas and *Thymus x-porlock* (Sharifi-Rad *et al*., 2017). Tolouee *et al*. (2010) proposed that the damage inflicted to the cell membranes of *Aspergillus niger* by EO isolated from the flowers of *Matricaria chamomilla* was the result of the increased cell permeability of the fungal plasma membrane after exposure. A reduction in the concentrations of ergosterol, the main sterol in the fungal cell membrane that maintains the integrity and the function of fungal cells, was measured after EO of *Cinnamomum jensenianum* was applied to *Aspergillus flavus* (Tian *et al*., 2015). In the same study, a disruption of the mitochondria in its internal structure was reported with a simultaneous decrease in the mitochondrial cristae. Other effects, such as alterations of the plasma membrane, fibrillar layer and cytoplasm were also observed (Tian *et al*., 2015). Zhou *et al.* (2014) demonstrated that the volatile compounds (citral, octanal and α-terpineol) caused loss of cytoplasmic content and distortion of the mycelial growth, thereby altering the morphology of *Geotrichum citri-aurantii*. Citral was also reported to strongly inhibit the mycelial growth of *Penicillium italicum*. The observation with the transmission electron microscope revealed loss of the cytoplasm and the presence of materials from outside of the cell wall, which was proven by the release of cell constituents, the extracellular pH, and the leakage of potassium ions (Tao *et al*., 2014). The same observations were also made after exposure of *Penicillium digitatum* to citral (Tao *et al*., 2014).

2.3.3.4 The use of EOs in combination

Several studies have investigated the effectiveness of the combination of EOs to combat microbial pathogens such as bacteria (De Rapper *et al*., 2013; Fratinia *et al*., 2017) and fungi (Regnier *et al*., 2014; Hossain *et al*., 2016). The individual application of EOs most often results in their usage at high concentration, which might affect the flavour of the product (De Azeredo *et al*., 2011). In addition, it is desirable to develop an antimicrobial with a multi-target antimicrobial effect that can be attained through mixtures (Regnier *et al*., 2014).

The addition of EOs in mixtures may even be applied at sub-inhibitory concentrations with the potential of providing the balance between sensorial acceptability and antimicrobial efficacy (De Azeredo *et al*., 2011). De Medeiros Barbosa *et al.* (2016) reported on the combined application of rosemary and oregano EOs at their sub-inhibitory concentrations (1:4 MIC: 5 µL/mL and 1:4 MIC: 0.6 µL/mL, respectively) which resulted in the inhibition of *Listeria monocytogenes*, *Escherichia coli,* and *Salmonella enteritidis in vitro*. Moreover, the *in vivo* results on fresh leafy vegetables (Lactuca s*ativa* L., *Beta vulgaris* L. var. *cicla* and *Eruca sativa* L.) revealed that there was no negative impact of the oils on the sensory attributes (appearance, texture, taste, odour, general perception and overall browning) of the vegetables after 72 h of refrigerated storage with the application of the EOs in combination at their tested sub-inhibitory concentrations. Thus, the strong odour of oregano EO that had affected the flavour and aroma of the leaves when applied alone, was changed to a distinctive, yet pleasant mint-like flavour upon mixing with rosemary EO.

However, the combining of EOs must be applied with care since not all combinations of EOs may result in a desirable outcome. For example, the combination of *Cymbopogon citratus* and *Lavandula angustifolia* essential oils yielded antagonistic results when tested *in vitro* against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans* (De Rapper *et al*., 2013).

2.3.3.5 Advantages of EO application against postharvest pathogens of fruits

Essential oils have many attributes that make them ideal for application to food products to prevent decay. Researchers have given considerable attention toward the use of EOs as food preservatives, because they are biodegradable, eco-friendly, economical and safe for human consumption (Sivakumar & Bautista-Banos, 2014). In addition, EOs are also well known for their antimicrobial properties (Sivakumar & Bautista-Banos, 2014). They exert antimicrobial activity against a wide range of bacteria and fungi growing on food products (Pandey *et al*., 2017). The build-up of resistance by pathogenic microorganisms upon continuous application of chemical synthetic fungicides may be overcome by the application of EOs, because of the complexity of their chemical compositions (Pavela & Benelli, 2016). Although EO application on food may result in the presence of some residual phytoconstituents on the commodity (Vendan *et al*., 2017), these residues might likely be eradicated by aeration as reported by Lee *et al.* (2003), whereby 24 h aeration was required to remove residues of eucalyptol on stored wheat. Moreover, the chemical constituents of EOs are generally regarded as safe (GRAS) for any of their applications (Smith *et al*., 2005).

The wide availability of EOs also constitutes one of the major advantages toward their application as food preservatives. In a review on the biological activities of EOs published by Sharifi-Rad *et al*. (2017), it was mentioned that about 3000 EOs have been described so far. These EOs can be tested against groups of pathogens that affect a specific crop. The selection of EOs that contain one or two main active components make it easier to control for quality control purposes (Chamorro *et al*., 2012).

2.3.3.6 Constraints in the application of essential oils

Nevertheless, despite the multiple advantages that EOs offer to industry there are also some concerns regarding their application. Considering the volatility of EOs, limited persistence of these volatile compounds would be observed under field conditions, thereby providing low effectivity over time towards pathogens that could re-infect the treated crop (Koul *et al*., 2008; Pavela & Benelli, 2016). The application at high concentrations may interfere with the natural aroma compounds of the fruit, with esters, alcohols and ketones present in the EO affecting their flavour and aroma (Ponce *et al*., 2008). The risk for developing allergies is also increased for packhouse workers, from continuously inhaling EOs. Some cases of EOs causing allergic contact dermatitis have been reported (Bleasel *et al*., 2002; Sarkic & Stappen, 2017).

Moreover, the cost of some EOs is relatively high, therefore a limiting factor for their use.

Problems arising from the volatility of the EOs constituents can be circumvented by preparing products that encapsulate the EOs or entrain them in a suitable coating matrix to facilitate the slow release of the active components, thereby offering prolonged protection. If both the matrix and the EOs used exhibit antimicrobial activity, it could yield a superior crop protection product.

2.3.4 Nanotechnology using metals

2.3.4.1 Nanotechnology involvement in agricultural crop protection

Nanotechnology implies the use of nanoparticles (NPs) defined as particles with sizes ranging from 1 to 100 nm (Li *et al*., 2017). These particles are used to prepare materials that provide altered physical and chemical properties of a substance at molecular levels (Fakruddin *et al*., 2012; Prasad *et al*., 2014). When a particle reaches the nanoscale level, its biological, chemical and physical properties differ to some extent to the fundamental ones, thereby new ways of exploiting the resulting product can be explored (Singh & Rattanpal, 2014). For this reason, nanotechnology, a relatively recent scientific approach, has gained much interest over the past decade and has been applied in various fields such as in medicine, biotechnology, electronics, materials science and in the energy sector (Parisi *et al*., 2014). In the biology-related field, nanotechnology has contributed through the manipulation of substances on a molecular, genetic, as well as on a cellular level, by incorporating knowledge and techniques of biological processes for use in medicine and agriculture (Fakruddin *et al*., 2012). According to Prasad *et al.* (2014), nanotechnology possess the potential to revolutionise agriculture and link fields such as aquaculture and food sectors through the molecular management of diseases, rapid disease detection, and by enhancing the ability of plants to absorb nutrients. Nano-biotechnology can also lead to the development of improved systems for nutrient or pesticide delivery to crops (Tarafdar *et al*., 2013). The small sizes of nanoparticles

(nanoparticles 10 to 100 nm) make them useful in biology, because of their similar size to microorganisms, proteins and deoxyribonucleic acid (DNA) which enables them to become compatible with and bind to the biological objects (Pershina *et al*., 2014).

In the postharvest field, nanotechnology has been applied in supply chain management with regard to the aspect related to food quality, handling, packaging and safety (Singh & Rattanpal, 2014). Several researchers have demonstrated the increasing trend and patents in agricultural nanotechnology, especially for disease management and crop protection (Sastry *et al*., 2010; Ocsoy *et al*., 2013; Patel *et al*., 2014).

2.3.4.2 Metals and metalloids used for nanoparticle synthesis

Over the past decades, several researchers have shifted their attention to utilising pure metals as antimicrobial agents (Yasuyuki *et al*., 2010; Khalid *et al*., 2014; Choi *et al*., 2018). These metals include silver (Ag), titanium (Ti), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), zirconium (Zr), molybdenum (Mo), tin (Sn), and lead (Pb). In a study conducted by Yasuyuki *et al.* (2010), metals such as cobalt, nickel, copper, zinc, zirconium, molybdenum, and lead were found to exhibit antimicrobial activity against both Gram-positive and Gram-negative bacteria. Khalid *et al*. (2014) found that selenium (Se), germanium (Ge) and lithium (Li) had the ability to inhibit the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Some metals, such as lead and tin, however, have potentially toxic effects on humans (Yasuyuki *et al*., 2010). There are only a handful of publications that elaborate on the antifungal effects of ionic metals. Silver in the ionic form has been used as an antifungal agent (Jo *et al*., 2009), but is better known as an antibacterial agent. Silver ions were reported to inhibit the growth of both *S. aureus* (Gram-negative) and *E. coli* (Gram-positive) (Jung *et al*., 2008).

Of the many metals used against microbial pathogens and for the synthesis of nanoparticles, silver is the most prominent and most widely researched. In addition to the various applications in which silver nanoparticles are involved in such as electronics, cosmetics, coatings, packaging, biotechnology and medicine (Khatoon *et al*., 2017), the substance can also be used in the management of plant diseases (Elgorban *et al*., 2016). Scientific reports have demonstrated that silver nanoparticles (SNPs) are highly toxic towards microorganisms such as viruses, bacteria and other eukaryotes (fungi and yeasts) at low concentration (Savithramma *et al*., 2012). The concentrations required to inhibit prokaryotic cells were found to be non-toxic to humans and animals with no side-effects (Joeng *et al*., 2005). In fact, SNPs have been reported to be less toxic than silver ions to the host (De Lima *et al*., 2012; Choi *et al*., 2018).

2.3.4.3 Silver nanoparticles antimicrobial mode of action

Silver nanoparticles have been reported to exert an antimicrobial activity towards a wide range of bacteria (Vilas *et al*., 2016) and fungi (Kim *et al*., 2012; Xia *et al*., 2016). For example, SNPs synthesised using a mixture of 2 M trisodium citrate dehydrate, and 0.504 g of polyvinyl pyrrolidone and 1 M sodium tetrahydridoborate as reducing agents inhibited the growth of *Alternaria alternata, Penicillium digitatum* and *Alternaria citri* by 83.88, 78.88 and 81.11% at 150 mg/L (maximum tested concentration) on the surface of Potato Dextrose Agar (PDA) (Abdelmalek & Salahaldin, 2016). Barzegar *et al.* (2017) reported that phytosynthesized SNPs incorporating *Salvadora persica* leaf extract caused an inhibition zone of 15.00 ± 0.00 mm and 14.00 ± 1.00 mm against *Aspergillus niger* and *Penicillium digitatum* respectively, at a concentration of 64 µg/mL on the surface of PDA medium.

The antimicrobial activity of SNPs is dependent on both the concentration of the nanoparticles and the microorganism that is exposed. For example, an *in vitro* experiment conducted by Venat *et al*. (2018) reported on the complete inhibition of the mycelial growth of *Sclerotinia sclerotiorum, Alternaria brassicicola* and *Botrytis cinerea* in the presence of craft colloidal silver at 7.5, 15 and 15 mg/L, respectively. In addition, more than 70% inhibition was observed of

Penicillium digitatum (76.19%) *and both Fusarium oxyporum* and *F. culmorum* (72.22%) at concentrations of 3.75 and 7.5 mg/L, respectively, while both *Aspergillus flavus* and *A. niger* were less susceptible to the silver colloidal with only 40.48% and 57.14% of mycelial inhibition reported, respectively, at 15 mg/L. According to Kim *et al*. (2012), an increase in the concentration of SNPs is associated with greater fungal inhibition. This was demonstrated by Pulit *et al*. (2013) who reported that an increase in nanosilver concentration from 25 to 50 mg/L, increased the inhibition of fungal growth of *Cladosporium cladosporioides* from 70% to 90%, and that of *Aspergillus niger* from 30% to 70%.

Although the mode of action by which SNPs exert antimicrobial activity has not been clearly elucidated, there are several theories that have been put forward (Prabhu & Poulose, 2012). There is substantially more information available on the antibacterial mode of action (Prabhu & Poulose, 2012; Ahmed *et al*., 2016) than the antifungal one. This lack of information was highlighted by Abdelmalek and Salahaldin (2016).

What has been established is that there are some parallels between the action of SNPs against bacteria and fungi in terms of the damage to cell walls. A review article on the antibacterial mode of action of SNPs published by Prabhu and Poulose (2012) revealed that SNPs may attach to the bacterial cell wall, consequently penetrate it and then induce structural changes within the cell membrane. Such changes include the permeability of the cell membrane, thereby causing death of the cell. An electron microscopy analysis conducted by Xia *et al.* (2016) to assess the effect of SNPs against the yeast *Trichosporon asahii*, revealed that the particles damaged the cell wall and cell membrane, thereby penetrating inside the cells. Further damage to organelles such as mitochondria and ribosomes was observed, as well as condensation and margination of chromatin that induced apoptotic cell death.

Kim *et al.* (2007) elaborated on the electrostatic attraction between the negative charge of the cell membranes of microorganisms (bacteria, viruses, and fungi), and the positive charge of natural products that also plays a role in the antimicrobial mechanism of actions of natural product-mediated SNPs. The electrostatic interaction between SNPs (positively charged) and the functional groups (negatively charged) of the proteins of the bacterium *Escherichia coli* led to protein denaturation, thereby inducing cytotoxicity to the microorganism (Raffi *et al*., 2008).

2.3.4.4 Synthesis of SNPs using EOs as reducing and stabilising agents

There are various methods by which SNPs are synthesised. The physical methods make use of physical energies (e.g. evaporation condensation, laser ablation, radiation), while chemical approaches (mostly used for the production of SNPs) involve the reduction of metal salts in solution by means of various chemical reducing agents (e.g. sodium borohydride) (Shaik *et al*., 2018). The biological approach exploits available bio-resources such as algae, bacteria, fungi (Rauwel, 2015) and plant materials in preparing nanoparticles (Vilas *et al*., 2014). However, the uses of physical and chemical-based methods are less environmentally friendly and cost effective at the industrial scale (Jeyalalitha *et al*., 2016). The biological methods have been investigated by several researchers as a potential means for the synthesis of metal nanoparticles (Abdelghany *et al*., 2017). These methods offer advantages that include non-toxicity, cost-effectiveness and enhanced production (Vilas *et al*., 2016). Although microorganisms have been used in the biological synthesis of SNPs, they are less popular than the use plant materials. The main disadvantage of using microorganisms over plant materials in the synthesis of SNPs is the slowness of the process (Prabhu & Poulose, 2012), to which Abdelghany *et al*. (2017) added the high cost required for the isolation and maintenance of microbial cultures (Singhal *et al*., 2011). In contrast, the use of readily available plant materials to rapidly synthesise stable nanoparticles (Makarov *et al*., 2014; Abdelghany *et al*., 2017) in a single step is an attractive alternative (Abdelghany *et al*., 2017). The main compounds involved in the reduction of silver in the plant- mediated synthesis are phytochemicals such as terpenoids, flavones, ketones, aldehydes, amides, and carboxylic acids (Sukumaran & Poulose, 2012).

Plant EOs, being composed of a variety of phytochemicals, have been reported for the successful biosynthesis of nanoparticles (Vilas *et al*., 2014; Vilas *et al*., 2016). Vilas *et al*. (2014) outlined a process in which SNPs were produced using EO isolated from *Myristica fragrans*. They later (Vilas *et al*., 2016) exploited the same methods using oils extracted from fresh leaves of *Coleus aromaticus.* The volume of the oil dilution solution was reported to play an important role in improving SNP synthesis.

2.3.5 The use of chitosan as a tool for the slow release of EOs

2.3.5.1 Overview information on chitosan

Chitosan (CHI), known chemically as poly-β-(1-4)-2-amino-2-deoxy-*D*-glucopyranose (Figure 2.3) (Yogeshkumar *et al*., 2013), is a deacetylated form of chitin extracted from the skeleton of crustaceans, such as shrimps (*Pandalus borealis*), crabs, and fungal cell walls (Arbia *et al*., 2013).

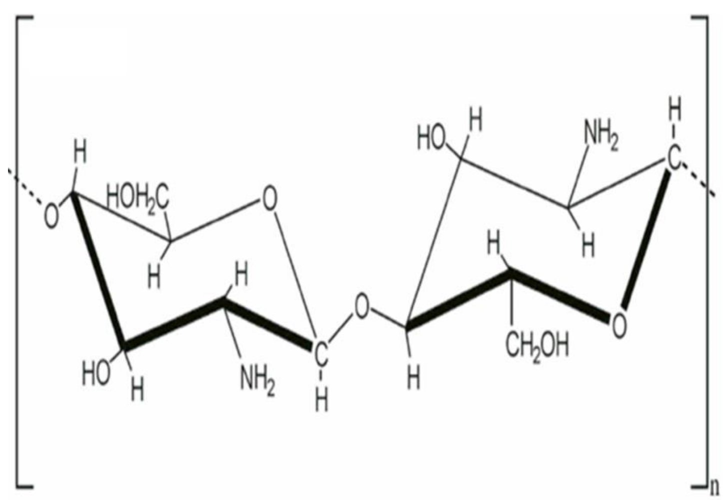

Figure 2.3: Chemical structure of chitosan[3]

Similar to cellulose and chitin polymers, chitosan polymers are also regarded as highly abundant natural polymers on the planet (Choudhary *et al*., 2016;

[3] https://www.researchgate.net/figure/263324738_fig2_Figure-2-Chitosan-chemical-structure

Hassan & Chang, 2017). Chitosan has been widely used, because of its well documented antimicrobial properties (Elsabee & Abdou, 2013). It is commercially available in different molecular weights, which include low molecular weight (LMW) of particles below 50 kDa, medium molecular weight (MMW) including particles between 50 and 150 kDa and high molecular weight (HMW), comprising particles bigger than 150 kDa (Jongsri *et al*., 2016; Sharma *et al*., 2016). Chitosan has been applied in many fields such as agriculture, medicine, tissue and bone engineering, the food sector, cosmetics, textiles, pharmaceutical nanostructure materials, biotechnology, the paper industry and in wastewater treatment (Sharma *et al*., 2016).

Some advantages of the use of chitosan include its biodegradability (Kean & Thanou, 2010), environmental friendliness, biocompatibility and non-toxicity (Castro & Paulin, 2012; Choudhary *et al*., 2016). Chitosan is edible without eliciting any adverse allergenic, mutagenic, or carcinogenic activities (Raafat *et al*., 2009). Furthermore, the US Environmental Protection Agency (EPA, 2008) has indicated that chitosan does not have an adverse impact on the environment. Chitosan polymers possess antimicrobial and film-forming properties (Badawy & Rabea, 2009; Xing *et al*., 2016) rendering it appropriate to surface protecting applications. All these advantages show that chitosan is an ideal substance for application to food products (Choudhary *et al*., 2016).

In the postharvest sector, chitosan is employed as an edible or film coating matrix and plays a major role in the food packaging industry. An edible coating is a thin layer of edible material applied in liquid form onto food, usually by immersing the product in a solution (Frazao *et al*., 2017). Chitosan has been applied to fruits such as citrus (Chien *et al*., 2007; Shao *et al*., 2015), blueberries (Yang *et al*., 2014), strawberries (Perdones *et al*., 2014; Khalifa *et al*., 2016), cherry tomato (Zhang *et al*., 2015). Fruits subjected to treatment with chitosan were immersed into the substance and air-dried at room temperature, as was the case for citrus (Mahmoud *et al*., 2017) and cherry tomato (Zhang *et al*., 2015), or on a nylon filter to drain excess liquid, as it was performed on grapes (Dos Santos *et al*., 2012; Guerra *et al*., 2016) and cherry tomato (Guerra *et al*., 2015).

It has been demonstrated that chitosan has antimicrobial activity against several fruit pathogens (Palou *et al*., 2015). On citrus, chitosan demonstrated an antimicrobial effect against *Botrydiplodia lecanidion*, *Botrytis cinerea* and both *Penicillium digitatum* and *P. italicum* (Chien *et al*., 2007). The coating of fruits with chitosan offers the advantages of retaining the nutritional quality of fruit and prolonging its shelf life (Kerch, 2015). Its acts by decreasing the response of the fruit to environmental conditions by reducing gas exchange, which retards ripening, loss of weight and water, respiratory rates and ethylene production (Jitareerat *et al*., 2007). In addition, it also provides a defensive mechanism through the induction of several defensive-related enzymes leading to the prevention of antagonist pathogen infection (Zhang *et al*., 2011; El Guilli *et al*., 2016).

2.3.5.2 Chitosan-coated EOs in postharvest application on fruits

The modern trends suggest the application of some natural polymers to be used as coating matrix instead of commercial wax to avoid formulations of synthetic components (e.g. polyethylene wax, ammonia, morpholine) (Palou *et al*., 2015). The use of chitosan incorporating natural additives such as EOs has been under examination (Dos Santos *et al*., 2012; Guerra *et al*., 2016). The traditional practice by which antimicrobial agents were added directly to foods have demonstrated some limitations which include a decrease in the efficacy as a result of interference by substances in the food itself, the poor solubility of EOs due to their lipophilic nature (Frazao *et al*., 2017), the volatility of the oil components, leading to the application of high concentrations, thereby negatively affecting the food product (e.g. affecting the taste and aroma) (Dos Santos *et al*., 2012). In addition, better results are generally reported for *in vitro* than *in vivo* experimental conditions (Frazao *et al*., 2017). The combination of chitosan and EOs has potential for application as a fruit coating to allow release of the EO over an extended period. As an illustration, Dos Santos *et al.* (2012) reported on the use of CHI and *Origanum vulgare* EO at sub-inhibitory concentrations, which significantly inhibited the postharvest pathogenic fungi *Rhizopus stolonifer* and *Aspergillus niger,* as well as normal fungal flora on table grapes stored at room and cold temperatures. The shelf-life of strawberries was increased by 21 days by coating with a chitosan-based formulation incorporating limonene (Vu *et al*., 2011). The application of chitosan encapsulated EO by Dos Santos *et al*. (2012) demonstrated that the sensorial impact resulting from the application of EOs alone at high concentrations on fruits was minimised. No negative effects on the physical and physicochemical aspects of the grapes were reported, while the sensory characteristics of the grapes improved during the storage period. The maintenance of quality and shelf-life extension of sweet peppers using chitosan coated cinnamon oil solution by Xing *et al.* (2011), led the team to conclude that such the combination of chitosan and EOs applied as a coating could be considered for commercial application and storage.

2.3.5.3 Antimicrobial mechanism of action

Since the mechanism of action through which chitosan exerts its antimicrobial activity is influenced by various factors such as the type of microorganism (Gram-positive bacteria, Gram-negative bacteria, chitosan-sensitive fungi and chitosan-resistant fungi), the molecular weight and the degree of deacetylation of the chitosan, the exact mechanisms of action remains elusive (Verlee *et al*., 2017). Other factors that affect the activity include derivative form, concentration and the source of origin (Hosseimnejad & Jafari, 2016), as well as experimental conditions such as pH, concentration, incubation medium, and method of chitosan sample preparation (Kulikov *et al*., 2014).

However, it has been confirmed that chitosan leads enhances the permeability of the cell membrane through electrostatic interactions between the positively charged protonated amino-groups of chitosan and the negatively charged molecules at the cell surface. The greater permeability causes leakage of intracellular material, inhibition of DNA/RNA synthesis and disruption of protein synthesis, leading to cell death (Verlee *et al*., 2017).

The antifungal activity obtained using low molecular weight (LMW) chitosan by Park *et al*. (2008) suggests that the major target site of chitosan is the plasma membrane of the microorganism. The cells of *Candida albicans* and *Fusarium oxysporum* treated with LMW chitosan for 4 h underwent disruption of the cell surface using scanning electron microscopy. Shao *et al.* (2015) supported their hypothesis after chitosan damage to the structure of the cell walls of *P. digitatum* hyphae was observed using transmission electron microscopy. They reported plasmalemma separation from the cell wall, disappearance of organelles and mitochondria, increased vacuolation, and loss of cytoplas, when compared to the control which exhibited a normal cell wall and organelle structures (uniform cellular cytoplasm, clearly visible internal organelles, and regularly-shaped mitochondria) (Shao *et al*., 2015).

In addition to the disruption of the cell membrane, Márquez *et al*. (2013) reported on the deletion of genes involved in membrane transport and cell wall functions upon application of LMW chitosan (0.75 µg/µL) against the yeast *Saccharomyces cerevisiae*. The membrane disruption properties of LMW chitosan was confirmed by mild leakage of carboxyfluorescein-loaded liposome. The carboxyfluorescein was encapsulated in large unilamellar vesicles to observe the membrane disruption potential of LMW chitosan in the yeast. The results were attributed to the electrostatic interactions that occur between negatively charged phosphate groups on DNA or carboxyl groups on proteins and positively charged amino-groups from the N-glucosamine of chitosan. Furthermore, the ability of chitosan to inhibit the growth of *Alternaria alternata*, *Penicillium expansum* and *Rhizopus stolonifer* by causing aggregation and morphological changes in the mycelia *in vitro* was proposed to be a consequence of the direct interaction of chitosan on the fungal cell wall, due to its polycationic nature (De Oliveira *et al*., 2012).

In fruits, chitosan inhibits pathogenic fungi by inducing the increased production of host defense-related enzymes in chitosan-coated fruits (Edirisinghe *et al*., 2014). For example, the chitinase activity was found to be higher in mandarin fruits cv.

“Ortanique” coated with chitosan and inoculated with green mould infection, than on the untreated control, chitosan-coated fruit (uninfected) and fruit without coating, but infected (El Guilli *et al*., 2016).

The mechanisms of action through which the EO-mediated chitosan exerts antifungal activity remain unclear (Mohammadi *et al*., 2016). Several articles discuss the antimicrobial activity of both substances (EO and chitosan) separately (Dos Santos *et al*., 2012; Guerra *et al*., 2016). However, Shao *et al*. (2015) provided evidence of the enhanced activity of the combination EOs and chitosan by observing the impact of both substances individually, and in a mixture, against green mould using transmission electron microscopy. The combined treatment caused greater damage to the structure of the fungal cell than the individual treatments. The antifungal mechanism of the EO combined with that of the chitosan provide a more complex action that increases the susceptibility of the fungal pathogen.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

The development of resistance by postharvest pathogens against synthetic fungicides, approved for application worldwide, is of concern for the citrus industry (Feng *et al*., 2011, Erasmus *et al*., 2015). Over the past two decades, numerous research teams have focused on the use of plant extracts, including volatile organic compounds in the form of essential oil (EOs), as alternatives to conventional chemicals for the inhibition of postharvest pathogens in citrus (Tripathi & Dubey, 2004; Liu *et al*., 2009; Regnier *et al*., 2014). Although the application of EOs were found to be a viable and cost-effective option for the control of sour rot and both green and blue moulds (Regnier *et al.,* 2014), the use of EOs is still limited due to the lack of research in commercial environments. The efficacy of adding encapsulated EOs to coatings must also be evaluated. Several studies have reported on the antifungal potential of various substances, such as chitosan (Chien *et al*., 2007; El Guilli *et al*., 2016) and silver nanoparticles (Jo *et al*., 2009; Xia *et al*., 2016). Guerra *et al*. (2016) reported the antifungal activity of EO-amended chitosan, while Vilas *et al*. (2016) found nanomaterials resulting from EOs (reducing and stabilizing agents) and silver nitrate ($AgNO_3$) solution to be safe and cost-effective antimicrobial agents. In this study, the antifungal activities of cinnamon leaf, lemongrass and spearmint EOs were tested *in vitro* and *in vivo* against sour rot and green mould. Various methods were applied to evaluate the potential effect of the EOs *in vitro* i.e. toxic media, microtitre and macrodilution assays. Chitosan and wax were used to establish the antifungal activities of EO-supplemented coatings. Moreover, the antifungal effect of silver nitrate alone, and in combination with EOs, as well as of silver nanoparticle-mediated EO structures were also assessed against both pathogens. The *in vivo* experiments were conducted at the Citrus Research International (CRI) facility using ‘Valencia’ oranges and *in vivo* trials on kumquat

(*Citrus japonica* var. *nagami*) were conducted at a commercial packhouse in Levubu. The methods are detailed in the following sections.

3.2. STANDARDS, SOLVENTS AND CHEMICALS

The list of standards, solvents and chemicals used in this study are summarised in Table 3.1. To ensure consistency in the quality of the EOs tested, all EOs were purchased from the same supplier (Holistic Emporium, Johannesburg, South Africa).

Table 3.1: Chemicals and solvents used in the study

Standards, solvents and chemicals	Company
Acetone Methanol Glacial acetic acid Ethanol Sodium hydroxide Hydrochloric acid Potato Dextrose Agar	Merck (Pty) Ltd, Johannesburg, RSA
Tween 80	Merck, Hohenbrunn, Germany
Ringer tablets Lactophenol Blue	Merck, Darmstadt, Germany
Silver nitrate Hexane Chitosan (Low molecular weight) Sabouraud Broth *p*-iodonitrotetrazolium violet (INT) Potato Dextrose Broth	Sigma Aldrich, Aston Manor, Johannesburg, RSA
Guazatine Imazalil	Makhteshim-Agan (Pty) Ltd, RSA
Chloramphenicol	Navartis SA (Pty) Ltd, Kempton Park, RSA
Cinnamon leaf EO Lemongrass EO Spearmint EO	Holistic Emporium, Johannesburg, RSA

All the chemicals and reagents purchased from Merck and Sigma Aldrich were analytical grade.

3.3 FUNGAL ISOLATES

The fungal cultures [*Galactomyces citri-aurantii* (sour rot) and *Penicillium digitatum* (green mould)] used in this study were obtained from Drs Arno Erasmus and Wilma Du Plooy [Citrus Research International (CRI), Nelspruit, Mpumalanga Province, South Africa], respectively. The isolates were sub-cultured on Potato Dextrose Agar (PDA, Oxoid, Johannesburg, South Africa) and incubated at 25 °C for eight days. Fungal spore suspensions were obtained by scraping the plate surface with a sterile L-shaped glass rod. Each inoculum was then filtered through a sterile cheese cloth placed on a sterile funnel for the removal of the hyphae. The filtrates were then collected and transferred to Schott bottles, containing a solution of one quarter (¼)-strength Ringer's solution. The inoculum concentration was subsequently adjusted to 1×10^8 and 1×10^6 spores/mL for sour rot and green mould, respectively, corresponding to an optical density (OD) of 0.14. The enumeration was carried out using an UV-visible spectrophotometer (Thermo-Spectronic, Helios, Gamma 9423 UVG 1002E, England) set at 425 nm, with ¼-strength Ringer's solution used as a blank. Spore suspensions of sour rot were supplemented with 20% freshly prepared orange juice obtained by squeezing the surface-sterilized (70% aqueous ethanol) orange fruit in a sterile beaker. The content of the beaker was transferred to Falcon tubes (50 mL) and centrifuged for 10 min at 5 °C (7000 rpm). Thereafter, the supernatant was filtered through a 0.45 µm filter disc (Minisart®; Sartorius Stedim, Germany).

The virulence of the isolates was regularly tested (Koch's postulate) on four surface-sterilised (70% aqueous ethanol) 'Valencia' oranges by dipping a steel rod with a 1 mm-wide and 2mm-long tip into the inoculum suspension, before making a single puncture in each fruit as described by Smilanick *et al*. (2008).

3.4 ESSENTIAL OIL ANALYSIS

The composition of the selected EOs (cinnamon leaf, lemongrass and spearmint) was performed using gas chromatography (GC; Model 6890N) coupled simultaneously to a 5973-mass spectrometer (MS) and a flame ionization detector (FID; Agilent Technologies, Illinois, USA) as described by Combrinck *et al*. (2010). A solution consisting of a 200 µL aliquot of each EO sample diluted with 800 µL hexane was prepared. A volume of 1.0 µL aliquot of each prepared sample was injected using an autosampler into the inlet of the GC, set to 250 °C and with a split ratio of 200:1 applied. The GC system was equipped with a HP-Innowax polyethylene glycol column (60 m x 250 µm internal diameter x 0.25 µm film thickness). The initial temperature of the oven was 60°C for 10 min, thereafter raised to 220 °C at a rate of 4 °C/min (held for 10 min), and then increased to 240 °C at a rate of 1 °C/min. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. Spectra were obtained over the range *m/z* 35 to 550 in electron impact mode by applying an ionization potential of 70 eV. The FID, set at 250 °C, was used to obtain the relative percentage areas of individual components present in the analysed oil. Identification of the sample components was confirmed by comparing their retention times to those of authentic standards and to mass spectra in the NIST®, Mass Finder® and Flavor® spectral libraries. The relative retention indices (RRI) for each compound were compared to those obtained for the authentic standards on the same stationary phase.

3.5 *IN VITRO* EVALUATION OF THE SELECTED ESSENTIAL OILS

3.5.1 Optimisation of the inhibitory activities of cinnamon leaf, lemongrass and spearmint EOs

3.5.1.1 Toxic medium technique

Potato dextrose agar medium amended with chloramphenicol (half a capsule for 1 L) [Novartis South Africa (Pty) Ltd, Kempton Park, South Africa] was prepared and supplemented with the three individual EOs at concentrations of 100, 250, 500, 750 and 1000 µL/L.

Essential oils were combined (lemongrass/spearmint, lemongrass/cinnamon leaf and cinnamon leaf/spearmint) in a 1:1 ratio at final concentrations of 250, 500, 750 and

1000 µL/L. The lowest concentration of the mixture, displaying complete inhibition, was further assessed at various ratios (9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8 and 1:9). The experiment was conducted to establish the optimum ratio at which the combined EOs would provide complete inhibition of the pathogens.

All test substances were pre-mixed with two drops of surfactant (Tween 80) using a Pasteur pipette (Deltalab, Barcelona, Spain). The mixture was added to the lukewarm PDA and mixed thoroughly before preparing Petri dishes (N=5). Negative controls consisted of PDA plates containing surfactant, but without any supplementation, whereas guazatine and imazalil (250 and 500 µL/L) were added to the medium and served as positive controls. An aliquot (10 µL) of the spore suspension was placed on the center of the set agar and the Petri plate was sealed with a strip of Parafilm [Merck (Pty) Ltd, South Africa] as described by Regnier *et al*. (2014). The experiment was repeated four times to assure the consistency of the results. For each repetition, five replicate (N=5) were used for each oil tested. After incubation for 6 - 7 days at 25 °C, the mycelial growth (mm) was measured with a 150 mm digital calliper-KTV150 (Major Tech Pty Ltd, Johannesburg, South Africa) (Regnier *et al*., 2008). The percentage inhibition of mycelial growth relative to the control was determined as described by Plaza *et al*. (2004). Average values and standard deviations for replicate measurements were determined using Microsoft Office Excel 2010 Version 14.0. No statistical analysis was performed to determine whether differences were significant or not, since total pathogen control was the sole criterium used to determine efficacy.

3.5.1.2 Microtitre assay

The experiment was performed to quantify the inhibitory activity of the EOs. The test substances (EOs) were diluted by pre-mixing with 100 µL Tween 80 to yield a concentration of 64 and 48 µL/L in Falcon tubes containing Sabouraud Broth [Sigma Aldrich (Pty) Ltd, Johannesburg, South Africa]. The final volume of the solution was 10.00 mL. The oils were tested individually, and in combination (1:1). After transferring 100 µL of the broth to each well, serial dilutions were performed by transferring 100 µL of each well content to the adjacent well, to yield concentrations of

32, 24, 16, 12, 8.0, 6.0, 4.0, 3.0, 2.0, 1.5, 1.0 and 0.75 µL/L. Subsequently, 50 µL of fungal spore suspension of sour rot (1 x 10^8 spores/mL) or green mould (1 x 10^6 spores/mL) were dispensed into the wells. The positive control (without antimicrobial agent) consisted of Sabouraud Broth amended with Tween 80 and 50 µL of fungal spore suspension. After covering with the lid and sealing with Parafilm, the microtitre plate was incubated at 25 °C for 24 h in a shaking incubator (Incoshake 355, Labotec, South Africa), which was allowed to shake at 40 rpm throughout the incubation period, to avoid spore deposition on the surface of the well. Thereafter, 0.4 mg/mL of *p*-iodonitrotetrazolium violet (INT) (Sigma Aldrich, Aston Manor, Johannesburg, RSA) solution was added to each well (40 µL) and left to incubate for a further 24 h at 40 rpm. The lowest dilution where no colour change was evident was considered as the minimum inhibitory concentration (MIC) for that oil. The assays were carried out in triplicate to ensure the accuracy of the results. The experiment was repeated twice every week within a period of two months. A duplicate (N=2) of each substance tested was performed upon repetition of the experiment.

3.5.1.3 Spore germination assay

The effect of the EOs on the germination of arthroconidia was determined using the microtitre assay technique as described in Section 3.5.1.2. After the 24 h period of incubation, 10 µL of the suspension was transferred to a glass slide. The spores were fixed using Lactophenol Blue (Merck KGaA, Darmstadt, Germany) and observed with a BH-2 light microscope (Olympus BX43F, Tokyo, Japan) at a 40x magnification. The experiment consisted of three replicate (N=3) over subsequent repetition (three times).

3.5.1.4 Fungicidal effect of the EOs

A portion of each well content (10 µL) was inoculated onto the surface of freshly prepared PDA medium. The plates were incubated for 4 days at 25 °C and examined for the presence of mycelial growth. The experiment consisted of three replicate (N=3) over successive repetition (three times).

3.5.2 Evaluation of the ability of silver nitrate and its combination with individual EO to inhibit both pathogens

Solutions of silver nitrate [0.10, 0.050 and 0.010% (m/v)] were prepared and used as solvent by adding PDA powder. After autoclaving, the medium was allowed to cool to 50 °C in a preset water bath [Lasec SA (Pty) Ltd, South Africa] and then transferred to pre-labeled Petri dishes. Lemongrass (100 µL/L), cinnamon leaf (250 µL/L) and spearmint (500 µL/L) EOs were added to the prepared agar-amended silver nitrate solutions (0.050 and 0.010%). The control consisted of PDA medium without any supplementation.

The agar-amended silver nitrate and silver nitrate/EO mixtures were allowed to set in Petri dishes. Thereafter, a fixed volume of a spore suspension (10 µL) prepared from an eight-day pure culture of the pathogens was placed aseptically onto the center of each plate (N=5). This experiment was performed three times. The plates were incubated at 25 °C for eight days and the mycelial growth was recorded as described in Section 3.5.1.1.

3.5.3 Synthesis of silver nanoparticles (SNPs) by means of EOs

The method of Villas *et al*. (2014) was followed, with minor modifications, for synthesis of SNPs. A concentration of 2.14×10^{-4} M of silver nitrate (AR grade) was prepared by dissolving 0.0364 g silver nitrate in 1000 mL of distilled water. The solution was stored in an amber bottle to avoid autoxidation of silver. The solution was left for an hour to stabilise. Each batch of SNPs was prepared from a fresh solution. The EOs of cinnamon leaf, lemongrass and spearmint were diluted using acetone (1:170 v/v) for the synthesis of the SNPs. Volumes of 1.0, 2.0, 3.0, 4.0 and 5.0 mL of the diluted EOs were added to separate measured volumes of 30 mL of 2.14×10^{-4} M silver nitrate at 100 °C and pH 7. After vigorous stirring, the colloids were obtained. The experiment was repeated individually at pH 8, 9 and 10 to obtain colloids. A colour change from colourless silver nitrate solution to yellow and then to brown, was observed, indicating the formation of SNPs by EO (Villas *et al*., 2014).

3.5.4 Characterization of the synthesised SNPs

3.5.4.1 UV spectrophotometer analysis

Samples were scanned over the wavelength range 200-600 nm using a UV-vis spectrophotometer (Helios, England) to determine the optimum wavelength for measuring the production of SNPs. Prior to sample analysis, unheated silver solution with EOs was used as the blank. Spectral analysis was done to confirm the formations of SNPs. Samples were placed in quartz cuvettes for the analysis.

3.5.4.2 Fourier Transform infrared (FTIR) analysis

The infrared spectra of the SNPs were recorded in the range 4000-400 cm^{-1} (wavenumbers) using a PerkinElmer spectrometer (Spectrum 65), mounted with a universal attenuated total reflectance (UATR) diamond crystal. The liquid sample was placed directly onto the surface of the diamond crystal, and the liquid was allowed to evaporate before acquiring the spectrum in the absorbance mode after background correction. Spectra were obtained and subsequently analysed using PerkinElmer Spectrum software. A total of four automated scans were accumulated for each sample with a spectral resolution of 4 cm^{-1}. The analyses were done in triplicate.

3.5.4.3 Scanning electron microscope

The suspension containing lemongrass-mediated synthesized SNPs was freeze-dried to perform the analysis with the scanning electron microscope (SEM). The freeze-dried powder was analysed at the National Metrology Institute of South Africa (Council for Scientific and Industrial Research, Pretoria, South Africa), using a Leo 1525 FE SEM. The size distributions of SNPs were determined on SEM image by means of imageJ software (Pillai *et al*., 2013).

3.5.4.4 *Energy-dispersive spectroscopy*

Energy-dispersive spectroscopy (EDS) was used to confirm the presence of silver in the particles. The analysis was carried at the National Metrology Institute of South Africa (Council for Scientific and Industrial Research, Pretoria, South Africa), using a Leo 1525 FE SEM.

3.5.5 *In vitro* anti-fungal assay to evaluate the synthesised EO-silver nanoparticles

Various volumes (100, 200, 300, 400 and 500 µL) of each synthesised SNP suspension were transferred to sterile Eppendorf tubes, containing 100 µL of the spore solution (green mould or sour rot) in Potato Dextrose Broth (PDB). The control was prepared from the same volume of spore suspension with 100 µL of the PDB alone. The Eppendorf tubes were incubated in a shaking incubator at 25 ± 1 °C for 24 h at 40 rpm. Thereafter, the suspensions were subjected to two analyses. The first consisted of inoculating the surface of the agar, set in Petri plates, with a 5 µL aliquot of spore suspension. The plates were incubated at 25 ± 1 °C, and evaluated after one and four days of incubation to determine the mycelia growth. The second analysis involved the immediate observation of possible spore germination using a microscope. A 10 µL portion of the suspension was transferred to the surface of a glass slide, mixed with a drop of Lactophenol Blue and covered with a glass slip. The samples were examined with a BH-2 light microscope (Olympus, Japan) at a 40x magnification. The experiment was performed in triplicate for every repeated (three times) attempt.

3.5.6 Preparation and testing of EO-coated chitosan solutions

3.5.6.1 *Determination of the minimum inhibitory concentration (MIC) for chitosan and EOs*

The MICs for chitosan and EOs, were determined using the broth macrodilution technique.

Chitosan solution was obtained by dissolving the polymer (30 mg/mL) in 1% (v/v) glacial acetic acid for 24 h at 25 °C (120 rpm). Serial dilutions (1:1) were performed in Sabouraud Broth to obtain solutions of 30.0, 15.0, 7.50 and 3.75 mg/mL, which were tested against green mould and sour rot.

Concentrations of 3000 and 2000 µL/L of the EOs (lemongrass and spearmint) were prepared in tubes containing Sabouraud Broth. The oils were pre-mixed with Tween 80 (100 µL in Eppendorf tubes) before dissolving in Sabouraud Broth. Serial dilutions (1:1) were performed using the same broth media to obtain solutions with concentrations of 3000, 2000, 1500, 1000, 750, 500, 375, 250 and 125 µL/L, which were tested against the pathogens in the absence of chitosan.

The final volume in the tubes containing different concentrations of the chitosan and EOs was 5.0 mL. Then, 1.0 mL of freshly prepared fungal suspension (1 x 10^8 spores/mL for sour rot and 1 x 10^6 spores/mL for green mould) and 4.0 mL of Sabouraud Broth were added to the tubes. The concentration was adjusted to 10 mL, meaning the final concentration to which the substances were tested against both pathogens were 15.0, 7.50, 3.75 and 1.88 mg/mL for chitosan and 1500, 1000, 750, 500, 375, 250, 187.5, 125 and 62.5 µL/L for EOs. The solution was incubated at 25 °C for 7 days. After the incubation period, test tubes were observed for turbidity in the medium. The lowest concentrations of chitosan and EO that exhibited no visible fungal growth (no turbid medium) were considered the MIC (Sharma & Tripathi, 2008). In the control experiment, the fungal suspension was inoculated in Sabouraud Broth without supplementation with chitosan or EO. The experiment was carried out in triplicate to ensure consistency in the results.

3.5.6.2 Antifungal assay of the oils coated with chitosan

The oil-coated chitosan solutions were prepared by making use of the MIC recorded from the chitosan and EOs. Chitosan solutions were obtained by dissolving 7.50 and 3.75 mg/mL of the polymer (both values represented the MIC and sub-MIC, respectively) in 1% (v/v) glacial acetic acid. The solutions were left to stir for 6 h at

25 °C. Thereafter, lemongrass and spearmint EOs were added at their sub-MIC values (value below the MIC) to the stirred chitosan depending on the pathogens. The final concentrations at which chitosan and EO solutions were prepared are presented in Table 3.2.

Table 3.2: Concentrations of EOs (lemongrass and spearmint) encapsulated in chitosan matrix

Test substances	**Concentration** CHI (mg/mL) / EO (µL/L)	
	Galactomyces citri-aurantii	***Penicilium digitatum***
Chitosan / lemongrass	7.50 / 125	7.50 / 250
	3.75 / 125	3.75 / 250
	7.50 / 188	7.50 / 375
	3.75 / 188	3.75 / 375
Chitosan / spearmint	7.50 / 250	7.51 / 375
	3.75 / 250	3.76 / 375
	7.50 / 375	7.50 / 500
	3.75 / 375	3.75 / 500

Tween 80 (1%) was then transferred to the solution to enable solubility of the EO. The solution was left to stir at 120 rpm for an additional 18 h at 25 ± 1 °C (Dos Santos *et al*., 2012).

The chitosan/EO solutions were tested by transferring 5 mL of each prepared chitosan/EOs solution into test tubes, followed by 1 mL of the pathogen and 4 mL of the Sabouraud Broth. The total volume in the tube was 10 mL. The tubes were incubated at 25 °C for 7 days. After, incubation period, the tubes were analyzed for the presence of turbidity. Three replicates (N=3) were used for each solution of chitosan/EO.

3.5.6.3 Effect on mycelial growth

Inhibition of fungal mycelial growth (dry mass weight) was determined by means of the toxic growth substrate technique (dilution in broth) in triplicate. A 10 mL volume of each spore suspension was transferred to a 250 mL Erlenmeyer flask containing 40 mL of Sabouraud Broth and supplemented with 50 mL of different oil-coated concentrations. The flasks were incubated at 25 °C and evaluated after 3, 5 and 7 days. The mycelia were obtained by filtering the suspension through a pre-weighed paper filter (Sartorious AG, Goettingen, Germany) using a peristaltic pump (Merck Millipore, Germiston, South Africa). The emptied flask containing the suspension was rinsed with a suitable volume of chloroform [Merck (Pty) Ltd, Modderfontein, Gauteng, South Africa], which was then filtered to rinse the mycelia on the surface of the filter paper. Thereafter, the filter paper was placed in an oven (55 °C) for 10 min, cooled and weighed. In the control experiment, the fungal suspension was inoculated in Sabouraud Broth without chitosan/EO. The results were expressed as percentage inhibition of the fungal mycelial mass relative to the control, obtained at the three different time intervals.

3.6 *IN VIVO* EXPERIMENTAL PROCEDURES

3.6.1 Semi-commercial trial using EOs

3.6.1.1 Efficacy of selected EOs applied as a dip

Untreated export-quality citrus fruit (Valencia orange) were obtained by Citrus Research International (CRI) during the 2015 season. The fruit were evenly sized and without visual damage. On the day of arrival, the freshly harvested fruit were washed with tap water; surface-sterilized using ozone as per the standard procedure followed by CRI, and allowed to dry. Thereafter, the fruits were kept overnight at ambient temperature (22 ± 1 °C).

In this experiment, fruits were treated curatively only. No preventative treated was applied. The term ‘curative’ is used when the treatment is applied after the artificial wounding and infection of the fruit have taken place. On the other hand, preventative treatment involves the applying of the protective substance before the infection of the fruit takes place.

The fruit were inoculated 24 h and 6 h prior to the application of the EO dips to evaluate the efficacy of the treatments towards green mould and sour rot. A cylindrical rod, with a 2 mm long circular tip and 2 mm in diameter, was used for wounding the rind, for the inoculation of green mould, (Figure 3.1a), while a screwing hook (13 mm protruding tip) was used for inoculation in the case of sour rot (Figure 3.1b). The hook was applied in such a way that the rind was perforated, without reaching the pulp. Four wounds were inflicted equidistantly around the calyx by dipping the rod and hook into a freshly prepared spore suspension of *P. digitatum* (1×10^6 spores/mL) (Kellerman *et al*., 2014) or *G. citri-aurantii* (1×10^8 spores/mL). Ethanol was used to sterilize the rod and hook before inoculation of the subsequent fruit. The wound-inoculated fruits were treated after six and twelve hours of infection. The negative control consisted of infected fruits left without treatment; while fruits treated with guazatine (1000 µL/L) and imazalil (500 µL/L) served as positive controls for sour rot and green mould, respectively. The fruits were dipped into tanks containing 50 L of water mixed with 3000µL/L of EOs (lemongrass or spearmint) and a mixture of lemongrass/spearmint (1:1) at 3000 µL/L each. The oils were pre-mixed with Tween 80 to ensure that the oils were miscible with the water. The fruit were submersed for 3 min in the dip at a temperature of between 23 and 24 °C.

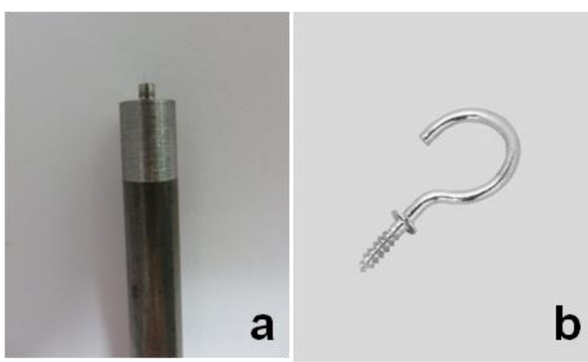

Figure 3.1: Materials used for wound inoculation of Valencia oranges: a) cylindrical rod (2 mm long circular tip and 2 mm in diameter), b) screw-in hook (13 mm protruding tip)

Following treatment, 12 fruits were packed on an indented cardboard insert placed on the bottom surface of a citrus carton (N=3 cartons/treatment), and left overnight to dry in the treatment facility. The following day, all cartons containing fruit inoculated with sour rot were wetted (80 mL water poured evenly over the cardboard insert without wetting the fruit directly) to provide a humid environment for the development of the pathogen. Each box was covered with transparent polyethylene bags, punctured twice on each end for gas exchange. Fruit were left to incubate at 25 °C (green mould) and 28 °C (sour rot) in the dark. Fruit were rated once the controls displayed high levels of infection (Erasmus *et al*., 2011). The results were compared to those obtained using the conventional fungicide.

3.6.1.2 Efficacy of EOs oils applied using conventional wax coating

Untreated export-quality citrus fruit (Valencia orange), obtained by CRI during the 2016 season, were used for this trial. The fruit were washed, ozonised under high pressure and wound-inoculated 18 h prior to treatment as explained (Section 3.6.1.1) since the time fitted with the practical work flow. The curative treatment included lemongrass-or spearmint-coated wax [Citrashine (Pty) Ldt, Booysens, South Africa] alone at an oil concentration of 2000 µL/L, as well as the mixture of these oils (1:1) at 3000 µL/L final concentration. The oils were blended with wax by pre-mixing the volume for the required concentration of the EO with a quarter of the total volume of the wax, prior to decanting the mixture into the bulk wax. Guazatine and imazalil were used as positive controls for sour rot and green mould, respectively. The fungicides were mixed with wax at concentrations of 1000 and 500 µL/L for guazatine and imazalil, respectively. These concentrations were equivalent to 2.4 mL/500 mL for guazatine (Citricure; ICA, Plankenburg, Stellenbosch, South Africa) and 0.34 g/500 mL for imazalil (Imazacure; ICA, Plankenburg, Stellenbosch, South Africa). For the successful blending of guazatine with wax, 100 µL of Tween 80 was mixed with 5 mL of ethanol, before adding guazatine with vigorous stirring using a spatula. Thereafter, 5 mL of wax was added whilst stirring. This solution was added to the bulk wax and the content was shaken.

Wax alone served as the negative control. All treatments were applied as a thin layer with a paintbrush dipped into the treatment solution. The fruit were further processed as described in Section 3.6.1.1. Fruit were left to incubate at 25 °C (green mould) and

28 °C (sour rot) in the dark. After incubation, they were examined and the extent of infection compared to that of the controls.

3.6.2 Pre-commercial trial using kumquats

Freshly harvested, untreated export-quality kumquats were obtained during the 2017 season from a packhouse near Levubu (Limpopo Province, South Africa). The fruit were packed into cartons and transported to the laboratory at Tshwane University of Technology (TUT); where they were immediately stored in the cold room (5 °C) and used on the following day.

Prior to the treatment, the fruit were surfaced-sterilized with 70% ethanol for 3 min and allowed to dry on the surface of the laboratory bench, covered with paper towel at room temperature (22 ± 1 °C). Each carton was filled with 100 fruit. Thereafter, kumquats in each box were wound-inoculated immediately with freshly prepared spore suspensions of sour rot (1×10^8 spores/mL) using the frame suspension hook (1 mm protruding tip) (Figure 3.2). The wounding and inoculation were done 24 h prior to the treatment, after which the fruits were stored at room temperature.

Figure 3.2: Frame wall hook (1 mm protruding tip) used for wound inoculation of kumquats (sour rot infection)

The *in vivo* experiment in a laboratory (TUT) consisted of two trials. The fruit were assessed for the incidence of decay. Fruit presenting with a water-soaked lesion and white and/or creamy mycelia were considered as decayed.

Trial 1

All the treatments used for this experiment are summarised in Table 3.3.

Table 3.3: Summary of the treatments applied in a TUT laboratory Trial 1

Treatment number	Code	Composition
1	LG/SM3 + Wax	Lemongrass/spearmint (3000 µL/L ratio 6:4)/Citrashine
2	LG3 + Wax	Lemongrass/Citrashine (3000 µL/L)
3	SM3 + Wax	Spearmint/Citrashine (3000 µL/L)
4	CIN3 + Wax	Cinnamon/Citrashine (3000 µL/L)
5	Wax	Wax citrashine

The control consisted of the wax without oil supplementation. All treatments were applied used the paintbrush. The fruit were allowed to dry prior to storage. All cartons were covered with black plastic bags punctured with two holes and containing wet paper towels in the form of balls. After seven days of storage at 25 °C, the fruit were assessed for decay and the results were expressed as percentage decay for each treatment. Each treatment was applied in triplicate [N=3 cartons (100 fruits/carton)].

Trial 2

The treatments applied in this trial are listed in Table 3.4.

Table 3.4: Summary of the treatments in a TUT laboratory Trial 2

Treatment number	Code	Composition
1	C	Control: untreated fruits
2	CHI	Chitosan 15 mg/mL
3	CHI/SM1	Chitosan (15 mg/mL)/spearmint (500 µL/L)
4	CHI/SM2	Chitosan (15 mg/mL)/spearmint (1000 µL/L)
5	CHI/LG1	Chitosan (15 mg/mL)/lemongrass (500 µL/L)
6	CHI/LG2	Chitosan (15 mg/mL)/lemongrass (1000 µL/L)

The control consisted of the untreated kumquats. All treatments were applied used the painting bush. The fruits were allowed to dry prior to storage. All cartons were covered with black bin plastic bag with two holes and containing wet paper towel in the form of balls. After seven days of storage at 25 °C, the fruits were assessed for decay and the results were expressed as percentage decay for each treatment. Each treatment was applied in triplicate [N=3 cartons (100 fruits/carton)]. The results obtained in this preliminary *in vivo* trial were used to select the treatments for the commercial trial.

3.6.3 Commercial packhouse trials

The trials were conducted at a packhouse near Levubu (Limpopo Province, South Africa) between July and September 2017. The fruit used were part of a consignment intended for export. Green mould infection was prevalent during the season.

3.6.3.1 Cleaning of the freshly harvested kumquats

All fruits were washed after harvesting by dipping in a freshly prepared solution of 1.2% Citrocide® PLUS [Citrosol South Africa (Pty) Ltd, Western Cape] mixed with water at room temperature for 30 s prior to storing in crates (Figure 3.3a) as usually practiced by the packhouse. The fruit were then immersed in a 0.2% chlorine bath (chloride dioxide; Biox 5000, South Africa) at 37 °C (Figure 3.3b) on the same day as packing, air-dried on the packline, brushed or sprayed with the appropriate treatment, dried again and packed into cartons for storage.

Figure 3.3: Fruit processing in the packhouse a) Fruit packed into crates after dipping in Citrocide® PLUS, b) chlorine dip, c) spray-application of selected treatment on the packline, and d) treated fruits packed into cartons

This procedure was identical to that followed by the packhouse. The commercial fruit destined for export served as controls.

Trial 1

The treatments used to test the efficacy of pathogen control on kumquat are listed in Table 3.5.

Table 3.5: Trial 1 treatments applied on kumquat using a sprayer or paintbrush

Treatment number	Code	Composition
1	C	Control: 0.2% chlorine bath at 37 °C
2	LG2	Lemongrass 2000 µL/L
3	LG3	Lemongrass 3000 µL/L
4	SM2	Spearmint 2000 µL/L
5	SM3	Spearmint 3000 µL/L
6	Mix (LG/SM)	Mixture: lemongrass/spearmint - ratio 1:1 (final conc. 3000 µL/L)
7	CN2	Cinnamon leaf 2000 µL/L
8	CN3	Cinnamon leaf 3000 µL/L
9	Wax	Apple Wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain)
10	Wax + Mix	Wax + mixture (lemongrass/spearmint oil- ratio 1:1) (3000 µL/L)
11	LG-SNPs	Lemongrass-mediated SNPs

All EO solutions were prepared using Tween 80 as surfactant (40%). Treatments 2 to 10 were applied using a sprayer (Figure 3.1c). The fruit were packed in cartons as portrayed in Figure 3.1d. In this trial, the sprayer was not yet linked to the packing-line. It was set aside and used after the fruits went through the chlorination and drying process.

For Treatment 11, the nanoparticle solution was applied manually onto the rind of each fruit using a brush. After drying, a random number of fruits (120-150) were packed in each carton. Each treatment consisted of 30 cartons, except for Treatment 11, which consisted of only 20 cartons since the nanoparticles available were limited. All cartons were left in the packhouse overnight, before storage. Of the 30 cartons, 25 cartons of each treatment and 15 cartons of Treatment 11 were stored at 5 °C in the packhouse facility. All the fruit were evaluated for decay after 26 days of storage. The results were recorded as percentage decayed fruit for each treatment.

Five cartons of each treatment were transported to the laboratory at TUT where two cartons were stored in a cold room (5 °C), while three cartons were stored at 22 ± 1 °C. The initial weight of each carton was recorded prior to storage. The cartons at 22 ± 1 °C °C were left for 14 days, while those in cold storage (5 °C) were stored for 34 days with an additional four days at 22 ± 1 °C. The weights of all cartons were recorded at the end of each storage period. All treatments were examined for the incidence of decay and fruit that exhibited a water-soaked lesion, white mycelium or presented with green spores on the rind were considered as decayed. The results were recorded as percentage decayed fruit for each treatment and percentage weight loss per treatment.

Trial 2

The treatments applied to the kumquat at the packhouse in Levubu are summarised in Table 3.6.

Table 3.6: Trial 2 treatments applied on kumquat using a sprayer

Treatment number	Code	Composition
1	C	Control: 0.2% chlorine bath at 37 °C
2	SM8	Spearmint 8000 µL/L
3	CIN8	Cinnamon leaf 8000 µL/L
4	Mix8 (SM/CIN)	Mixture: spearmint/cinnamon leaf - ratio 1:1 (final concentration 8000 µL/L)
5	Wax	Wax
6	Wax + SM8	Wax + spearmint 8000 µL/L
7	Wax + CIN8	Wax + cinnamon leaf 8000 µL/L
8	Wax + Mix	Wax + mixture: spearmint/cinnamon leaf - ratio 1:1 (final conc. 8000 µL/L)
9	SM-SNPs	Spearmint-mediated SNPs
10	CIN-SNPs	Cinnamon leaf-mediated SNPs
11	Mix (SM/CIN)-SNPs	Spearmint/cinnamon leaf-mediated SNPs
12	Mix-SNPs + Wax	Spearmint/cinnamon leaf-mediated SNPs + Wax

All the EO solutions were prepared using Tween 80 as surfactant (40%). Treatments 2 to 12 were applied using the on-line sprayer. The fruit were packed in cartons. Each treatment consisted of 15 cartons and the initial weight of each carton was recorded. All cartons were left in the packhouse overnight before storage.

The following day, five cartons from each treatment were stored at 5 °C in the packhouse facility for 26 days, while 10 cartons of each treatment were transported to the laboratory at TUT on the same day. Once in the TUT facility, all cartons were immediately stored. Five cartons were stored in the cold room (5 °C) for 34 days, with an additional four days at 22 ± 1 °C. The remaining five were stored at 22 ± 1 °C for 10 days. The results were expressed as percentage decayed fruit for each treatment and percentage weight loss per treatment.

Trial 3

All treatments applied to kumquat at the packhouse in Levubu are summarised in Table 3.7. This time, fruits were exported *via* airfreight to Rotterdam.

Table 3.7: Trial 3 treatments applied on kumquats using a sprayer

Treatment number	Code	Composition
1	C	Control: 0.2% chlorine bath at 37 °C
2	SP3	Spearmint 3000 µL/L
3	CIN3	Cinnamon leaf 3000 µL/L
4	WSP	Wax + spearmint 3000 µL/L
5	NMix	Spearmint/cinnamon leaf-mediated SNPs

The EO solutions were prepared by the addition of 40% surfactant Tween 80. All treatments were applied following the procedure of the packhouse, which included the chlorine bath followed by drying, followed by spraying then drying, and finally by packing of fruit. Twenty litres of solution was prepared for each treatment and used to fill the tank of the commercial sprayer. The fruit were dried and selected (small fruit and those with defects such as punctured rind, spot and smoothing, were discarded) using the commercial packline process. Thereafter, fruit were packed in cartons. The trial consisted of 160 cartons per treatment. All cartons were stored in the packhouse overnight before shipment to Europe. The fruit were evaluated for firmness, colour, decay, rind pitting and shriveling by an independent importer upon reaching the shipping destination.

3.6.4 Evaluation of points of potential fungal contamination in the packhouse

The packhouse was examined to identify points where infection was likely to occur. Samples were collected by passing sterile swabs over the surface of at least three parts of the plastic crates used for harvesting. A sample (100 mL) of the freshly prepared chlorine bath at 37 °C was collected in a sterile Schott bottle immediately prior to the first batch of fruit being processed for the day. The same volume of the chlorine bath was also collected at the end of the day. In addition, swabs were taken in different areas of the packline, including the ‘passage’ after the chlorine bath, the roller of the dryer, on randomly selected fruit after drying and in the collection area as illustrated in Figure 3.4.

Figure 3.4: Points on the packline where inoculum samples were collected to identify potential infection sources a) crate, b) chlorine water bath, c) ‘passage’ after chlorine bath, d) roller of the dryer, e) selected fruit after drying, and f) collection area

The chlorine water and swabs were taken to the laboratory at TUT for analysis. For the sample representing the chlorine bath, a serial dilution was performed directly into test tubes by transferring 1 mL of the sample into a test tube containing 9 mL of (¼)-strength Ringer's solution. The suspensions of each sample were subjected to 10 000 fold dilutions (10^0 to 10^{-4}). A 100 µL aliquot of each solution was spread evenly onto the surface of PDA amended with chloramphenicol. The plates were incubated for two days at 25 ± 1 °C and the CFU/mL was determined. The CFUs were calculated using the equation (Eq1) (Willey *et al*., 2014).

$$\mathrm{CFU/mL} = (numberofcells \times dilutionfacter) \div 0.1 \qquad \text{Equation 1}$$

The swabs were placed in test tubes containing 10 mL of Sabouraud Broth and incubated for two days at 25 ± 1 °C. A serial dilution of the suspension was performed in test tubes containing 9 mL of (¼)-strength Ringer's solution by transferring aseptically 1 mL from one tube to another until a dilution of 10 000-fold was achieved. Each dilution (100 µL) was spread onto the surface of a PDA plate as described. The plates were incubated for two days at 25 °C before determining the total CFU/mL.

3.6.5 Effect of a warm water dip on the quality of kumquats

3.6.5.1 Effect of warm water bath at different temperature on kumquat decay

The experiment was performed to assess the effect of different temperatures (30, 35, 37, 40, 45 and 50 °C) on decay. For each treatment, 60 fruit were dipped in the warm water bath set at each of the stated temperatures for one minute. Then the fruit were allowed to dry at room temperature. Thereafter, 30 fruits were stored at 25 °C for 14 days, while the other 30 were kept in a cold room (5 °C) for 34 days. Fruit subjected to cold storage were after stored at 25 °C for an additional four days. The number of decayed fruit per carton was recorded and expressed as percentage of decayed fruit using Excel 2010. Fruit were considered as decayed when they exhibited one of the following, a water-soaked lesion, white mycelium or green spores presenting on the rind.

3.6.5.2 Physiological studies

Rind colour

The peel colour was measured using a Konica Minolta CR- 400 colorimeter (Tokyo, Japan). The instrument was calibrated using the white calibration tile and set with a C-illuminant. An average of two readings around the equatorial region per fruit was obtained (five fruit per treatment). The parameters of the CIE L*, a*, b* colour space were recorded and used to determine the peel colour. Lightness (L) ranges from black (0) to white (100). Positive a* is red and negative a* is green. Positive b* is yellow and negative b* is blue (Bora *et al*., 2015). The total colour difference (∆E*) was calculated using Equation 2 (Eq 2):

$$\Delta E = (\Delta \mathrm{L} * 2 + \Delta \mathrm{a} * 2 + \Delta \mathrm{b} * 2)½ \quad \text{Equation 2}$$

Total colour difference (∆E*) expressed the magnitude of difference between the control (fruit not subjected to the water bath dipping) and samples (dipped fruit into warm water bath at different temperatures) (Choi *et al*., 2002).

The Chroma (Equation 3) and the hue angle (Equation 4) were calculated (Choi *et al*., 2002):

$$C *: (a * 2 + b * 2)½ \quad \text{Equation 3}$$

$$Hangle: (arctan\ b */a *) \quad \text{Equation 4}$$

Fruit firmness

The TA.XT plus instrument (Stable Micro Systems, Haslemere, United Kingdom) was used in conjunction with the Instron Bluehill 2 Version 2.25 software to determine the firmness of the kumquat fruit. Each fruit out 20 per treatment including the control was placed horizontally on the curved platform (stem axis parallel to plate). A probe of 3 mm diameter was selected to perform two measurements per fruit sample, on opposite sides of the equatorial region. The cross-head speed was set at 2 mm/s.

The result was expressed as N/mm (Guerra *et al*., 2016) and the percentage hardness of the peel against the untreated control calculated using Equation 5.

$$H\ (\%): [(Average\ sample - average\ control) \div averagesample] \times 100 \qquad \text{Equation 5}$$

3.6.6 Statistical analysis

In this study, all data obtained were subjected to Analysis of Variance (ANOVA) using the STATGRAPHICS Centurion software, version 16.01.2017 (Statpoint Technologies Inc.). Mean values among treatments were compared using the Fisher's least significant difference (LSD) procedure and Duncan's Multiple Range test. The level of statistically significant difference between variables was determined at the 95.0% confidence level.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

Citrus fruits are widely consumed and the most traded horticultural commodity in the world (Ajila *et al*., 2016). They contain beneficial nutrients to human health, such as vitamin C and bioactive phytochemicals with disease-preventive properties (e.g. flavonoids and limonoids) (Ajila *et al*., 2016). Postharvest decay causes considerable losses of export fruits. In addition, the application of synthetic fungicides are of concern to consumers and therefore to the citrus industry. There is increasing interest in the use of EOs to control postharvest pathogens as alternatives to synthetic fungicides. Several researchers have reported on the potential of some EOs to control citrus postharvest pathogens (Liu *et al*., 2009; Regnier *et al*., 2014). Essential oils have been tested *in vivo* to control pathogens on citrus fruits, such as orange (Regnier *et al*., 2014), mandarin (Jhalegar *et al*., 2015) and lime (Tripathi *et al*., 2004). However, to the best of our knowledge, there are no reports on the application of EOs on kumquats fruits. Some research has been done on the encapsulation of EOs in chitosan matrices (Dos Santos *et al*., 2012; Guerra *et al*., 2016) and inclusion in commercial wax (Du Plooy *et al*., 2009). This was done to retain the volatile components on the rind throughout the export duration. In addition, EOs have been used in the synthesis of SNPs (Vilas *et al*., 2016). The antifungal potential of chitosan and SNPs are well established (Romanazzi & Feliziani, 2016; Xia *et al*., 2016). In this study, EOs were applied in these forms *in vitro* and *in vivo* on Valencia oranges and kumquats. In addition, the influence of dipping kumquats at different temperatures was investigated with regard to decay and physiology (rind firmness and rind colour).

4.2 CHEMICAL COMPOSITION OF CINNAMON LEAF, LEMONGRASS AND SPEARMINT OILS

The six most abundant compounds present at concentrations above 1% were identified and are presented in Table 4.1.

Table 4.1: Relative percentage peak areas of the six most abundant compounds (above 1%) that were identified in selected EOs by GC-MS and quantified by GC-FID

Essential oil	**Percentage peak areas of compounds**					
	1	**2**	**3**	**4**	**5**	**6**
Lemongrass	geranial (37.0%)	neral (32.6%)	limonene (10.0%)	α-terpineol (5.1%)	geraniol (3.5%)	citronellol (3.2%)
Spearmint	carvone (76.5%)	limonene (11.1%)	borneol (2.0%)	β-bourbonene (1.3%)	3-octanol (1.1%)	-
Cinnamon	eugenol (76.7%)	α-phellandrene (4.01%)	β-caryophyllene (3.37%)	linalool (2.78%)	cinnamaldehyde (2.70%)	α-terpinene (1.09%)

The main component of the cinnamon leaf oil was eugenol (76.7%), a compound known to have good antifungal activity (Campaniello *et al*., 2010; Regnier *et al*., 2014). Geranial (37.0%) and neral (32.6%), geometric isomers collectively known as citral, were determined as the main components of lemongrass oil (Shahi *et al*., 2005). As expected, carvone was present in a high concentration in the spearmint oil. These major constituents have been reported to exhibit antifungal activities against green mould and sour rot. Du Plooy *et al*. (2009) reported the total inhibition *in vitro* of mycelial growth of green mould after exposure to R-carvone at 1000 µL/L. The compound still caused a 60% inhibition of the pathogen at 500 µL/L. Regnier *et al.* (2014) reported that eugenol and geraniol at a concentration of 500 µL/L completely inhibited the growth of *G. citri-aurantii.* In addition to the main components found in the selected EOs, lemongrass contained geraniol (3.5%) as a main component. Citral (geranial and neral) tested at 500 µL/L was reported to inhibit radial mycelial growth of the sour rot *in vitro* by 75.9% (Regnier *et al.*, 2014). Recently, Wu *et al*. (2017) reported on the *in vitro* potential control of sour rot using cinnamaldehyde, one of the constituents of cinnamon leaf oil. The MIC and the MFC of the substance against *G. citri-aurantii* were both 500 µL/L.

Although pure compounds have demonstrated inhibition against both pathogens, their use would be costly for application in the packhouse (Regnier *et al*., 2014). It is more prudent to apply EOs containing these pure compounds at high concentrations, since EOs in bulk would be affordable and cost effective for citrus producers. The occurrence of eugenol, R-carvone, as well as geraniol and citral in cinnamon leaf, spearmint and lemongrass oils, respectively, lead to the selection of these EOs as promising for the control of both green mould and sour rot in citrus.

Even though the selected EOs used in this study were purchased from the same commercial outlet (Holistic Emporium, Johannesburg) as those used by Combrinck *et al.* (2010), the chemical compounds differed in concentrations. However, the major compounds of these oils remained the same. It is of importance to have knowledge of the chemical composition of the EO prior to *in vitro* and *in vivo* trials to ensure reproducible results and to explain discrepancies in findings (Table 4.1). R-carvone in spearmint differed from 87.9% (Combrinck *et al.,* 2010) to 76.5% in the current study. In addition to differences in the concentration, three components (dihydrocarvone, dihydrocarveol acetate and dihydrocarveol) previously reported (Combrinck *et al.*, 2010) were not present at concentrations above 1%, but instead, borneol, β-bourbonene and 3-octanol were found in higher concentrations (Table 4.1). Synergistic interactions between oil compounds may also influence the effectiveness of the EO against the microorganism (Van Vuuren, 2008).

4.3 *IN VITRO* EVALUATION OF THE SELECTED ESSENTIAL OILS AGAINST BOTH PATHOGENS

4.3.1 Optimisation of various ratios of the inhibitory activities of cinnamon leaf, lemongrass and spearmint EOs

4.3.1.1 Toxic medium technique

The three selected EOs completely inhibited the mycelial growth of both pathogens at 1000 µL/L (Table 4.2).

Table 4.2: Effects of individual EO (cinnamon leaf, lemongrass and spearmint) at various concentrations on the mycelial growth of sour rot and green mould

Test substance	**Concentration (µL/L)**	**Pathogen inhibition (%)**	
		Sour rot	***Green mould***
Guazatine	250	**100**	-
	500	**100**	-
Imazalil	250	-	**100**
	500	-	**100**
Cinnamon leaf	100	0.00	1.1 ± 0.8
	250	0.00	35.9 ± 2.7
	500	47.7 ± 3.5	**100**
	750	**100**	**100**
	1000	**100**	**100**
Lemongrass	100	10.1 ± 3.3	7.9 ± 3.3
	250	74.8 ± 1.2	8.8 ± 3.7
	500	**100**	31.0 ± 7.3
	750	**100**	**100**
	1000	**100**	**100**
Spearmint	100	1.2 ± 0.9	0.00
	250	10.1 ± 1.9	20.2 ± 3.0
	500	40.3 ± 1.3	35.9 ± 2.7
	750	70.2 ± 1.3	**100**
	1000	**100**	**100**

Guazatine and imazalil were used as positive control for sour rot and green mould, respectively. All results are expressed as percentage inhibition ± standard deviation (%) (N = 5). The values in bold are indication of 100% inhibition for the particular concentration

Lemongrass EO was the most active towards sour rot with 100% inhibition attained at 500 µL/L. spearmint was the least effective requiring 1000 µL/L for complete inhibition. Cinnamon leaf oil was the most active against green mould (500 µL/L), while lemongrass and spearmint were moderately active (750 µL/L). As expected, guazatine and imazalil-based fungicide, used as positive control, completely inhibited the fungal growth of both sour rot and green mould, respectively. Both fungicides inhibited the corresponding pathogen at 250 µL/L. The antifungal efficacy of guazatine and imazalil upon application against sour rot and green mould, respectively, is well known. In the packhouse, guazatine was previously (before discontinuation) applied to citrus to combat sour rot, while imazalil-based products are still used against green mould infection (Serfontein, 2018).

The results differed slightly from those reported (Combrinck *et al*., 2010; Regnier *et al*., 2014) against these pathogens regarding the concentration required for inhibition of the pathogens. In the present study, lower concentrations were needed for total inhibition of the pathogens. However, the isolates used were not the same. Lemongrass, tested at a concentration of 500 µL/L, was found to provide only 52.2% inhibition of sour rot (Regnier *et al*., 2014). The investigation by Combrinck *et al*. (2010) on the antifungal properties of twenty EOs revealed that the lowest inhibition concentrations for green mould (Q370), isolated from orange, were 1000 and 2000 µL/L for spearmint and cinnamon, respectively. The results demonstrate the potential of these EOs as fungicide to use against both pathogens. It is probable that the pathogenic virulence of the pathogens is not the same and/or that the difference in activity may be attributed to differences in the EO composition as discussed.

From these results, it can be expected that a combination of lemongrass and cinnamon leaf oil should give adequate protection against both pathogens. The effect of combined EOs on the mycelial growth of green mould and sour rot is summarised in Table 4.3.

Table 4.3: Inhibitory effects of EOs mixtures (1:1 ratio) at various final concentrations on the mycelial growth of sour rot and green mould

Test substance	Concentration (µL/L)	Pathogen inhibition (%)	
		Sour rot	**Green mould**
Lemongrass/spearmint (1:1)	250	50.7 ± 2.4	42.4 ± 8.3
	500	**100**	53.1 ± 8.6
	750	**100**	**100**
	1000	**100**	**100**
Lemongrass/cinnamon leaf (1:1)	250	13.5 ± 2.7	36.1 ± 2.1
	500	51.0 ± 8.9	**100**
	750	**100**	**100**
	1000	**100**	**100**
Cinnamon leaf/spearmint (1:1)	250	34.4 ± 4.5	47.2 ± 3.4
	500	89.1 ± 1.5	67.3 ± 6.3
	750	**100**	**100**
	1000	**100**	**100**

All results are expressed as percentage inhibition ± standard deviation (%) (N = 5). The values in bold are indication of 100% inhibition for the particular concentration

The combination of EOs did not influence the level of inhibition of both pathogens when compared with the individual application. The mixture lemongrass/spearmint inhibited completely the mycelial growth of sour rot and green mould at final concentrations of 500 µL/L and 750 µL/L, respectively. Lemongrass/cinnamon leaf oil inhibited at concentrations of 750 µL/L and 500 µL/L respectively; and cinnamon leaf/spearmint at 750 µL/L against both pathogens (Table 4.3). The same inhibitory effects of the mixture lemongrass/spearmint and lemongrass/cinnamon leaf were observed with the individually tested lemongrass and cinnamon (Table 4.2). The combination of cinnamon leaf and spearmint displayed the poorest activity. Regnier *et al*. (2014) reported on the combination lemongrass/spearmint (1:1) oils, which completely suppressed the spore germination of sour rot at 500 µL/L each. In their study, mixing lemongrass and spearmint oils (1:1, v/v) at 750 µL/L each, resulted in 100% growth inhibition of both green and blue mould, as well as sour rot, when applied as a toxic medium. Moreover, the mixture (1:1) at 500 µL/L for each oil completely inhibited *Penicillium spp* but caused only 66.8 ± 0.8% of growth inhibition against sour rot. The final inhibitory concentration obtained in the current study is lower than the concentration reported by Regnier and co-workers (2014).

In addition, in this study green mould was more resistant to the lemongrass/spearmint combination than sour rot.

Since the 1:1 combination of the EOs did not improve their efficacy, it could be concluded that there were no synergistic interactions taking place between oil components. Instead, the interaction appeared to be additive in nature. It was therefore decided to test other ratios. The outcome on the assessment of the mixture of EOs at different ratios is summarised in Table 4.4.

Table 4.4: Inhibitory effects of oil mixtures combined at various ratios by applying the lowest inhibitory concentration determined for the individual oil against sour rot and green mould

	Lemongrass/ Spearmint		**Spearmint/ cinnamon leaf**		**Lemongrass/cinnamon leaf**	
Final conc (µL/L)	**Sour rot**	**Green mould**	**Sour rot**	**Green mould**	**Sour rot**	**Green mould**
	500	**750**	**750**	**750**	**750**	**500**
Combination	**Pathogen inhibition (%)**					
9:1	**100**	**100**	10.4±5.2	40.2±6.8	72.3±2.9	53.2±2.3
8:2	**100**	**100**	20.6±4.8	51.8±3.4	74.6±3.1	65.0±6.7
7:3	**100**	**100**	34.2±6.0	61.3±4.7	76.7±2.0	72.8±1.8
6:4	93.9±12.2	**100**	56.1±5.9	**100**	81.4±0.7	84.7±0.0
5:5	64.9±4.6	91.2±12.1	61.1±7.1	**100**	**100**	**100**
4:6	54.7±6.7	79.0±5.3	68.5±1.9	**100**	**100**	**100**
3:7	49.1±0.9	57.4±5.5	**100**	**100**	**100**	**100**
2:8	37.8±7.0	55.5±4.7	**100**	**100**	**100**	**100**
1:9	31.2±5.5	44.06±3.8	**100**	**100**	**100**	**100**

All results are expressed as percentage inhibition ± standard deviation (%) (N = 5). The values in bold are indication of 100% inhibition for the particular ratio

The mixture lemongrass/spearmint at a ratio of 7:3 and higher inhibited completely the growth of sour rot, while a ratio of 6:4 upward inhibited green mould completely. Spearmint/cinnamon leaf inhibited completely sour rot at a ratio of 3:7 and lower and green mould at a ratio of 6:4 and lower. Both sour rot and green mould were inhibited at ratio 5:5 and lower for the mixture lemongrass/cinnamon leaf.

Furthermore, the observation was such that the increase in concentration of cinnamon leaf oil, when combined with lemongrass and spearmint, resulted in an enhancement of the inhibition towards both pathogens. However, increasing the concentration of spearmint in the mixture did not improve the inhibition of green mould. The more spearmint was added, the less effective was the mixture against green mould mycelial growth. Both lemongrass and spearmint have been reported for their effectiveness against sour rot and green mould respectively (Regnier *et al*., 2014). Ideally, the expectation is that an increase in the concentration of these two EOs in a mixture would increase their effectiveness against fungi. An increase in the effectiveness may result from the interaction of the EO constituents.

4.3.1.2 Microtitre assay

The antimicrobial activity of the oils was not clearly observed with the application of the *p*-iodonitrotetrazolium violet solution (INT). The pink colour was apparent in all the wells. However, the colour change was significantly less intense with the increased concentration of the EOs as illustrated in Figure 4.1. In addition, different results were observed after every week that the assay was repeated.

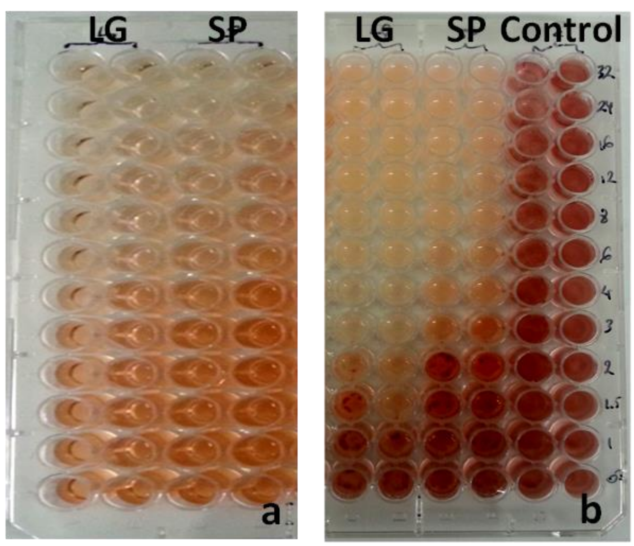

Figure 4.1: Microtitre assay (32 000-750 µL/L) indicating the variation in the antifungal activity towards sour rot of lemongrass and spearmint oils immediately after preparation (left) and two weeks later (right)

Initially, the results were not reproducible. For this reason, four replicate (four microtitre plates for each pathogen) were prepared. One plate was examined for spore germination and mycelial growth without being subjected to the INT. The results of the microtitre plates in which INT was added, were compared to those of microscope observation and the inoculated suspension onto PDA (Figure 4.2).

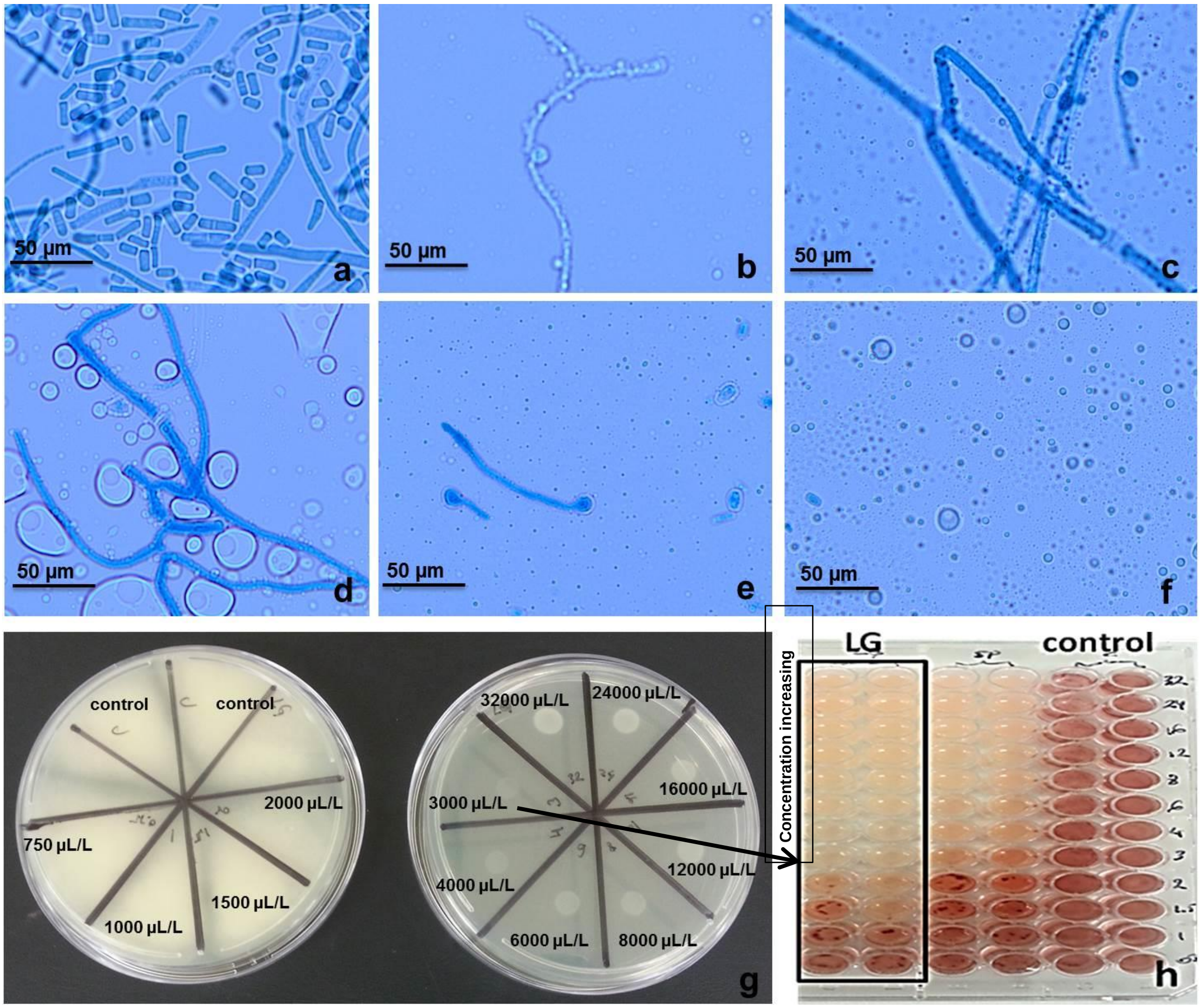

Figure 4.2: Micrograms of well contents, and photographs of cultures grown from well content and microtitre plate, following exposure of sour rot to essential oils. Micrograms of a) negative control sour rot and sour rot exposed to lemongrass oil b) 750 µL/L; c) 1000 µL/L; d) 1500 µL/L; e) 2000 µL/L; f) 3000 µL/L. g) Fungal growth on PDA of well content exposed to lemongrass oil. h) Microtitre plate used to determine MICs for lemongrass (LG) over the concentrations 32 000 to 750 µL/L. The arrow from g at 3000 µL/L points toward the corresponding concentration tested on the microtitre plate (h)

The colour of the well content decreased from 3000 µL/L and higher (Figure 4.2h). Microscope observation revealed that no germination occurred when the pathogens were exposed to 3000 µL/L of lemongrass oil (Figure 4.2f). The result was confirmed when the suspension was cultured on the surface of a PDA plate. After incubation for four days at 25 $\pm$ 1 °C, no mycelial growth was visible indicating the complete inhibition of the pathogen at concentrations of 3000 µL/L and above (Figure 4.2g).

Essential oils can inhibit the main metabolic activities related to conidial germination such as respiration, RNA and protein synthesis, as well as the breakdown of trehalose (Grbić *et al*., 2011). Many EO constituents act as regulators of intermediary metabolism and lead to the alteration of membrane structures. They also affect nutrient uptake from the medium, thereby influencing the enzyme synthesis occurring in the nucleus or in ribosomes. A degeneration of the fungal hyphae of *Aspergillus niger* exposed to *Cinnamon zeylanicum* EO was attributed to the leakage of the cytoplasm content resulting in reduced conidial production (Carmo *et al*., 2008).

The results obtained after inoculation (4 days incubation) of the petri-plates with the microtitre well contents are presented in Table 4.5. The concentration of the EOs in the suspension ranged from 32 000 to 750 µL/L.

Table 4.5: Final concentrations of selected essential oils and their mixtures to which sour rot and green mould were exposed prior to inoculation, yielding complete inhibition

Essential oils	**Final concentrations for pathogens inhibition (µL/L)**	
	Sour rot	**Green mould**
Cinnamon	2000	3000
Lemongrass	3000	4000
Spearmint	12000	8000
Cinnamon/lemongrass (1:1)	4000	4000
Cinnamon/spearmint (1:1)	4000	6000
Lemongrass/spearmint (1:1)	3000	4000

Cinnamon leaf EO provided the lowest concentration for the inhibition of both sour rot and green mould at concentrations of 2000 µL/L and 3000 µL/L, respectively. The worst performer was spearmint EO which required concentrations as high as 12 000 µL/L to inhibit sour rot and 8000 µL/L for green mould. Complete control of both pathogens by lemongrass was reached at 3000 µL/L for sour rot and 4000 µL/L for green mould. The mixture lemongrass/spearmint performed equally or better than the other mixtures against sour rot and green mould. However, the performance was no better than lemongrass alone. The mixture cinnamon leaf/spearmint required a higher concentration (6000 µL/L) to inhibit green mould, while the remaining mixtures (cinnamon leaf/lemongrass and lemongrass/spearmint) inhibited the same pathogen at a concentration of 4000 µL/L.

4.3.2 Evaluation of the ability of silver nitrate ($AgNO_3$) and its combination with the oils to inhibit both pathogens

The results displayed in Table 4.6 demonstrate the potential of silver nitrate to inhibit green mould and sour rot. The results also indicate that the combination of EOs and silver nitrate provides a way of using both the silver nitrate and essential oils at a lower concentration than when applied on their own.

Table 4.6: Inhibitory effects of selected essential oils, silver nitrate as well as a mixture of oils and silver nitrate at various concentrations [0.1, 0.05 and 0.01% (m/v)] on the mycelial growth of sour rot and green mould

Test substance	**Concentration**	**Pathogen inhibition (%)**	
		Sour rot	**Green mould**
Silver nitrate	0.01%=0.0064%Ag^+	32.8 ± 1.0	9.6 ± 1.1
	0.05%=0.032%Ag+	37.4 ± 1.4	27.3 ± 4.7
	0.1% = 0.064% Ag+	**100**	**100**
Silver nitrate + lemongrass	**% + µL/L**		
	0.01 + 100	35.5 ± 11.0	42.7 ± 8.0
	0.05 + 100	**100**	**100**
Silver nitrate + spearmint	**% + µL/L**		
	0.01 + 250	34.4 ± 9.0	27.4 ± 7.4
	0.05 + 250	88.5 ± 23.1	94.4 ± 11.2
	0.01 + 500	**100**	**100**
	0.05 + 500	**100**	**100**
Silver nitrate + cinnamon leaf	**%+ µL/L**		
	0.01 + 250	**100**	**100**
	0.05 + 250	**100**	**100**
	0.01 + 500	**100**	**100**
	0.05 + 500	**100**	**100**

All results are expressed as percentage inhibition ± standard deviation (%) (N = 5)

Silver nitrate alone inhibited both pathogens at 0.10% (0.064% of Ag^+). However, silver nitrate at only 0.05% (0.032% Ag^+), combined with lemongrass (100 µL/L), cinnamon leaf (250 µL/L) and spearmint (250 µL/L) inhibited the growth of both pathogens. The best performer was the combinations of silver nitrate and cinnamon leaf oil because all the combinations yielded complete inhibition of the pathogens. The lowest concentration would therefore be the preferred choice (0.01% + 250 µL/L). Spearmint oil combined with silver nitrate was not as effective as the other combinations. The concentrations of the EOs required for complete inhibition were substantially lower than those determined for the oils alone (Table 4.2). This result indicates a synergistic or additive effect of the two tested substances. The combination of EOs and silver nitrate also reduced the concentration of silver nitrate needed for pathogen control than silver nitrate alone (Table 4.6). Silver ions (Ag^+) have proven their antimicrobial activity against a wide range of microorganisms including bacteria, fungi and viruses (Gong *et al*., 2007). An *in vitro* investigation by Jo *et al*. (2009) revealed that silver ionic forms of silver nitrate had a broad spectrum antimicrobial activity in the inhibition of spore-producing phytopathogenic fungi that cause plant diseases. They reported that silver nitrate reduced significantly the colony formation of *Bipolaris sorokiniana* at an EC_{50} of 2.2 mg/L and *Magna porthegrisea* at EC_{50} of 0.9 mg/L after 1 h of exposure.

The promising data obtained using silver nitrate prompted further investigation into the use of SNPs for postharvest application. Garcia-Reyeroa *et al*. (2015) proposed a mechanism of action for both silver nitrate and SNPs, involving the release of silver ions.

4.3.3 Synthesis of silver nanoparticles by means of essential oils

In this study, the preparation of SNPs solution was mediated by different EOs (cinnamon leaf, lemongrass and spearmint) as reducing and stabilising agents. The formation of SNPs was evaluated using different pH environments and using a variety of volume ratios. The optimum conditions for SNP formation was pH 10 with the addition of 5 mL oil-acetone solution to the silver nitrate, from which the colour change was successfully observed as illustrated in Figure 4.3. The samples were then further characterised using UV spectrophotometry, Fourier Transform infrared spectroscopy, scanning electron microscopy, as well as energy-dispersive spectroscopy.

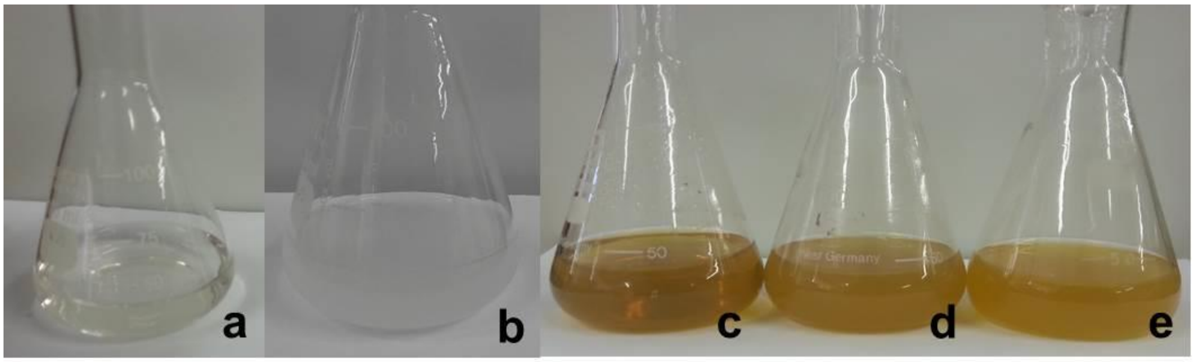

Figure 4.3: Synthesised silver nanoparticles (SNPs) using individual oils and their combinations as reducing and stabilizing agents. a) Silver nitrate (pH 10); b) unheated silver nitrate mixed with acetone-oil (lemongrass) solution; c) cinnamon leaf-mediated SNPs; d) lemongrass-mediated SNPs and e) spearmint-mediated SNPs

In general, colour change is the primary prerequisite through which, a successful formation of SNPs is reached regardless of the method used. Colour change from colourless to light brown was observed when preparing SNPs using the fungus *Fusarium moniliforme* as reducing agent (Abdelghany *et al*., 2017). In the current study, the colour change was regarded as the signal for SNPs formation. A pale yellow colour signal the formation of SNPs when Vilas *et al*. (2016) used EO from fresh leaves of *Coleus aromaticus* as reducing and stabilizing agent.

The formation of SNPs was influenced by the ratio of the EO to the mixture (Vilas *et al*., 2016). Volumes lower than 5 mL of the diluted EO solution into acetone did not result in the formation of nanoparticle. The intensity of the colour of each solution was regarded as a reflection of the SNP concentration obtained.

4.3.4 Characterization of the synthesized SNPs

4.3.4.1 UV spectrophotometry

Prior to the characterisation of the synthesised nanoparticles samples using UV-spectrophotometry, a blank composed of unheated silver nitrate and EO was analysed, yielding only a baseline (Figure 4.4a). However, the UV-spectrum of the SNPs displayed a prominent peak at λ_{max}= 419.5 nm as illustrated in Figure 4.4b for synthesised lemongrass-mediated SNPs.

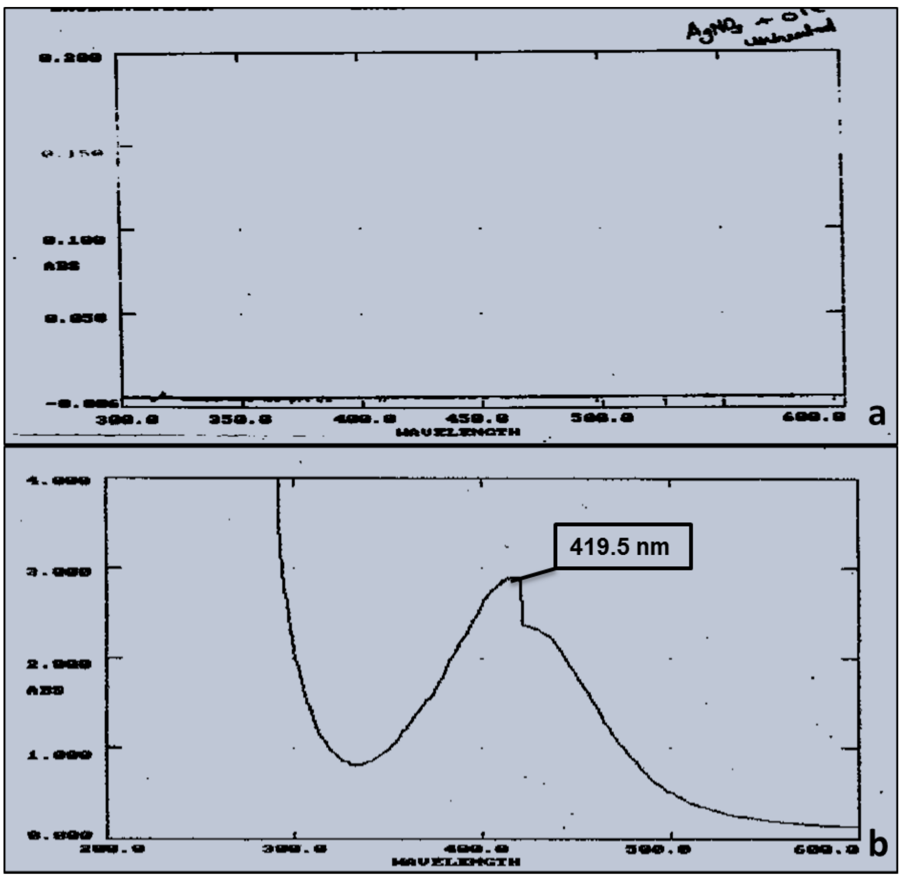

Figure 4.4: UV-spectrum of a) blank consisting of $AgNO_3$/lemongrass (unheated) + surfactant and b) lemongrass-mediated SNPs (λ_{max}= 419.5 nm) at pH 10

Several researchers have reported that the formation of SNPs can be observed at wavelengths ranging from 400-425 nm (Abdelmalek & Salaheldin, 2016; Vilas *et al*., 2016; Sithara *et al*., 2017). Vilas *et al*. (2014) observed the formation of photochemically synthesised SNPs at a wavelength of 420 nm. In the current study, the formation of all the synthesised SNPs was also confirmed by a strong absorbtion around 420 nm. Thus, the results from the UV on the EOs-mediated SNPs demonstrate that there is a formation of SNPs based on the wavelength observed.

4.3.4.2 FT-IR (Fourier Transform Infrared spectroscopy)

All SNPs were further characterised using FTIR spectroscopy. The difference between the spectra of the pure oils and the spectra of EO enhanced nanoparticles was attributed to the formation of SNPs. The IR spectra of lemongrass alone displayed three weak absorption band around 3000 cm^{-1} due to -CH and $-CH_2$ stretching (Figure 4.5a). The strong peak at 1600 cm^{-1}, represents C=C bonds. Medium peaks at 1470 and 1490 cm^{-1} indicate the two C-O- vibrations of compounds present in the lemongrass oil. The spectra of all the synthesised EO-mediated-SNPs exhibited similar absortion regions. The weak absorption peak at 3000 cm^{-1} (Figure 4.5b) is smaller due to the evaporation of water taking place. The peak at 1639.5 cm^{-1} is weak following nanoparticle formation in comparison to a strong peak observed in the EOs. Almost all peaks in the fingerprint region disappeared with the formation of SNPs.

Turek & Stintzing (2013) observed that terpene hydrocarbons are ready to change valency at high temperatures in the presence of molecular oxygen thereby causing the cleavage of double bonds, autoxidation and dehydrogenation. Thereafter, the process leads to the formation of free radicals, hydroperoxides and aromatic systems. The free radicals formed are able to reduce heavy metal ions such as Ag. Sheny *et al*. (2012) reported that the degradation of hydroperoxides of terpenes leading to the formation of secondary oxidation products are regarded as suitable in reducing and stabilizing Ag, at high temperatures in the presence of acetone.

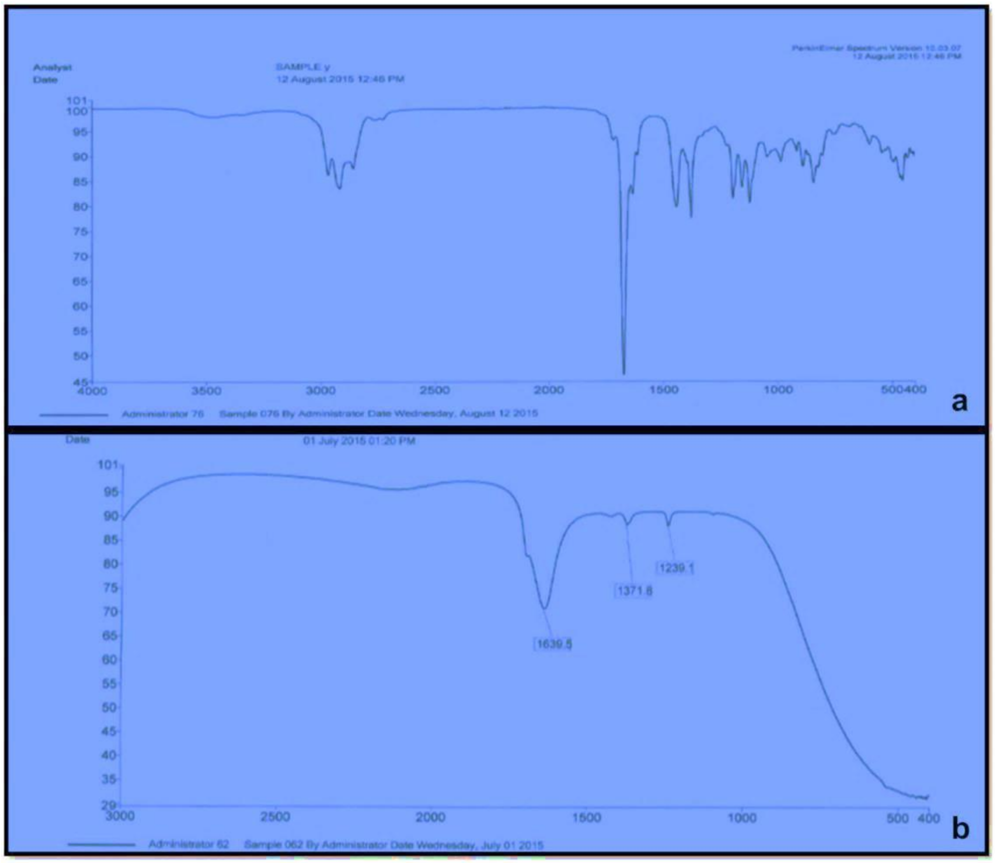

Figure 4.5: FTIR spectrum of a) diluted lemongrass oil in acetone at ratio 1:170 (v/v) and b) lemongrass-mediated SNPs at pH 10

4.3.4.3 Scanning electron microscope (SEM) and energy-dispersive spectroscopy (EDS)

The scanning electron microscope revealed that the particle sizes ranged from 37.6 - 91.2 nm particles and have a rod to spherical shape (Figure 4.6a). The size distribution of the synthesised SNPs was determined from the SEM images (Figure 4.6a) using ImageJ software. Nanotechnology entails materials that possess a dimension of approximately 1-100 nm (Duncan, 2011; Sambale *et al*., 2015). Thus, the resulting SNPs may be included in the range of successfully generated nanoparticles. The energy-dispersive spectroscopy analysis detected the presence of silver in the nanoparticles synthesised (Figure 4.6b).

The result of the SEM is demonstrated in Figure 4.6.

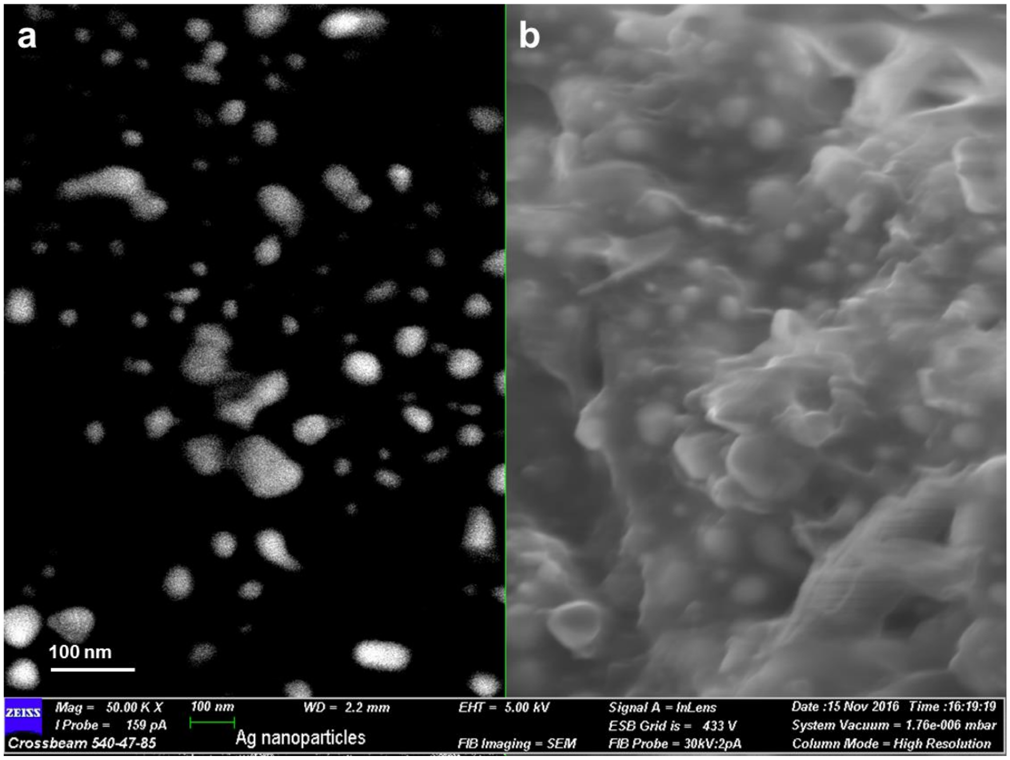

Figure 4.6: Scanning electron microscope (SEM) and energy-dispersive spectroscopy (EDS) illustration of SNPs using lemongrass as reducing and stabilizing agent: a) SEM depiction of SNPs size distribution (37.6 - 91.2 nm) and b) EDS detection of silver

The toxicity of SNPs against microorganisms depends on their size and concentration (Alananbeh *et al*., 2017). Smaller size SNPs, such as 7 nm were found to be highly toxic against bacteria (Martinez-Castanon *et al*., 2008), are regarded effective because they are deemed able to reach the bacterial nuclear content and can interact with a larger surface area (Lok *et al*., 2006). However, Alananbeh *et al.* (2017) reported that rod-shaped SNPs of 50 nm inhibited the growth of both *Aspergillus niger* and *Aspergillus terreus*. They suggested that the inhibition of microbes by larger sized SNPs may be the result of their long persistence, and that they consequently serve as a continuous source of Ag^+ as described by Dobias *et al*. (2013). Therefore, a smaller sized nanoparticle may have yielded better results in the current study.

In this study, we were unable to isolate the nanoparticles through filtration or centrifugation due to a lack of suitable equipment. The sodium hydroxide could therefore not be removed and would be present after drying. The inconsistent particle sizes (Figure 4.6a) may be explained by the presence of NaOH crystals between the nanoparticles. The NaOH was used to adjust the pH of the silver nitrate solution. The centrifugation method for the collection of SNPs was used in some studies (Masurkar *et al*., 2011; Amin *et al*., 2012). In the case of this study, no pellet could be collected due to low rotation speed for subsequent washing. The collection of SNPs by centrifugation posed problems to several researchers as stated by Prabu and Johnson (2015). In the current study, it might be because of the lack of a centrifuge since at least 13 000 rpm is recommended for successful SNP collection derived from EOs (Masurkar *et al*., 2011).

4.3.5 *In vitro* antifungal assay of EO-mediated silver nanoparticles

All the EO-mediated SNPs (cinnamon leaf, lemongrass, spearmint and mixtures) yielded the same results towards green mould and sour rot after 24 h of exposure to different volumes (100, 200, 300, 400 and 500 µL) of the SNP suspensions in a test tube, followed by plating out of an aliquot of the content onto the surface of a PDA plate. An example is given in Figure 4.7 and 4.8 with lemongrass-mediated SNPs tested against green mould and sour rot respectively. The synthesised SNPs delayed the growth of green mould after 24 h of incubation whereas growth was observed on the control (Figure 4.7a and b). The results, after extension of the incubation period to four days, revealed that the synthesised SNPs did not completely inhibit the growth of green mould, but instead, prevented the sporulation of the pathogen as demonstrated in Figure 4.7c and d for lemongrass-mediated SNPs. Increasing the volume tested of the EO-mediated SNPs did not improve the inhibitory effect against the pathogen. All volumes tested showed similar results in term of the radial growth. However, the EO-mediated SNPs were not effective against sour rot. Growth was already observed on the surface of PDA plate after 24 h of incubation. There was no difference between the control and the tested SNP suspensions (Figure 4.8a and b), even when the plates were observed after 4 days of incubation (Figure 4.8c and d).

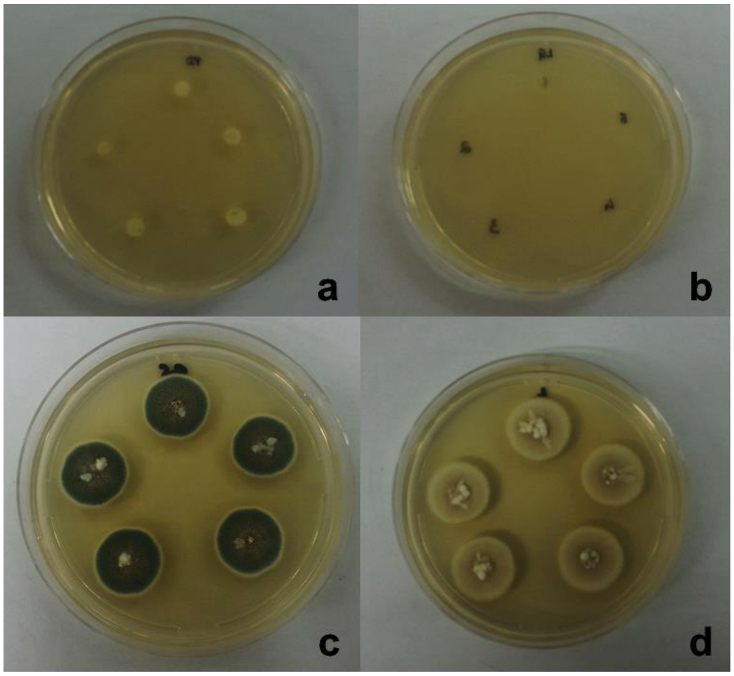

Figure 4.7: Radial mycelial growth of green mould following exposure for 24 h to lemongrass-mediated SNPs and then plated out onto PDA a) control and b) SNPs after 1 day of incubation; c) control and d) SNPs after four days of incubation

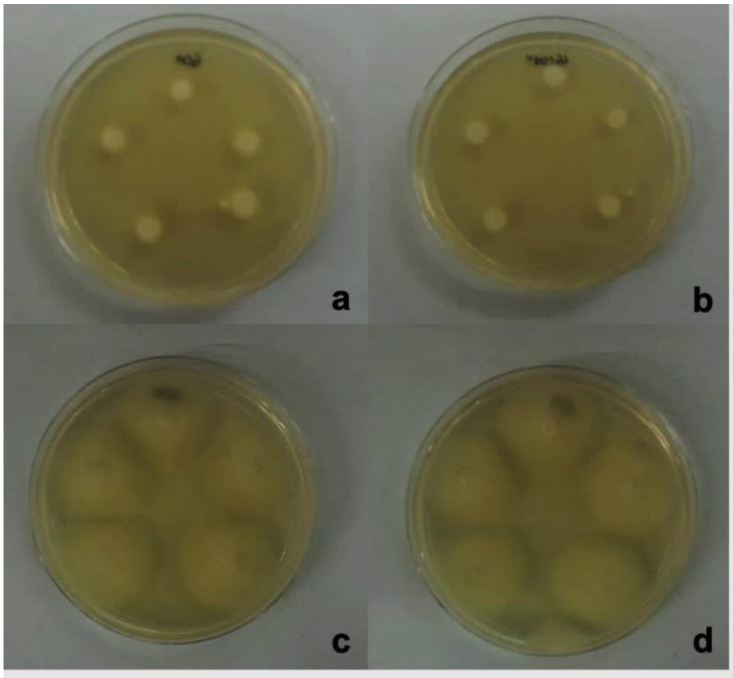

Figure 4.8: Radial mycelial growth of sour rot following exposure for 24 h to lemongrass-mediated SNPs and then plated out onto PDA a) control and b) SNPs after 1 day of incubation; c) control and d) SNPs after four days of incubation

The microscopic observation revealed that the spores of green mould did not germinate upon challenging with the SNP suspension. On the other hand, spore germination occurred after the samples were challenged with sour rot suspension, indicating selected control by the EO-mediated SNPs.

The effect of the EO-mediated SNPs on spore germination immediately after 24-h exposure of both pathogens is illustrated in Figure 4.9.

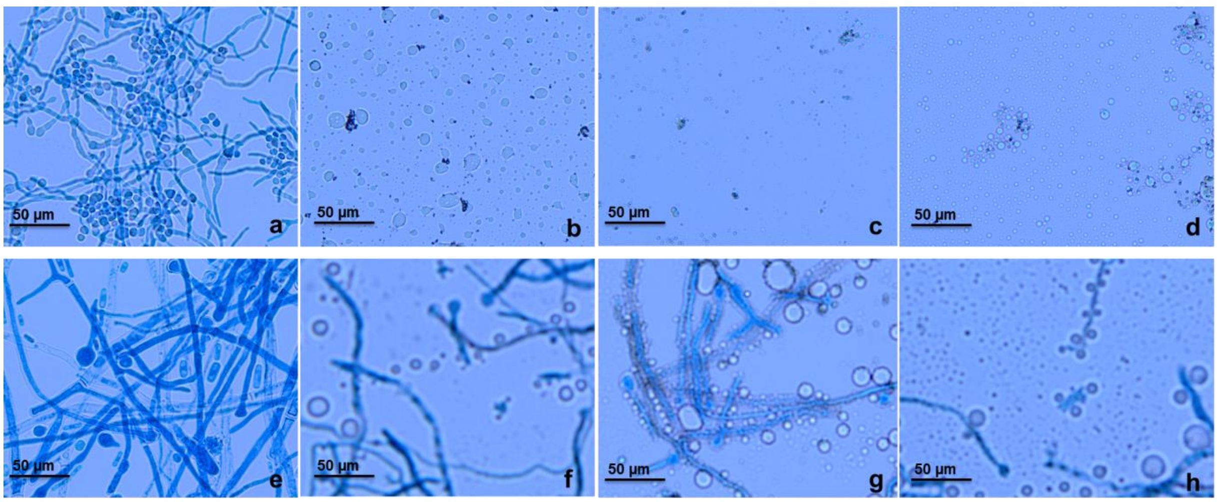

Figure 4.9: Effect of the lemongrass-mediated SNPs on the spore germination of green mould and sour rot following 24 h exposure in test tubes to different volumes of SNPs a) control green mould b) 100 µL of green mould spore suspension exposed to 100 µL SNPs, c) 100 µL green mould spore suspension exposed to 200 µL SNPs, d) 100 µL green mould spore suspension exposed to 300 µL SNPs e) control sour rot, f) 100 µL of sour rot spore suspension exposed to 100 µL SNPs, g) 100 µL sour rot spore suspension exposed to 200 µL SNPs, h) 100 µL spores suspension exposed to 300 µL SNPs

A study on the effect of different amounts (0.1, 0.2, 0.3, 0.4 and 0.5 mL) of MgO and ZnO nanoparticles on the spore germination of *Rhizopus stolonifer* revealed that there was a sharp decrease in germination of the challenged pathogen as the amounts exposed to increased (Wani & Shah, 2012). Maximum inhibition of spore germination was observed at a volume of 0.5 mL, followed by 0.4 mL. In the current study, no difference in either mycelial growth (Figures 4.7 and 4.8) or spore germination (Figure 4.9) was observed as the volume increased, indicating that factors such as the size and shape of nanoparticles had an influence in the inhibitory effect against the tested pathogens. Siddiqi *et al*. (2018) postulated that the cytotoxic effect of nanomaterials is dependent on their size, shape and the type of pathogens against which the nanomaterials are tested. The size and shape of nanoparticles exhibit different physical and chemical properties; therefore, SNPs size distribution is generally an important matter (Rauwel *et al*., 2015). The results given were identical for all the tested EO-mediated SNPs (cinnamon leaf, lemongrass, spearmint, lemongrass/spearmint, lemongrass/cinnamon leaf and cinnamon leaf/spearmint), as well as for the silver nitrate solution at pH 10.

The results obtained in this study are different to those of Kim *et al.* (2012). They suggested that more often SNPs activity against fungi increases with the increase of the concentration. These researchers were of the opinion that an increase in the SNP concentration may result in a high density of the particles that saturate the fungal hyphae, thereby increasing the ability of the SNPs to penetrate and deactivate plant pathogenic fungi (Kim *et al*., 2012). The size of nanoparticles influences the activity against fungal pathogens. Although Alananbeh *et al.* (2017) suggested that larger particles may be more active because of their long persistence and as a result serve as continuous source of silver ions (Ag^+). The current study did not substantiate the theory. The larger size (37.6 - 91.2 nm) EO-mediated SNPs had a poor activity towards green mould and no activity against sour rot. Kotzybik *et al*. (2016) demonstrated that smaller-sized SNPs (0.65 and 5 nm) were more effective in inhibiting the growth of *Penicillium verrucosum* than larger particles of 50 and 200 nm. They reported that SNPs were attached to the mycelial cell surface, but that only smaller-sized particles could penetrate the fungal filaments to some extent. They deduced that this resulted in the agglomeration of silver in the interior of the cytoplasm, thereby disturbing important mechanisms within the cell. However, larger particles resulted in the poor inhibition of *P. verrucosum* with all tested concentrations.

In the current study, the prevention of sporulation observed with the green mould (Figure 4.9) demonstrates that there is some antifungal activity using larger particles, in this case 37.7 - 91.2 nm, as determined through SEM (Figure 4.6a). This may be the result of impairment of some organelles in the cell structure as reported by some studies such as the blocking of the respiratory and metabolism processes (Xia *et al*., 2016). However, the inability to provide a response against sour rot growth is a concern.

4.3.6 *In vitro* antifungal activity of EO-coated with chitosan

4.3.6.1 Determination of the minimum inhibitory concentration (MIC)

The results obtained after exposing the pathogens to the chitosan alone and individual chitosan-coated EOs using the macrotube assay are presented in Table 4.7. Figure 4.10A and B illustrates the macrotube assay with the absence of turbidity used as a reflection of the MIC. Chitosan alone yielded an MIC of 7.50 mg/mL against both sour rot and green mould (Figure 4.10A and B; Table 4.7). The MIC values of lemongrass and spearmint were 0.250 µL/mL and 0.500 µL/mL against sour rot, and 0.375 µL/mL and 0.750 µL/mL against green mould, respectively (Table 4.7).

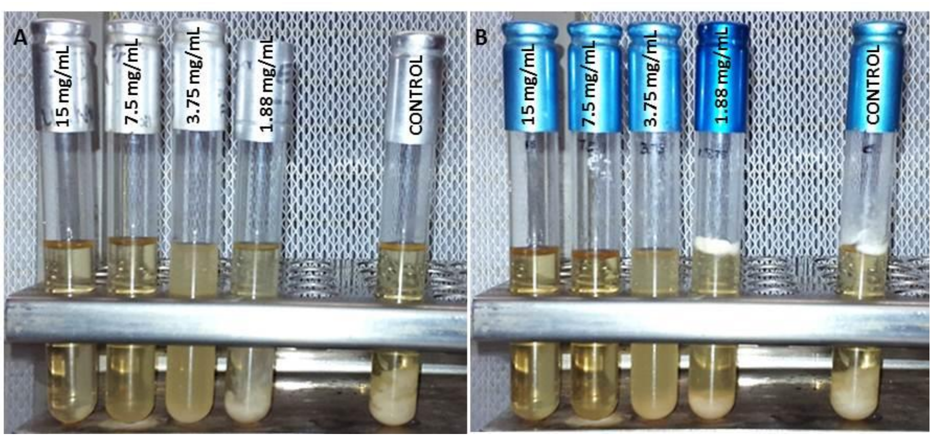

Figure 4.10: Macrotubes containing chitosan tested at 15.0, 7.50, 3.75 and 1.88 mg/mL against A) sour rot and B) green mould. Clear tube indicates complete inhibition of growth

Table 4.7: Minimum inhibitory concentrations for chitosan and EOs alone, against green mould and sour rot by means of macrotube assay. Results expressed as visible growth (+) and no visible growth (-)

Test substances	**Concentration**	**Pathogen inhibition**	
		Galactomyces citri-aurantii	***Penicillium digitatum***
Chitosan (mg/mL)	15.0	-	-
	7.50	-	-
	3.75	+	+
	1.88	+	+
Lemongrass (µL/L)	1500	-	-
	1000	-	-
	750	-	-
	500	-	-
	375	-	-
	250	-	+
	188	+	+
	125	+	+
Spearmint (µL/L)	1500	-	-
	1000	-	-
	750	-	-
	500	-	+
	375	+	+
	250	+	+
	188	+	+
	125	+	+

The antifungal effect of chitosan and EOs are well-known. The mode of action through which chitosan exerts its antifungal activity is not fully understood (Gutiérrez-Martínez *et al*., 2016). Some studies suggest that the permeability of the cell plasma membrane is increased through interaction between NH_3^+ groups of chitosan and phosphoryl groups of the phospholipids present in the cell membrane of the fungus. This results in damage to the cell membrane (García-Rincón *et al*., 2010).

An *in vitro* study (El Guilli *et al*., 2016) revealed that chitosan provided a maximum inhibition of 69% at 3.5 g/L (v/v) against green mould when the PDA medium was amended with chitosan. They found that the mycelial growth was concentration-dependent, since fungal inhibition was greater as the concentration of the chitosan increased. Our study yielded similar results to those of El Guilli *et al*. (2016), since the growth inhibition was greater (less turbid) for the 3.75 mg/mL than for the 1.88 mg/mL concentration. The *in vitro* inhibitory effect of chitosan against *G. citri-aurantii* has not yet been reported. In the current study, the pathogens were equally susceptible to chitosan as reflected by the MIC (7.50 mg/mL). However, better results (lower MICs) were obtained for sour rot than for green mould using chitosan-coated EOs, indicating the potential use of this mixture for the postharvest control of sour rot.

The antifungal activity of the EOs has been attributed to their highly lipophilic nature and low molecular weight, which enable EOs to disrupt the fungal cell membrane, causing either cell death or the inhibition of sporulation and germination of food spoilage fungi (Nazzaro *et al*., 2017). The plasma membrane was reported as an important antifungal target of spearmint oil (Kedia *et al.*, 2014). The EO was found to cause substantial impairment of the ergosterol biosynthesis by *Aspergillus flavus*, consequently leading to a decrease in biomass as the EO concentrations increased. Citral, one of the major compounds of lemongrass, has been proposed to target the cell plasma membrane (Tao *et al*., 2014). Citral was found to significantly reduce lipid levels in *Penicillium italicum* by impairing ergosterol biosynthesis in the cells of the organism. Moreover, Zhou *et al*. (2014) reported that citral inhibited the mycelial growth of sour rot as a result of cell membrane disruption, followed by loss of cellular components.

4.3.6.2 Antifungal assay of the EO coated with chitosan against both pathogens

The chitosan-encapsulated EOs exhibited complete inhibition of the mycelial growth of both pathogens at all tested concentrations (no turbid medium in the tubes) as presented in Table 4.8. It is important to recall that the concentrations used for the encapsulation of the EOs (lemongrass and spearmint) were at their sub-minimum inhibitory concentration (concentration at which visible growth has been observed in the test tubes). Both the encapsulation at the MIC and sub-MIC values of the chitosan totally inhibited both pathogens. This result indicates that the use of chitosan-coated EOs is more active than the chitosan or the oils alone.

Table 4.8: Effect of oil encapsulated in chitosan against sour rot and green mould by means of macrotube assay. Results expressed as visible growth (+) and no visible growth (-)

Test substances	Concentration	Pathogen inhibition
		Sour rot
Chitosan (mg/mL) + lemongrass (µL/L)	7.50 + 125	-
	3.75 + 125	-
	7.50 + 188	-
	3.75 + 188	-
Chitosan (mg/mL) + spearmint (µL/L)	7.50 + 250	-
	3.75 + 250	-
	7.50 + 375	-
	3.75 + 375	-
		Green mould
Chitosan (mg/mL) + lemongrass (µL/L)	7.50 + 250	-
	3.75 + 250	-
	7.50 + 375	-
	3.75 + 375	-
Chitosan (mg/mL) + spearmint (µL/L)	7.50 + 375	-
	3.75 + 375	-
	7.50 + 500	-
	3.75 + 500	-

In a study conducted by Dos Santos *et al*. (2012), the combined application of chitosan and *Origanum vulgare* EO at sub-inhibitory concentrations caused greater inhibition of *Rhizopus stolonifer* and *Aspergillus niger* in liquid medium than the substances alone. They found significant morphological changes in the spores of both *R. stolonifer* and *A. niger* following exposure. The positive results of the current study suggest that the application of chitosan-coated EOs may be a good option for pathogen control on citrus since it appears to effect spores rather than merely affecting mycelial growth.

4.3.6.3 Effect of chitosan-encapsulated EOs solution on mycelial growth using mycelial dry mass technique

Although the chitosan-encapsulated EOs solution has demonstrated antifungal effect on the previous section, it was essential to prove the efficacy of the solution using another technique. The mycelial dry mass technique has been exploited by several researchers (Zeng *et al*., 2015; Ghoneem *et al*., 2016) to examine antimicrobial activity of EOs on mycelial growth. Dos Santos *et al*. (2012) successfully used the technique to evaluate the antifungal effect of the combination of chitosan and *Origanum vulgare* L. essential oil to inhibit *Rhizopus stolonifer* and *Aspergillus niger*. The technique offers the advantages of observing the inhibitory influence that the chitosan encapsulated EOs has on the pathogens over time. In addition, it provides a quantitative measurement of growth inhibition, whereas the previous test was based solely on visually assessing the degree of turbidity.

The chitosan-encapsulated EO solutions at different concentrations affected the mycelial growth of both pathogens considerably, as indicated in Table 4.9.

Table 4.9: Effects of the combination chitosan/EOs at different concentrations on mycelial growth of sour rot and green mould in broth media over three, five and seven days by means of mycelial dry mass technique

Combination CHI (mg/mL) + EO (µL/L)	**Days of exposure**		
	3	**5**	**7**
Galactomyces citri-aurantii		**Inhibition (%)**	
CHI (7.50) + LG(0.188)	97.2	**100**	**100**
CHI (7.50) + LG (0.125)	98.1	**100**	**100**
CHI (7.50) + SM (0.375)	96.6	97.5	**100**
CHI(7.50) + SM (0.250)	97.0	97.2	99.0
CHI (3.75) + LG (0.188)	97.1	98.6	**100**
CHI (3.75) + LG (0.125)	97.1	98.5	**100**
CHI(3.75) + SM (0.375)	96.5	97.6	98.5
CHI(3.75) + SM (0.250)	96.8	97.5	**100**
Penicillium digitatum		**Inhibition (%)**	
CHI (7.50) + LG (0.375)	98.3	99.9	**100**
CHI (7.50) + LG (0.250)	98.7	99.9	**100**
CHI(7.50) + SM (0.500)	99.9	**100**	**100**
CHI(7.50) + SM (0.375)	**100**	**100**	**100**
CHI (3.75) + LG (0.375)	99.9	**100**	**100**
CHI (3.75) + LG (0.250)	99.9	**100**	**100**
CHI(3.75) + SM (0.500)	**100**	**100**	**100**
CHI(3.75) + SM (0.375)	99.9	99.9	**100**

LG = lemongrass, SM = spearmint and CHI= chitosan

It was observed that the mycelial growth was reduced over time as measured by the reduction in the mycelial dry mass. The same results were observed by Dos Santos *et al*. (2012). In the current study, total inhibition was observed for all concentrations tested after seven days of incubation. The combination of chitosan and lemongrass at the highest concentration of chitosan (7.5 mg/mL), resulted in 100% inhibition of sour rot mycelial growth on the fifth day of incubation, while green mould was highly susceptible to all the treatments within the same timeframe (fifth day of incubation) (Table 4.9).

4.4 *IN VIVO* EFFECT OF EOs ON THE DECAY INCIDENCE OF BOTH PATHOGENS

4.4.1 Semi-commercial trial using EOs

4.4.1.1 Efficacy of EOs applied as a dip and in the form of EO-amended coating on wound-inoculated Valencia

All the treatments applied as a dip did not differ from the control (untreated Valencia oranges). Even the conventionally used fungicides (guazatine and imazalil) were ineffective. One positive finding was that the fruit artificially inoculated with green mould developed decay at a slower rate, indicating that the sporulation was delayed by the spearmint oil when applied as a dip. The results are not provided since the decrease was not significant.

The observations of the second trial were the same as for the first trial. However, no delay in sporulation was observed with any treatment applied. The poor results were attributed to the harsh inoculation method applied, which provided the pathogen with an unfair advantage over the fungicides. Such an inoculation technique does not reflect natural infection. This method of challenging the pathogen was, at that time, used by CRI for their in-house trials.

Similar results to those of the present study were obtained by Souri and Bakhtiarizade (2018) after using a sharp-tip pin to artificially inoculate

Valencia with the spores of blue mould (*Penicillium italicum*). Their treatments consisted of three EOs, which included rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*) and tarragon (*Artemisia dracunculus*) oils at 500 to 1000 µL/L. However, the fruits inoculated by dipping them into the fungal spores without artificially wounding, were protected by the EOs. This underlines the importance of method of inoculation in the ability of the EOs to protect the fruit.

4.4.2 Pre-commercial packhouse trial on kumquats

At this point in time, the kumquat exporters (Premier Fruit Exports) approached the postharvest innovation group to assist them with postharvest control. Several producers were lucratively exporting kumquats as air freight to the Netherlands (Rotterdam) and the Middle East. However, repacking of the fruit was necessitated at the destination due to high incidence of decay, particularly due to sour rot. This time, the freshly harvested kumquats were artificially inoculated with a spore suspension of sour rot.

Trial 1

The results obtained after the application of the EOs and/or wax coating to the artificially wound-inoculated kumquats are presented in Figure 4.11. The fruits were stored for 7 days at 25 °C.

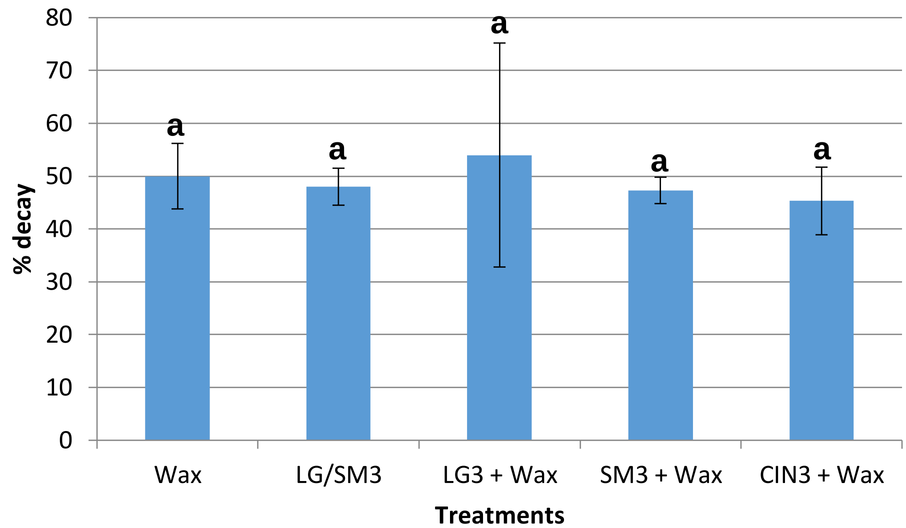

Figure 4.11: Effects of essential oil treatment on the decay incidence of kumquat artificially inoculated with sour rot (result expressed as percentage decay per treatment)

Wax = Citrashine alone; LG/SM= Lemongrass/spearmint (3000 µL/L); LG3= Lemongrass (3000 µL/L); SM3= Spearmint (3000 µL/L); CIN3= Cinnamon leaf (3000 µL/L). *Means indicated with the same letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

The statistical analysis of variance revealed that there was no significant difference between the means of the five variables (treatments) at the 95% confidence level, because the P-value (0.8741) obtained for the F-test was greater than 0.05. Nevertheless, the application of lemongrass mixed with wax provided the worst scenario, as depicted in Figure 4.11 with 54 ± 21% of decay incidence. Treatment with wax, a mixture of lemongrass/spearmint/wax (60/40 at 3000 µL/L, final concentration), spearmint/wax (3000 µL/L) and cinnamon/wax (3000 µL/L) resulted in 50.0 ± 6.2, 48.0 ± 3.5, 47.3 ± 2.50 and 45.3 ± 6.4% decay incidence, respectively. The poor results may be attributed to the rapid evaporation of the EOs when stored at room temperature. In addition, the wounding of the fruit was possibly too severe because the wound reached the fruit pulp.

Trial 2

The outcome of the effect of chitosan alone and mixture chitosan/EO on the artificially wound inoculated kumquats with sour rot infection are demonstrated in Figure 4.12.

This time, the wounding was done by making a minor lesion on the fruit rind, stopping at the albedo rather than penetrating it.

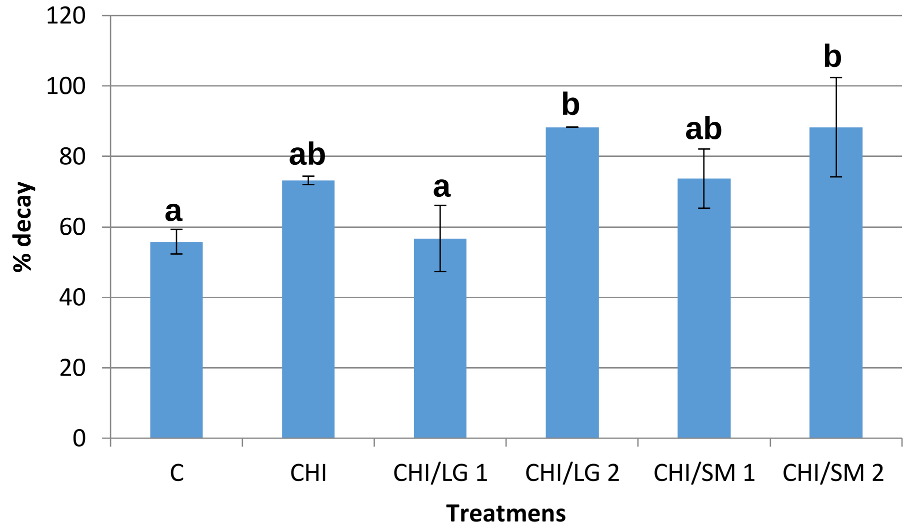

Figure 4.12: Percentage decay of artificially inoculated kumquats with sour rot infection after treatment with chitosan and essential oils combined with chitosan (result expressed as percentage decay per treatment)

C = control; CHI = Chitosan (15 mg/mL); LG1= Lemongrass (500 µL/L); LG2= Lemongrass (1000 µL/L); SM1= Spearmint (500 µL/L); SM2= Spearmint (1000 µL/L). *Means with one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

There was a statistically significant difference between some of the means of the six treatments at the 95% confidence level. The results revealed that the control fruit had the least decay and that none of the treatments were able to reduce pathogen infection. The worst scenarios were observed with the application of both chitosan combined with 1000 µL/L of spearmint and lemongrass oils. The decay incidents were 88.3 ± 14.1 and 88.3 ± 0.0%, respectively. These results showed that the increase in EO concentration increased the impact of decay incidence. The poor result would be attributed to the inability of the chitosan to form a film on the rind of the fruits when exposed to higher temperature. This resulted in a high humidity environment as chitosan solution formed moisture on the carton, which favoured the growth of the pathogen.

Previous studies have used the concentration of 4 mg/mL (chitosan) when combining EOs with chitosan as coating matrix on fruits such as grapes (Guerra *et al*., 2016) and cherry tomato fruit (Barreto *et al*., 2016), because it was the lowest concentration capable to form a viscous solution with coating features on those fruits. Moreover, concentrations higher than 8 mg/mL were found unable to form continuous films when they were applied to fruit due to the formation of highly viscous dispersions (Guerra *et al*., 2016). Chien *et al*. (2007) reported that low molecular weight chitosan (LMWC) applied on mature yellow Murcott tangor at concentration 0.1% (equivalent to 1 mg/mL), not only maintained the quality and extend the shelf life of fruits but also effectively reduced the incidence decay of *P. digitatum, P. italicum, B. lecanidion* and *B. cinerea* by 20% after 56 days of storage. In addition, a study conducted by Shao *et al*. (2015) revealed that 1% chitosan combined with 0.5 mL/L clove oil was effective against the development of green mould on mature satsuma mandarin by reducing the lesion diameter and decay incidence. Mixing the same concentration of chitosan with 1 mL/L clove oil or higher concentration of the same oil resulted in the improvement of disease severity.

In Trial 2, a concentration of 15 mg/mL chitosan was applied to the kumquats. Such a high concentration is definitely linked to the inability of the chitosan to form a film on the fruit rind. Furthermore, at higher EO concentrations the results are in accordance with those of Shao *et al.* (2015). High concentrations of EOs in the chitosan matrix resulted in an undesirable effect when applied *in vivo*, whereby the oil reduced the ability of chitosan to bind as a film onto the fruit rind.

Based on the poor results obtained after application of the EO-encapsulated chitosan, these treatments were removed from further *in vivo* trials.

4.4.3 Commercial packhouse trials

Initially, the exporters informed the postharvest group that throughout the previous year, sour rot was the most important infection causing kumquats rot. For this reason, sour rot was mainly targeted in the previous trial as described in Section 4.4.2.

However, during the trials it was found that green mould was a bigger problem and attention was subsequently shifted to green mould control.

Trial 1

In this trial, all treatments were applied using a sprayer, except for lemongrass mediated SNPs synthesis solution which was applied manually onto the rind of each fruit using a brush.

Freshly harvested kumquats were not subjected to artificial inoculation. This trial relied on natural infection of the fruits. After treatment, most of the cartons were stored in the packing house facility at 5 °C and the remainder were stored at 22 ± 1 °C or at 5 °C at TUT. The results of the 14 days storage at 22 ± 1 °C in a TUT laboratory facility are illustrated in Figure 4.13.

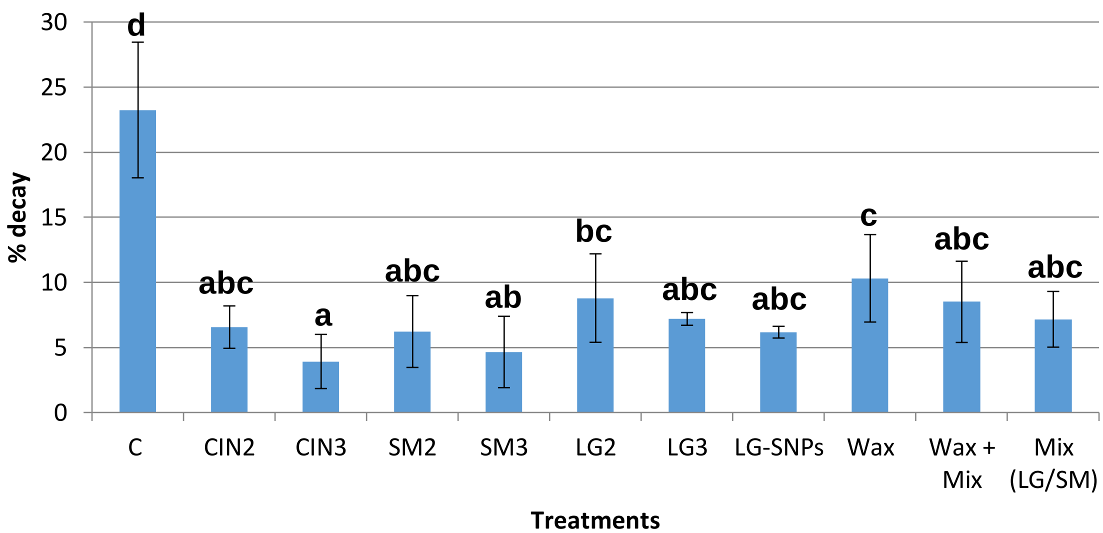

Figure 4.13: Effect of the treatment on fruit decay after 14 days storage at 22 ± 1 °C (TUT) (result expressed as percentage decay per treatment)

C= control; CIN2= cinnamon leaf (2000 µL/L); CIN3= cinnamon leaf (3000 µL/L); SM2= spearmint (2000 µL/L); SM3= spearmint (3000 µL/L); LG2= lemongrass (2000 µL/L); LG3= lemongrass (3000 µL/L); LG-SNPs= lemongrass-mediated SNPs; Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); Mix (LG/SM)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 3000 µL/L). *Means indicated presence of at least one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

The analysis of variance showed that there was a significant difference between the means of the 11 treatments at the 95% confidence level. The control displayed significantly higher levels of decay (23.2 ± 5.2%) than any of the treatments. Treatment using cinnamon leaf oil at 3000 µL/L (CIN3) resulted in 3.92 ± 2.1% of decay, while kumquat treated with spearmint oil at 3000 µL/L (SP3) presented with 4.65 ± 2.7% of decay. This was the best outcome with a decay reduction of about 83.1 and 80.0%, respectively, compared to the control. All the other treatments reduced fungal infection significantly compared to the control. Treating the fruits with lemongrass-mediated SNPs, spearmint (2000 µL/L), cinnamon leaf (2000 µL/L), mixture lemongrass/spearmint (3000 µL/L), lemongrass (3000 µL/L), wax and mixture (lemongrass/spearmint) (3000 µL/L), lemongrass (2000 µL/L) and wax decreased the decay incidence by 73.45, 73.23, 71.77, 69.19, 69.06, 63.42, 62.18 and 55.64%, respectively, relative to the control. The treatments applied indicated the potential to reduce the pathogen development and increase the shelf life of the naturally infected kumquats.

The only treatment to reduce weight loss was the application of cinnamon leaf oil (CIN2) at 2000 µL/L (Table 4.10). Only 2.02 ± 0.15% weight loss was recorded for CIN2, when compared to the control, which had 4.03 ± 1.59%. The highest percentage on weight loss was recorded with treatment lemongrass-mediated SNPs with 6.80 ± 1.41%. Although the weight loss determined for the other treatments were higher than that of the control, the difference is not of concern and were quite acceptable. The weight loss of the fruit for each treatment is summarised in Table 4.10.

Table 4.10: Percentage weight loss per treatment after 14 days of storage at 22 ± 1 °C (TUT) (Result expressed as percentage weight loss per treatment)

Code	% Weight loss	SD
C	4.03^{a}	1.59
CIN2	2.02^{a}	0.15
CIN3	5.32^{a}	1.7
LG2	4.99^{ab}	0.49
LG3	6.80^{ab}	1.41
SM2	4.67^{ab}	0.65
SM3	4.58^{a}	1.53
Mix (LG/SM)	5.91^{ab}	0.88
LG-SNPs	6.22^{ab}	0.89
Wax	5.14^{ab}	1.91
Wax + Mix	5.39^{ab}	2.09

SD = standard deviation

C= control; CIN2= cinnamon leaf (2000 µL/L); CIN3= cinnamon leaf (3000 µL/L); SM2= spearmint (2000 µL/L); SM3= spearmint (3000 µL/L); LG2= lemongrass (2000 µL/L); LG3= lemongrass (3000 µL/L); LG-SNPs= lemongrass-mediated SNPs; Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); Mix (LG/SM)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 3000 µL/L). *Values with the same letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

The results of the fruit in cold room storage at TUT are displayed in Figure 4.14. This time the fruits were assessed over a storage period of 34 days in cold (5 °C) followed by four days storage at room temperature (22 ± 1 °C). The percentage decay was below 5% for the control and all the treatments stored at 5 °C, with the exception of lemongrass EO applied at 2000 µL/L (6.31 ± 0.7%) after 34 days in cold storage. Treatment with cinnamon EO at 3000 µL/L provided the lowest percentage decay (0.71 ± 0.07%). It is important to underline that for the farmer/exporter, a decay percentage below 5% is regarded as acceptable (Personal communication S. Whitworth, 2017).

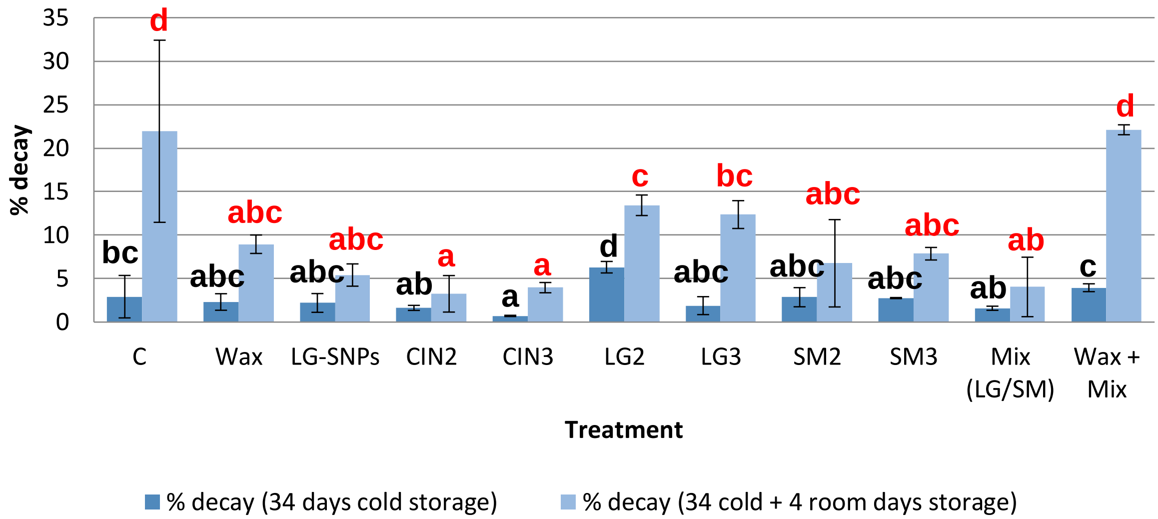

Figure 4.14: Effect of the treatment on fruit decay after 34 days at 5 °C and an extended 4 days at 22 ± 1 °C in TUT (Result expressed as percentage decay per treatment).

C= control; CIN2= cinnamon leaf (2000 µL/L); CIN3= cinnamon leaf (3000 µL/L); SM2= spearmint (2000 µL/L); SM3= spearmint (3000 µL/L) ; LG2= lemongrass (2000 µL/L); LG3= lemongrass (3000 µL/L); LG-SNPs= lemongrass-mediated SNPs; Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); Mix (LG/SM)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 3000 µL/L) . *Means with at least one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test. The black letters indicate the significance level between the treatment of 34 days cold storage and the red for the extended four days at room temperature.*

However, it was observed that storing the fruits for four days at 22 ± 1 °C after a long storage period in the cold (5 °C for 34 days) resulted in the rapid development of the pathogen on the fruits. The decay incidence increased drastically with all the treatments of the trial. A 86.7% increase in the decay incidence of the control was observed. It rose from 2.92 ± 2.4% in cold storage to 21.94 ± 10.5% on the fourth day of the room temperature storage. The same trend was observed with the mixture of EO in wax (Wax + Mix) where the incidence increased by 82.1%. All of the other treatments displayed decay of less than 15%. Once again, cinnamon leaf oil performed the best, with the oil reducing decay by 85.2% (CIN2) and by 82.0% (CIN3).

Table 4.11 provides the percentage weight loss recorded after 34 days cold storage, and after further four days storage of the fruit at room temperature. For the treatments under both storage conditions, the P-values were greater than 0.05

(0.3573 for the 34 days and 0.6250 for the extended four days storage). Therefore, the analysis of variance revealed that there were no statistical significant differences between the means of the 11 variables at the 95% confidence level.

Table 4.11: Percentage weight loss per treatment after 34 days at 5 °C and an extended 4 days at 22 ± 1 °C (TUT) (Result expressed as percentage decay per treatment)

Code	% Weigh Loss 34 days	SD	% Weigh Loss 34 + 4 days	SD
C	4.15^{ab}	0.22	7.97^{A}	1.01
CIN2	4.75^{ab}	0.74	9.40^{A}	0.65
CIN3	4.86^{ab}	0.94	7.98^{A}	0.08
LG2	3.83^{a}	1.12	9.57^{A}	1.33
LG3	5.67^{b}	0.80	10.93^{A}	2.82
SM2	3.94^{a}	0.75	9.42^{A}	1.29
SM3	4.06^{ab}	0.46	8.96^{A}	1.21
Mix (SM/LG)	5.21^{ab}	0.02	9.54^{A}	1.18
LG-SNPs	4.44^{ab}	1.00	8.87^{A}	1.99
Wax	5.13^{ab}	1.09	9.01^{A}	2.04
Wax + Mix	5.03^{ab}	0.04	11.08^{A}	1.69

SD = standard deviation

C= control; CIN2= cinnamon leaf (2000 µL/L); CIN3= cinnamon leaf (3000 µL/L); SM2= spearmint (2000 µL/L); SM3= spearmint (3000 µL/L); LG2= lemongrass (2000 µL/L); LG3= lemongrass (3000 µL/L); LG-SNPs= lemongrass-mediated SNPs; Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); Mix (LG/SM)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 3000 µL/L). *Values with one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test. Small later indicate significance level between the values of 34 days storage and capital letter for the extended four days at room temperature.*

The results revealed that cold storage provides resistance to rapid weight loss of the kumquats. The exposure of the fruit to room temperature for the extended period of four days, resulted to a drastic weight loss. The weight loss was not directly proportional to the level of decay incidence. For example, the control showed a lower percentage weight loss compared to the treatments that performed best for the control of the pathogen in both scenario of storage (Table 4.11).

The percentage decay recorded for the kumquats stored in the cold room (5 °C) of the packhouse are presented in Figure 4.15.

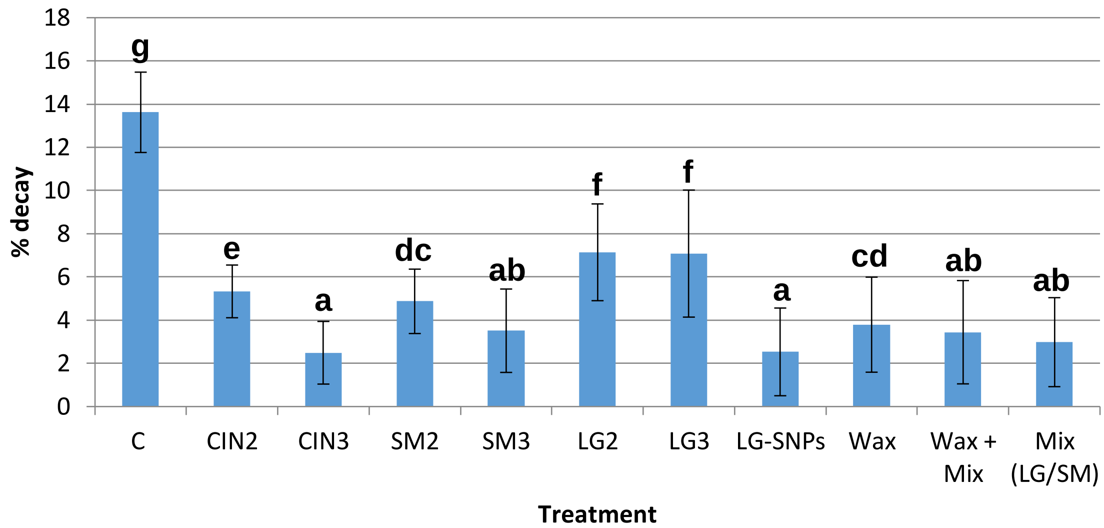

Figure 4.15: Effect of the treatment on fruit decay after 26 days storage at 5 °C in Levubu (Result expressed as percentage decay per treatment)

C= control; CIN2= cinnamon leaf (2000 µL/L); CIN3= cinnamon leaf (3000 µL/L); SM2= spearmint (2000 µL/L); SM3= spearmint (3000 µL/L); LG2= lemongrass (2000 µL/L); LG3= lemongrass (3000 µL/L); LG-SNPs= lemongrass-mediated SNPs; Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); Mix (LG/SM)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 3000 µL/L). *Means with at least one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

All the treatments reduced decay relative to the controls (13.6%). The best performer was cinnamon leaf oil at 3000 µL/L (CIN3) (2.49 ± 1.5% decay) and lemongrass mediated (LG-SNPs) (2.53 ± 2.0%) applied as an aqueous spray. Lemongrass was unable to reduce infection, since both treatments LG2 (7.08 ± 2.9% decay) and LG3 (7.14 ± 2.2%) were unable to reduce the decay to below 5%. Spearmint oil was also able to reduce infection since its application at 2000 µL/L (SM2) yielded 4.87 ± 1.5% decay and 3.51 ± 1.9% decay at 3000 µL/L (SM3). Both lemongrass and spearmint were effective against green mould with a complete inhibition at 750 µL/L using toxic media. However, lemongrass (4000 µL/L) outperformed spearmint (8000 µL/L) in the microtitre assay (4000 µL/L against 8000 µL/L, respectively) against green mould and when using the macrodilution technique (375 µL/L against 750 µL/L, for lemongrass and spearmint, respectively). It is therefore surprisingly that such a good outcome was achieved with the application of spearmint oil at this stage of the trial.

Although the outcome of the fruit stored at the packhouse may be a more realistic environment than laboratory storage, because the laboratory has a more controlled environment, storage at both facilities indicated that both treatments with cinnamon leaf oil at 3000 µL/L (CIN3) and 2000 µL/L (CIN2) yielded less decay compared to the other treatments applied. The performance of spearmint applied at 3000 µL/L was also satisfactory, since it produced the second least incidence of decay (after CIN3) during the TUT laboratory storage at 22 ± 1 °C for 14 days, and the third least decay after storage at the packhouse (after CIN3 and LG-SNPs). Lemongrass oil presented an overall poor activity compare to other treatments and therefore was the least effective of the treatments applied.

Trial 2

In this packhouse trial, higher concentrations of EOs were used. This was done to determine if the EOs would be phytotoxic at high concentrations and if the decay control would improve. The concentration applied was increased from 3000 µL/L to 8000 µL/L. Since the application of SNPs yielded fairly good results, the cinnamon leaf- and spearmint-mediated nanoparticle suspensions were applied in this trial. The fruit were stored at various conditions at TUT (22 ± 1 °C for 10 days and 5 °C for 34 days with four extended storage at 22 ± 1 °C) and at the packhouse facility at 5 °C for 34 days.

The data recorded for the fruit decay after storage at 22 ± 1 °C for 10 days in the TUT facility are represented in Figure 4.16. The evaluation of the fruits demonstrated that the application of treatments on this trial was less effective compared to the first trial, since there were no significant differences in the percentage decay between most of the treatments and the control (Figure 4.16).

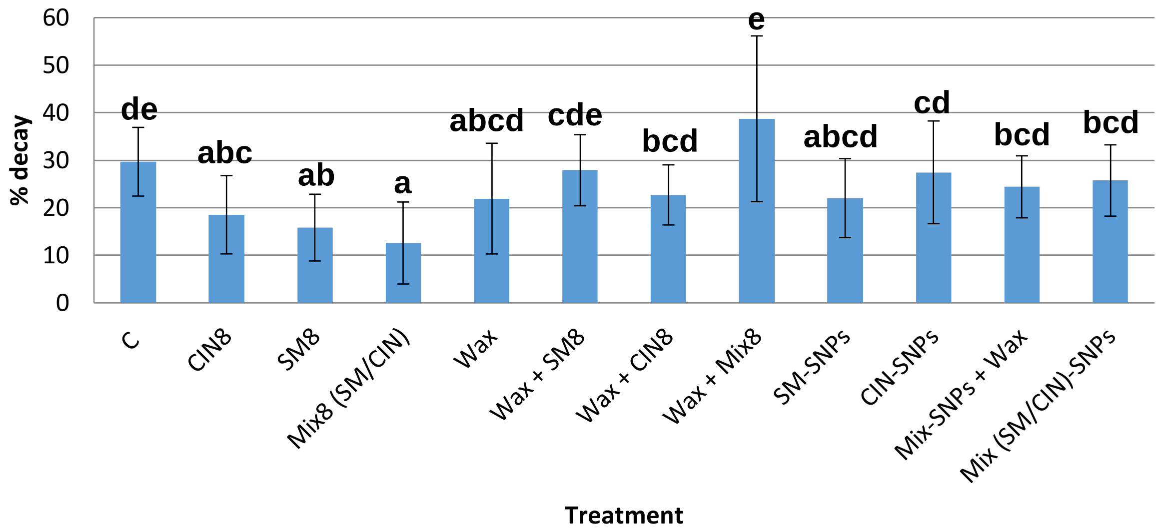

Figure 4.16: Effect of the treatment on fruits' decay after 10 days storage at 22 ± 1 °C in TUT (Result expressed as percentage decay per treatment)

C= control; CIN8= cinnamon leaf (8000 µL/L); SM8= spearmint (8000 µL/L); Mix8 (SM/CIN)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 8000 µL/L); Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); SM-SNPs= spearmint-mediated SNPs; CIN-SNPs= cinnamon leaf-mediated SNPs; Mix (SM/CIN)-SNPs= mixture of spearmint and cinnamon leaf-mediated SNPs. *Means with at least one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

However, treatment with the mixture of spearmint and cinnamon leaf at 8000 µL/L [Mix8 (SM/CIN)], as well as spearmint oil and cinnamon oils both at 8000 µL/L moderately reduced the decay incidence to 12.6 ± 8.6%, 15.8 ± 7.0% and 18.5 ± 8.2%, respectively compared to the control, with a corresponding decay incidence of 29.7 ± 7.2%. The nanoparticles combined with essential oils (24.4 ± 6.5%) did not reduce the decay significantly when compared to the control. The wax treatment (21.9 ± 11.6%) also did not perform better than the control. The worst result was obtained for the treatment with wax containing spearmint and cinnamon oil (Wax + Mix8) (38.8 ± 17.4%), where the percentage decay was even higher than that of the control (29.7 ± 7.2%).

The percentage weight loss related to the TUT storage at 22 ± 1 °C for 10 days is presented in Table 4.12.

Table 4.12: Percentage weight loss per treatment after 10 days of storage at 22 ± 1 °C (TUT) (Result expressed as percentage weight loss per treatment)

Code	% Loss (10 days)	SD
C	10.17^{abcd}	2.95
CIN8	13.63^{e}	2.96
SM8	8.52^{ab}	1.65
Mix (SM/CIN)	9.62^{abc}	1.33
Wax	8.25^{a}	2.36
Wax + CIN8	9.46^{abc}	0.66
Wax + SM8	12.57^{de}	1.95
Wax + Mix	11.66^{cde}	2.83
SM-SNPs	8.30^{a}	2.28
CIN-SNPs	9.28^{abc}	1.26
Mix (SM/CIN)-SNPs	10.32^{abc}	2.35
Mix-SNPs + Wax	11.11^{bcde}	2.00

SD= standard deviation

C= control; CIN8= cinnamon leaf (8000 µL/L); SM8= spearmint (8000 µL/L); Mix8 (SM/CIN)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 8000 µL/L); Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); SM-SNPs= spearmint-mediated SNPs; CIN-SNPs= cinnamon leaf-mediated SNPs; Mix (SM/CIN)-SNPs= mixture of spearmint- and cinnamon leaf- mediated SNPs. *Values with at least one identical letter do not significantly differ at $p > 0.05$, according to Duncan's multiple range test*.

There was a significant difference at the 95% confidence level between the weight loss of the various treatments. The high level of decay might contribute to the high rate of weight loss, when comparing with weight loss data from previous trial.

In addition, the physiology of kumquat may have been affected by the high concentrations of EOs applied. The fruit is small and has a larger surface area than most citrus when compared to the fruit volume. Applying high concentrations of EOs may therefore affect kumquat than in the case of other citrus. Regnier *et al*. (2014) reported on the application of seven essential oils (*Mentha spicata*, *Cymbopogon aurantium* var. *amara*, *Cymbopogon citratus*, *Cymbopogon martinii*, *Geranium graveolens roseum Bourbon*, *Oreganum vulgare* and *Thymus vulgaris*) in their aqueous solutions forms at concentration as high as 10 000 µL/L on Valencia oranges without causing blemishes or desiccation. However, Garcia *et al.* (2008) warned on the phytotoxic effects that fruit may exhibit as result of direct contact between pure EO and delicate fruits skin. Therefore, high concentration of EOs in bulk may increase the concentration of active ingredient, thereby leading to phytotoxic effect on fragile fruit rind. In addition, the high decay incident resulted from the application of wax combined with EO at high concentration, may definitely influence the fruit physiology by stopping respiration and also increase contact between EO and the fruit rind.

The results on the fruits decay for the storage at 5 °C for 34 days and at 22 ± 1 °C for 4 days are provided in Figure 4.17.

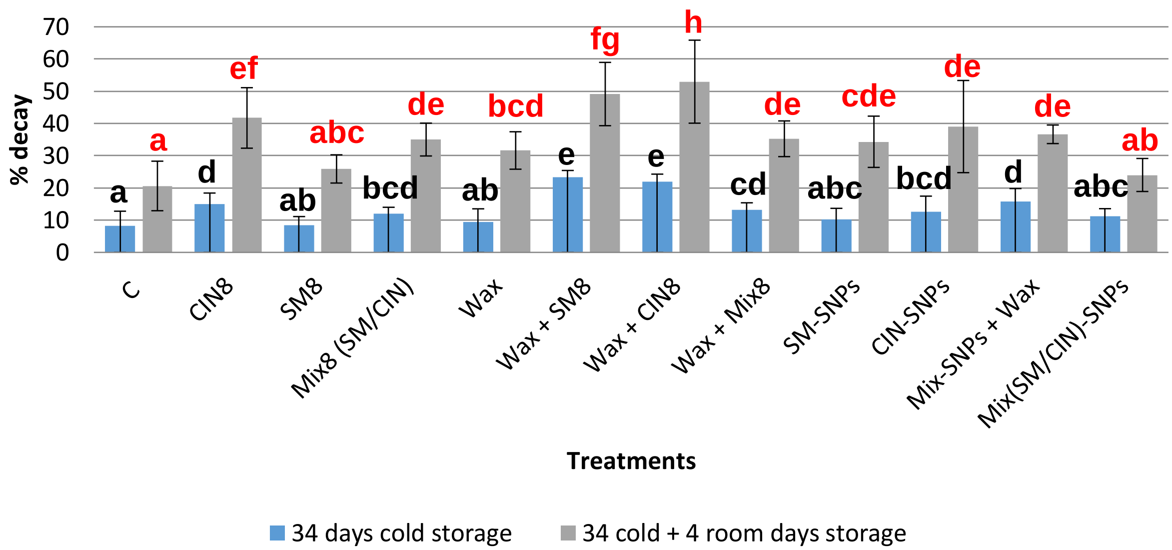

Figure 4.17: Effect of the treatment on fruit decay after 34 days at 5 °C and an extended 4 days at 22 ± 1 °C in TUT (Result expressed as percentage decay per treatment)

C= control; CIN8= cinnamon leaf (8000 µL/L); SM8= spearmint (8000 µL/L); Mix8 (SM/CIN)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 8000 µL/L); Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); SM-SNPs= spearmint-mediated SNPs; CIN-SNPs= cinnamon leaf-mediated SNPs; Mix (SM/CIN)-SNPs= mixture of spearmint and cinnamon leaf-mediated SNPs. Values with the same letter do not significantly differ at p > 0.05, according to Duncan's multiple range test. *Means indicating at least one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test. The black letters indicate the significance level between the treatment of 34 days cold storage and the red for the extended four days at room temperature*.

Both storage conditions resulted in high levels of decay in the treated fruit than in the control. The control showed the lowest percentage of fruits decay (8.25 ± 4.53% and 20.62 ± 7.69%, for the 34 days at cold and extended four days at room temperature, respectively). As before, the wax treatments yielded the poorest results.

Treatments WSP and WCIN provided decay above 21% (about three times more than the control) for the 34 days of storage at 5 °C and over 49% when the fruits were stored for an additional four days at 22 ± 1 °C. In spite high decay incidence recorded at the end of the extended storage period, unaffected fruit did not exhibit signs of rind burning or blemishes. However, rapid ripening was observed at the end of four days storage, explaining the increased fruit susceptibility to pathogen development. This indicates alteration of fruit respiration, and increased ethylene production (Bangerth *et al*., 2012). The increase in ethylene production is therefore in response to abiotic stress induced in the citrus fruit (Ballester & Lafuente, 2017). The high concentration of EO applied may have affected the rind structure thereby increasing sensitivity to physical factors such as high temperature to which fruits were exposed after cold room storage.

The weight loss data for both storage conditions are recorded in Table 4.13.

Table 4.13: Percentage weight loss per treatment after 34 days at 5 °C and an extended 4 days at 22 ± 1 °C (TUT) (Result expressed as percentage weight loss per treatment)

Code	% Loss (34 days)	SD	% Loss (34 + 4 days)	SD
C	4.82^{abc}	1.03	13.47^{A}	9.89
CIN8	5.02^{abc}	0.58	12.09^{A}	1.57
SM8	5.41^{bc}	0.65	9.33^{A}	1.8
Mix8 (SM/CIN)	5.43^{bc}	0.4	10.16^{A}	1.12
Wax	4.01^{a}	0.23	9.33^{A}	1.63
Wax + CIN8	4.54^{abc}	1.66	10.03^{A}	6.13
Wax + SM8	7.97^{c}	6.35	12.85^{A}	8.33
Wax + Mix	5.43^{bc}	0.68	9.88^{A}	1.52
SM-SNPs	5.54^{bc}	0.37	9.90^{A}	4.72
CIN-SNPs	4.94^{abc}	0.41	9.25^{A}	1.9
Mix (SM/CIN)-SNPs	4.81^{abc}	0.64	10.14^{A}	1.2
Mix-SNPs + Wax	3.86^{a}	0.33	9.91^{A}	1.04

SD= standard deviation

C= control; CIN8= cinnamon leaf (8000 µL/L); SM8= spearmint (8000 µL/L); Mix8 (SM/CIN)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 8000 µL/L); Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); SM-SNPs= spearmint-mediated SNPs; CIN-SNPs= cinnamon leaf-mediated SNPs; Mix (SM/CIN)-SNPs= mixture of spearmint and cinnamon leaf-mediated SNPs. *Values with at least one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test. Small later indicate significance level between the values of 34 days storage and capital letter for the extended four days at room temperature*.

Again, fruit lost more weight when removed from the cold storage. However, the storage at 5 °C for longer period preserved the fruit from losing more weight as it was the case in Trail 2.

In the packhouse, treatment with spearmint at 8000 µL/L (SP8) yielded the lowest decay incidence at 10.6 ± 2.0% followed by the wax alone 11.8 ± 2.0% (Figure 4.18). The control displayed 16.4 ± 2.0% decay. All treatments consisting of wax combined with EOs or SNPs with wax resulted on a high decay incidence, which was almost double that of the control. The mixture cinnamon and wax provided the highest decay incidence with 32.4 ± 6.0% (Figure 4.18).

The outcomes of fruits stored in the packhouse are presented in Figure 4.18.

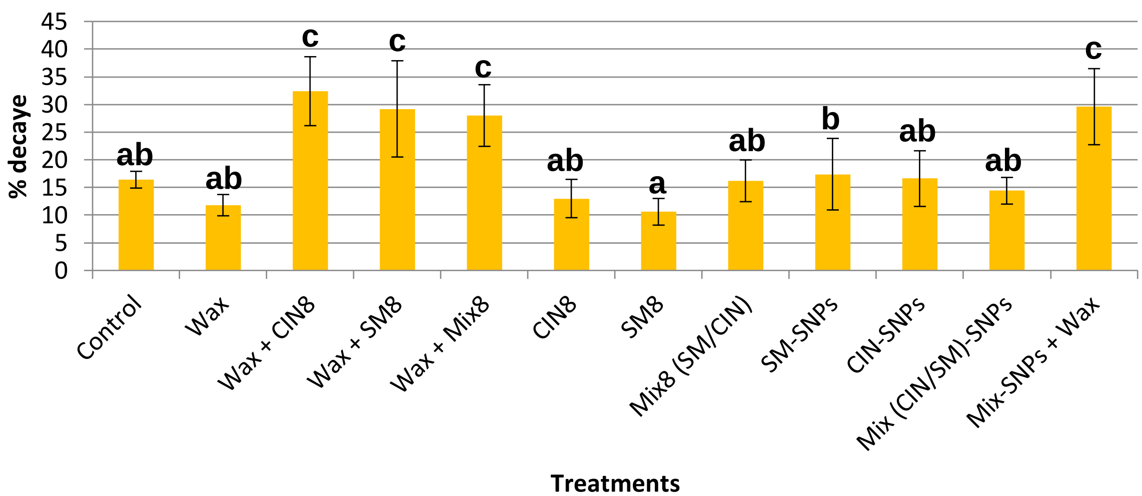

Figure 4.18: Effect of the treatment on fruit decay after 34 days storage at 5 °C in Levubu (Result expressed as percentage decay per treatment)

C= control; CIN8= cinnamon leaf (8000 µL/L); SM8= spearmint (8000 µL/L); Mix8 (SM/CIN)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 8000 µL/L); Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); SM-SNPs= spearmint mediated SNPs; CIN-SNPs= cinnamon leaf mediated SNPs; Mix (SM/CIN)-SNPs= mixture of spearmint and cinnamon leaf mediated SNPs. *Means with one identical letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

The fruits stored in the packhouse were also examined for weight loss and the results are presented in Table 4.14. The analysis of variance showed no statistical significant difference between the means of the 12 treatments at the 95% confidence level, since the P-values were greater than 0.05.

Table 4.14: Percentage weight loss per treatment after 22 ± 1 °C at 5 °C at the packhouse (Result expressed as percentage weight loss per treatment)

Code	% Weight loss	SD
Control	5.44^{ab}	1.43
Wax	4.53^{a}	0.49
Wax + CIN8	5.85^{ab}	1.63
Wax + SM8	6.11^{ab}	2.09
Wax + Mix8	4.99^{a}	1.67
CIN8	8.30^{b}	6.14
SM8	4.33^{a}	0.85
Mix8 (SM/CIN)	4.86^{a}	0.57
SM-SNPs	3.77^{a}	0.29
CIN- SNPs	5.76^{ab}	3.00
Mix (SM/CIN)-SNPs	4.26^{a}	0.74
Mix-SNPs + Wax	5.30^{a}	1.80

SD= standard deviation

C= control; CIN8= cinnamon leaf (8000 µL/L); SM8= spearmint (8000 µL/L); Mix8 (SM/CIN)= mixture of lemongrass and spearmint at ratio 1:1 (final conc. 8000 µL/L); Wax= apple wax (Naturcover conservacion extra; Decco Ibeerica Post-Cosecha, Valencia, Spain); SM-SNPs= spearmint mediated SNPs; CIN-SNPs= cinnamon leaf mediated SNPs; Mix (SM/CIN)-SNPs= mixture of spearmint and cinnamon leaf mediated SNPs. *Values with the same letter do not significantly differ at p > 0.05, according to Duncan's multiple range test*.

The lowest weight loss percentage recorded in this storage was of treatment spearmint-mediated SNPs (SM-SNPs) which accounted 3.77 ± 0.29% followed by the mixture cinnamon/spearmint mediated SNPs [Mix (SM/CIN)-SNPs] with 4.26 ± 0.74% (Table 4.14). Treatment SP8, which provided a better control for the packhouse storage (Figure 4.18) had a weight loss of 4.33 ± 0.85%. The results on the weight loss may be influenced by the position of the boxes in the cold room.

Trial 3

This trial constituted the true test because everything was conducted exactly as done in real life, following all conditions required for the packaging and export of kumquat. In this trial, fruits were shipped by airfreight to Rotterdam (Europe) where the evaluation of the fruits was conducted. The concentration of EOs used in this trial was the same as in Trial 1 (3000 µL/L). The performance of treatments on exported fruits is summarised in Table 4.15. The fruits were evaluated for firmness, rind colour, decay, rind pitting and shrivelling on the second day after arrival in Rotterdam.

Table 4.15: Quality of kumquat, treated with various essential oil applications following a chlorine bath in the packline, after export to Rotterdam

Treatments	Firmness	Colour	Decay	Rind pitting	Shriveling
Control (commercial fruit)	80% slight sensitive/ 20% sensitive	Light dark orange	2%	1%	1%
Spearmint	80% slight sensitive/ 20% sensitive	Light dark orange	-	-	1%
Cinnamon leaf	100% slight sensitive	Light dark orange	-	1%	-
Nano (mixture EO)	80% slight sensitive/ 20% sensitive	Light dark orange	1%	1%	1%
Wax spearmint	80% slight sensitive/ 20% sensitive	Light dark orange	2%	1%	1%

Only green mould infection was present and all treatments were able to control decay during and after export to Rotterdam. Even the commercial treatment presented with only 2% disease. Minimum rind pitting and shriveling were observed. In general, treatment with 3000 µL/L cinnamon leaf (CIN3) and 3000 µL/L spearmint (SM3) essential oils both at 3000 µL/L were the most effective with no decay as illustrated in Figure 4.19.

Moreover, for cinnamon leaf (CN3) and spearmint (SM3) EOs, less shrivelling or rind pitting was recorded than for the commercial fruit. The assessment on the firmness revealed that cinnamon leaf oil exhibited the best outcome with 100% fruit described as slightly affected, compared to 80% for the commercial control, with 20% described as sensitive. None of the treatments affected the rind colour. Therefore, cinnamon leaf oil followed by spearmint oil showed the potential of being the best treatment of the trials. However, the sharp odour of cinnamon leaf oil may affect the sensorial quality of the fruit (Brnawi, 2016), and therefore the ultimate option to control green mould incidence on kumquat dwells in the application of spearmint oil.

Figure 4.19: Photographs of kumquats following air transport to Rotterdam.
A) Control (fruit treated only with 0.2% chlorine bath at 37 °C): a) rotten kumquat with green spores development; b) kumquat rind pitting; c) decayed kumquats with white mycelial developed; d) fruit appearance in the box. B) Fruit treated with spearmint at 3000 µL/L: a) and c) whole box of fruit's appearance; b) Closer observation of fruit without phytotoxic effect of the oil; d) fruit sliced showing intact quality of the albedo and carpel. C) Fruit treated with cinnamon leaf at 3000 µL/L: a) fruit sliced showing intact quality of the albedo and carpel; b), c) and d) Closer observation of fruit without phytotoxic effect of the oil.

Plaza *et al*. (2004) attributed the failure in the implementation of EOs as crops protective products to the fact that successful results are obtained *in vitro* while failing when applied *in vivo*. In the case of this study, the theory is found to be true for lemongrass. Although, the substance was found very effective against both green mould and sour rot *in vitro*, it performed poorly when applied *in vitro*. In contrast, spearmint oil failed to impress in the in vitro assays, yet provided good protection from green mould *in vivo* on kumquat.

In most cases during the three trials, the application of wax alone and EO-amended wax did not lead to a decrease in the incidence of decay as expected. Instead, the number of rotten fruit was higher than the number recorded for treatments such as EOs (CIN and SM) applied alone by spraying. Commercial waxes are generally used to enhance shine, reduce water loss and act as a vehicle for fungicides (Contreras-Oliva *et al*., 2011). Hassan *et al*. (2014) reported on the importance of adjusting the thickness of the wax coating according to the type of fruit, storage condition and intended market requirements. They emphasised that decay may not be controlled when too thin wax layers are applied, whereas too thick wax emulsion might be the cause of fruit rot, leading to the development of off-flavours. Wax coatings can have a detrimental effect on the fruit by creating a barrier between the external and internal atmosphere, thereby restricting the exchange of respiratory gases (CO_2 and O_2). This could result in fermentation and development of off-flavours (Hassan *et al*., 2014) from the development of anaerobic bacteria. The application of apple wax to the kumquat, as used commercially, possibly made the fruit more prone to infection by disturbing the fruit physiology.

Throughout the trials (*in vitro* and *in vivo*) the spearmint and cinnamon leaf oils yielded the best results in terms of decay control. The performance of lemongrass EO was, however, disappointing. The spray application of the EO produced better disease control than EO-amended wax coatings. The final trial carried out in the packhouse and during export is a true reflection of the real-life situation. In the laboratory, conditions are far more controlled than in the packline and cold-storage, while conditions may be variable during air transport, affecting the concentration of the EO on the rind. Nevertheless, both cinnamon and spearmint oil performed well under commercial conditions. The commercial outcome of spearmint oil in this study corresponds to the findings of Du Plooy *et al* (2009). They reported that spearmint oil-amended commercial coatings (StaFresh series SF865MS and Carnauba Tropical®) were effective for the inhibition of green mould on Tomango oranges during a commercial export–simulting trial.

4.4.4 Evaluation of chlorine bath, crates and packline for the potential fungal contamination

There are different stages at which citrus fruits are infected and contaminated by pathogenic fungi, which includes the orchard and after harvest through mechanical injury and/or subsequent handling and storage (Talibi *et al*., 2014). Swabs were taken at various parts of the packline and from the chlorine bath, to identify potential infection sources. The packing crates, ‘passage’ after the chlorine bath, the rollers of the dryer, the channel for fruit selection after drying, and the collection area were sampled.

Chlorination is used commercially for disinfection of fruit surfaces (Kassim *et al*., 2016). Chlorine is an effective sanitiser of most pathogenic fungi associated with deciduous fruit decay (Fourie, 2013). Although sanitisers such as chlorine reduce the spore load, it is unable to control infections that are already established in the fruit tissues (Fourie, 2013). Therefore, the action of chlorine is confined to the fruit surface and it is unable to control wound pathogens such as sour rot and green mould (Lesar, 2003; González-Candelas *et al*., 2010). Initially, the chlorine bath was suspected of being a source of green mould contamination, since the solution was not renewed throughout the washing of the fruit from early morning until the closing of the packline in the late afternoon. The outcome on the evaluation of possible contamination of the fruits in the packing-line is illustrated in Table 4.16.

Table 4.16: Identification of potential points of contamination throughout the packline

Section	**Colony forming units/mL (CFU)**
Crates (C)	8.0×10^3
Chlorine bath before	0
Chlorine bath after	0
First (1st)	3.3×10^2
Roller drier (RD)	1.0×10^1
After roller (AR)	6.0×10^4
Collection place (CP)	6.0×10^4

The outcome revealed that the chlorine bath effectively killed all microorganisms in the first phase of the packline. The efficacy of the bath was retained throughout the day, since no CFUs were recorded in the sample taken from the bath at the end of the day. However, the packline and crates had some pathogenic load. The collection section was the most contaminated area, while green mould was mostly found at the first channel (passage on which fruit pass through after chlorine bath and prior to the roller drier). This results indicate that the decay developing on the fruit after packing stems either pre-havestly from orchard contamination, or post-harvestly after the chlorine bath or during handling. The application of chlorine to kumquats only delays the onset of infection. Kassim *et al*. (2016) demonstrated that the chlorine bath could only suppress the growth of *P. digitatum* on kumquats for 14 days, but thereafter infection became apparent with visible signs of the mould on the 28th day.

4.4.5 Effect of warm water dip on the quality of kumquats at different temperatures

4.4.5.1 Effect of warm water bath at different temperature on decay

At the packhouse where our commercial trials were conducted, kumquat were subjected to chlorine warm water bath set at 37 °C. However, literature suggested dipping in hot water at temperature range of 50-53 °C to be suitable for the treatment of kumquat to inactivate potential fungal infection present in the fruit (Schirra *et al*., 1995; Vázquez *et al*., 2015). Heat treatment has been investigated as one of the alternatives to chemical treatments for postharvest application on citrus fruit (Erkan *et al*., 2005; Palou, 2009; García *et al*., 2016). It was hypothesised that physical treatment such as heat treatment may act directly and indirectly on the pathogen infecting the fruit (Palou, 2009). Moreover, hot water has been associated with the increase of phenolic contents (Shen *et al*., 2012), delay of ripening and rigidity of fruit rind (Paull & Chen, 2000), which play an important in the defence mechanisms of citrus fruit against pathogens (Ballester & Lafuente, 2017). However, exposure of some fruit with sensitive rinds to high temperatures may cause physiological disorders such as rind blemishes on fruit (Erkan *et al*., 2005; García *et al*., 2016). Therefore, it is necessary to provide the optimum temperature that is adequate to use in the integrated postharvest treatment of kumquat.

After 14 days storage at 22 ± 1 °C °C they were no differences between the decay incidence of the control (7%: 2 of 30 fruit) and the treatments at 35, 40 and 45 °C. Fruit treated at 30, 37 and 50 °C were free of decay. After 34 days of storage at 5 °C, 7% of the control and the fruit treated at 37 and 45 °C were infected with green mould (Table 4.17). The decay incident was observed to be low including in the control after

34 days of storage at 5 °C. Fruit sample treated at 35 °C was free of decay and remained free of decay after an additional four days storage at 22 ± 1 °C. This result was unexpected, since storage at room temperature caused a 7% decay incidence. Extending the storage period for four days at room temperature increased the incidence of decay slightly.

Table 4.17: Percentage decay following 34 days storage at 5 °C and after an additional storage period of 4 days at 22 ± 1 °C

Temperature	% decay 34 days	% decay 34 + 4 days
Control (untreated)	7	10
30 °C	3	7
35 °C	0	0
37 °C	7	10
40 °C	3	10
45 °C	7	7
50 °C	3	10

The optimum temperature of growth for *P. digitatum* was reported to be 25 °C (Palou, 2014). Higher temperatures applied to the fruit would be expected to inhibit decay by green mould. Therefore, hot water bath are commonly used in commercial pack lines for citrus. A hot-water dip for 2 min at 52 - 53 °C (heat-treated 1 day after inoculation) has found to inhibit (no decay within 6 days) the development of the artificially inoculated *P. digitatum* decay in lemons, whereas 100% decay was observed after four days for fruit dipped in water at 25 °C (Nafussi *et al.*, 2001). Furthermore, thermal treatment at 45 - 55 °C for 2 - 5 min of oranges and mandarins were reported to reduce green mould infection in artificially inoculated fruit (Palou, 2013). The lack of control at many of the higher temperatures in the current study may be related to the time of exposure to the heat (1 min for all the treatments). García *et al*. (2016) reported on the influence of duration exposure of citrus fruits (oranges and mandarins) in the heat treatment. They observed that the decay incidence of green mould was reduced as the duration of heat treatment increased. However, longer exposure time and higher temperature of some citrus would result in negative effect on the appearance of the fruits, as it was the case for citrus Navel, Fortune, Elendale and Lanelate, treated at 53 °C for 2 min (García *et al*., 2016).

Those fruits exhibited slight and moderate rind blemishes. Kumaquat are small and have a thin rind and albedo. For this reason, the treatment was restricted to 1 min to avoid blemish formation. The treatment at 35 °C yielded the most promising results and should be further investigated.

4.4.5.2 Effect of warm water bath on Peel firmness

The results on the firmness of the rind are illustrated in Table 4.21. The strength of the rind was evaluated by calculating the percentage hardness compared to the control.

Table 4.18: Impact of warm water dip set at different temperature (30, 35, 37, 40, 45 and 50 °C) on the rind firmness of kumquat stored for 34 days at 5 °C with additional 4 days storage at 25 °C

Temperature	Firmness (N/mm)	% hardness (relative to control)
Control	1006 ± 17^{a}	-
30 °C	1063 ± 12^{ab}	5.4
35 °C	1128 ± 17^{b}	11
37 °C	1037 ± 16^{ab}	2.9
40 °C	1042 ± 16^{ab}	3.5
45 °C	1052 ± 14^{ab}	4.3
50 °C	1009 ± 14^{a}	0.3

Values with the same letter do not significantly differ at p > 0.05, according to Duncan's multiple range test.

The fruit rinds of all the treatments besides 50 °C were firmer than the controls. García *et al*. (2016) reported that high temperature exposure of some species of citrus fruits may have a negative impact on the appearance of the fruits. The rinds of fruit dipped at 35 °C were the most firm (11% firmer than the control) (Table 4.18). This is attributed to the lower rate of infection found in the fruit treated at the same temperature.

4.4.5.3 *Effect of warm water bath on Rind colour*

The results on the evaluation of the rind colour are presented in Table 4.19. The total colour differences were calculated relative to the control.

Table 4.19: Influence of warm water dip set at different temperature (30, 35, 37, 40, 45 and 50 °C) on the colour parameters of kumquat stored for 34 days at 5 °C followed by 4 days storage at 25 °C

Temperature of exposure	L	A	b	Hue	Chroma	∆E
Control	61.37	30.44	51.26	59.29	59.62	0.00
30 °C	62.30	31.38	54.58	60.10	62.96	3.57
35 °C	62.23	30.24	51.49	59.57	59.71	0.91
37 °C	62.21	28.75	49.34	59.77	57.11	2.69
40 °C	60.03	27.88	44.99	58.21	52.93	6.90
45 °C	62.97	28.98	52.51	61.10	59.98	2.50
50 °C	60.71	26.97	45.86	59.54	53.20	6.45

There were no significant differences between any of the results obtained. The data on the hue angle are similar to those reported by Kassim *et al*. (2016) for the peel colour after different treatments of kumquats following 28 days of storage. The hue angle can be used to indicate the ripening of the fruits (Kassim *et al*., 2016). The hue angle results in the current study showed that the fruits had more and less similar degree of rind ripening after the storage period. However, some differences have been observed with the total colour difference of the fruits (∆E*). The total colour difference values ranged from 0 to 7. These differences were calculated based on colour differences compared to the control samples (Table 4.19). The lowest ∆E* value (0.91) was obtained from the fruits sample exposed at 35 °C and the highest value was 6.9 from sample 40 °C, followed by 6.45 data of sample 50 °C. This is an indication that visual changes in colour could be perceived between samples with lower ∆E* value and those with higher values (Choi *et al*., 2002).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 ACHIEVEMENT OF OBJECTIVES

The main purpose of the study was to assess the *in vitro* and *in vivo* ability of essential oils (EOs) in various forms (aqueous solution, encapsulated in chitosan and wax, and as EO-mediated SNPs) to control *Penicillium digitatum* and *Galactomyces citri-aurantii*. Both pathogens are amongst the most destructive of citrus worldwide. Although, much research effort has gone into studying the postharvest protective effects of EOs, their application has been limited due to constraints including the volatility of the chemical constituents (Pavela & Benelli, 2016) and the imparting of off-flavours to the product when applied at high concentration (Ponce *et al*., 2008). For this reason, the encapsulation of EOs into matrices such as chitosan (CHI) and commercial wax coating, and the use of EOs to mediate the synthesis of silver nanoparticles (SNPs) were considered as potential solutions to retain the antimicrobial benefit resulting from EOs.

All the listed objectives in accordance with Section 1.4 were achieved, using published methods with modifications where applicable.

To confirm the efficacy of the essential oils, several oils previously reported to have good activity against green mould and sour rot were evaluated in both the toxic medium and microtitre assays. The toxic medium assay revealed that among the individual applications of EO, lemongrass and cinnamon leaf exhibited the best activity (500 µL/L) by completely inhibiting the mycelial growth of sour rot and green mould. Spearmint oil was the least effective. The combination of EOs (cinnamon leaf/lemongrass; cinnamon leaf/spearmint; lemongrass/spearmint) did not markedly improve the activity when compared with the individual application of EOs. A slight enhancement in activity was obtained for the mixture of cinnamon leaf and spearmint

EOs against sour rot, providing a complete inhibition at a total concentration of 750 µL/L, whereas cinnamon leaf and spearmint alone, inhibited completely sour rot growth at concentrations of 750 and 1000 µL/L, respectively. The combination of EOs at different ratios (9:1; 8:2 etc.) with a final concentration equal to the lowest concentration where complete inhibition of the pathogens had been observed, was used to identify the oil mixture with the greatest efficacy. Results indicated that a higher relative concentration of cinnamon leaf, when combined with lemongrass and spearmint oil, led to an enhanced inhibitory effect against both pathogens. On the contrary, increasing the concentration of spearmint in the mixture did not improve the inhibition of green mould. Since cinnamon leaf oil has a strong odour, an increase in either lemongrass or spearmint would have been a more desirable outcome.

The microtitre assay initially led to inconsistent results. In addition, the intensity of indicator (INT) was inconsistent upon repetition of the experiment. Therefore, additional tools were applied to confirm the fungicidal activity of the EOs, including conducting light microscopy and plating out the suspension (EO/pathogen) on the surface of PDA to verify that spore germination does not take place. The results of the microscope observations and the fungicidal effects correlated, since the concentration at which spore germination was inhibited, also resulted in no growth on the plate. The MICs obtained were substantially higher than the concentrations required to completely inhibit mycelial growth in the toxic medium method. The lowest MIC value was obtained for cinnamon leaf oil against both sour rot and green mould at total concentrations of 2000 µL/L and 3000 µL/L, respectively, followed by lemongrass (total concentration 3000 µL/L and 4000 µL/L, respectively) when applied individually. As far as the mixtures were concerned, the lemongrass/spearmint (1:1) EO mix performed better than the other mixtures with MICs of 3000 µL/L and 4000 µL/L against sour rot and green mould, respectively, indicating that no benefit was derived from the use of mixtures.

Silver nitrate alone completely inhibited the mycelial growth of both pathogens at a concentration of 0.10% (equivalent to only 0.064% of Ag^+), using the toxic medium technique. Nevertheless, the combination of silver nitrate and EOs (cinnamon leaf, lemongrass and spearmint) further reduced the concentrations of the silver nitrate and EOs required, compared to those required when testing the substances alone. The lowest concentration was obtained with the mixture lemongrass and silver nitrate (100 µL/L and 0.05% (equivalent to 0.032% Ag^+), respectively], whereas silver nitrate combined with cinnamon leaf or spearmint resulted in complete inhibition at 0.01% (0.0064% Ag^+) and 250 µL/L, respectively. The use of silver ions to inhibit fungal growth must be further investigated, since this technique yielded excellent results.

The synthesis of SNPs was successfully attained at pH 10, with a colour change from colorless to golden yellow signaling the formation of nanoparticles. This was confimed through further characterisation of the particles. The *in vitro* experiment using SNPs demonstrated antifungal activity against green mould by retarding the growth (after one day of incubation), suppressing sporulation of the pathogen on the plate after four days of incubation on the surface of PDA and inhibiting the germination of spores as observed under the microscope. However, the synthesised SNPs were unable to inhibit sour rot.

The macrodilution technique used to determine the MICs of chitosan-encapsulated EOs provided much lower inhibitory concentrations than the other techniques tested. Lemongrass exhibited the lowest MIC at 0.25 µL/mL (250 µL/L) and 0.375 µL/mL (375 µL/L) when tested against sour rot and green mould, respectively.

Different techniques used for evaluating the EOs *in vitro* resulted in different concentrations required for the inhibition of the pathogens. This phenomenon was also reported by Thippeswamy *et al.* (2013) when testing solvent extracts from various plant species against *Fusarium verticillioides*. In their study, the poisoned food technique to assess the radial mycelial growth and two-fold broth microdilution method to determine the MICs were used. They found that the concentration (2 mg/mL) at which the extracts were effective (60% and above) against the mycelial growth was relatively lower than for the MIC recorded (0.25 to 1 mg/mL). For example, the chloroform extract of *Albizia amara* inhibited 81.2 ± 0.40% of mycelial growth at 2 mg/mL, while its MIC was obtained at 0.25 mg/mL. However, in spite of different assays yielding different values, the current study showed consistency in the reproducibility in term of EO performance. In both the toxic medium and microdilution assays it was found that cinnamon leaf oil had the highest efficacy. In addition, lemongrass repeatedly outperformed spearmint EO against both pathogens throughout the toxic medium, microdilution and macrodilution assays.

The lowest concentration of chitosan that exhibited antifungal activity was 7.5 mg/mL. However, a combination of the EOs and chitosan at their sub-inhibitory concentrations was found to provide complete inhibition of mycelial growth of both pathogens, indicating potential for *in vivo* use.

The *in vivo* experiments encompassed laboratory, semi-commercial and commercial packhouse trials. The semi-commercial trials involved treating artificially wounded Valencia oranges by dipping the first group in EO solutions, while the second group was brushed with a mixture of EOs and conventional wax. The poor results for both treatments and controls were attributed to the deep wounds inflicted during inoculation. However, the fruits dipped in spearmint solution demonstrated a delay in sporulation after seven days of storage, although this was not proven, since no data was recorded due to the poor results.

In the laboratory, two trials were conducted using kumquats artificially inflicted (wound and inoculated) with sour rot infection. The first trial involved treating the fruit with a combination of wax and EOs. The combination cinnamon/wax treatment exhibited a slightly lower incidence of decay than the controls (wax alone). The worst scenario was observed with the lemongrass/wax combination. The second trial included treating the artificially wounded and inoculated (sour rot inoculum) kumquats with EOs/chitosan coating. Fruit coated with EOs/chitosan and chitosan alone exhibited a higher decay incidence than the control (uncoated fruit). It was observed that higher decay incidence correlated with high concentrations of EOs in the coating. The poor result was attributed to the inability of the chitosan to form a film on the surface of the fruit, thereby creating a high moisture environment that, instead, favoured pathogen growth. The concentration of chitosan used for the coating was higher than that previously used by Guerra *et al*. (2016).

In response to a request by an exporter, three commercial trials were conducted in a packhouse. The control, in these three trials, consisted of fruit subjected to the chlorine bath (0.2% at 37 °C) without further treatment. The first trial involved treating the fruit with cinnamon leaf, lemongrass and spearmint EOs at concentrations of 2000 and 3000 µL/L, as well as a mixture of lemongrass/spearmint at a final concentration of 3000 µL/L (1:1). In addition to the EOs, fruit was also treated with apple wax, a suspension of lemongrass-mediated SNPs and a mixture of EOs (lemongrass/spearmint at ratio 1:1) at 3000 µL/L combined with the apple wax. Cinnamon leaf (3000 µL) and spearmint (3000 µL) oils provided the best outcome with a decay reduction of 83.13% and 79.99%, respectively, compared to the control, for the storage period of 14 days at 22 ± 1 °C. Moreover, cinnamon leaf oil (3000 µL) yielded the best result with the lowest percentage decay (0.71 ± 0.1%) for storage at 5 °C, while the highest decay incidence was recorded for the combination wax and mixture (lemongrass/spearmint) at 3000 µL (3.95 µL/L ± 0.45%). A decay incidence of 2.92 ± 2.4% was recorded for the control. The results of the extended four days storage at 22 ± 1 °C were such that both treatments with cinnamon leaf oil at 2000 µL and 3000 µL/L yielded the best results with a decay incidence of 3.25 ± 1.5% and 3.96 ± 0.6%, respectively.

Fruit stored at the packhouse facility demonstrated that treatment with cinnamon leaf (3000 µL) (2.49%) and lemongrass-mediated SNPs (2.53%) performed best compared to the control (13.62%), providing a decay reduction of 82.16% and 81.42%, respectively. Lemongrass EO was the least effective but still yielded better results than the control.

After the first commercial trial it was concluded that treatment with cinnamon leaf oil at 3000 µL/L provided the best overall outcome under all storage conditions used. The SNP suspension led to an average of 76.57% decay reduction for all the storage conditions assessed in this trial (Trial 1), indicating that SNPs are effective *in vivo*.

In the second commercial trial, the concentration of the EOs was increased to 8000 µL/L. The increase in concentration was to assess if high concentration would yield better outcomes than recorded in Trial 1. The substantial increase in the concentration of the EOs did not reduce the decay incidence. All the combinations of wax with either EOs or SNPs had a negative impact on the fruit, thereby resulting in a higher percentage decay than for the untreated control. It was concluded that a high concentration of EOs might affect the physiology of the delicate kumquat rind and that the wax together with the EOs may restrict the exchange of respiratory gases, thereby causing adverse effects on the fruit physiology.

The third trial involved treatment followed by transport *via* airfreight to Rotterdam, The Netherlands, where they were evaluated for firmness, colour, decay, rind pitting and shriveling by an independent importer as the fruit reached its destination. The treatments consisted of cinnamon leaf (3000 µL/L) and spearmint (3000 µL/L) oils, apple wax/spearmint (3000 µL/L) and spearmint-mediated SNPs. The control consisted of untreated fruit (chlorine bath only). The best outcomes in terms of decay were recorded after treatment with cinnamon leaf and spearmint alone. Both treatments provided zero percent decay incidence against the 2% recorded for the control during export to Rotterdam. The outcome of SNPs was not negative. In fact, it outweighed both the control (2% decay) and spearmint/wax (2% decay) by providing 1% decay. Only low levels of rind pitting or shriveling (1%) was recorded with cinnamon leaf or spearmint, respectively. Since the strong odour of cinnamon leaf oil may restrict its use for the export of kumquat, spearmint was concluded to be the best alternative to synthetic fungicides.

However, it is clear that the results do not accurately reflect the previously reported effect of these EOs. Lemongrass and spearmint oils as well as their mixture were reported to provide successful inhibition both *in vitro* and *in vivo* against sour rot and green mould (Regnier *et al*., 2014). Moreover, although lemongrass was highly effective *in vitro*, it did not prove effective in *in vivo* experiments, while the contrary was observed with spearmint oil.

Upon evaluation on the possible fungal contamination that might occur from the crates, chlorine bath and different sections of the packline, none of the tested areas indicated a source of contamination, except for the passage after the chlorine bath, where green mould was found. The results from the chlorine bath demonstrated that the sanitizer was able to kill the pathogen on fruit rind. However, like any other sanitizer, chlorine is not able to control infections that already established in the interior of the fruit tissue (Fourie, 2013). Therefore, the near absence of green mould throughout the packline suggests that fruit infection occurs primarily in the orchard.

Kumquats are routinely immersed in water at 37 °C in the packhouse where our trials took place. However, this temperature selection is not based on scientific evidence. The effects of exposure to various temperatures were assessed on the decay and physiology (rind colour and firmness) of the fruit. The results revealed that treatment at 35 °C was best, with no decay and better rind firmness recorded relative to the control. The lowest ΔE^* value (0.91) was also recorded for fruit exposed to 35 °C, indicating that the rind colour of the sample could be visibly distinguished from the other samples. However, the result on the hue angle and chroma revealed that all the fruit undergo a similar degree of ripening (Kassim *et al*., 2016). It was concluded that the warm water dip of kumquat at 35 °C for 1 min is potentially good to use in the integrated postharvest control of kumquat in the packhouse. However, further investigation is needed to confirm its application.

5.2 CONTRIBUTION OF THE STUDY

Researchers studying the effects of natural fungicides as alternatives to their synthetic counterparts, use a variety of *in vitro* techniques to evaluate their efficacy. In this study it was clearly demonstrated that the effective concentration values determined to control the pathogens differ substantially, depending on the technique applied (toxic medium, microtitre and macrotitre assays). However, the different techniques consistently indicated the same trend in efficacy i.e. cinnamon leaf oil > lemongrass oil > spearmint oil. It is therefore not necessary to use several techniques to test the effectiveness of the oil in *in vitro* studies of this nature. The ideal method to use is the toxic medium. The method is simple and avoids the use of multiple further tests to confirm results as in the case of microtitre assay.

The current study made use of cinnamon leaf, lemongrass, spearmint and their mixtures (cinnamon leaf/lemongrass, cinnamon leaf/spearmint and lemongrass/spearmint) as reducing and stabilising agents of silver in the preparation of SNPs. The generated SNPs presented some antifungal activity with the reduction of green mould decay incidence *in vivo*, but was no better than the EOs alone.

The biggest contribution of the study was to identify a natural treatment that will allow a reduction in decay on kumquat during export. The application of synthetic fungicide to the fruit rind is prohibited, since the rind is consumed together with the fruit and used in beverages (Peng *et al*., 2013). The fruit is also used in the candied form or preserved in sugar syrup (Lou & Ho, 2016). Fruit exported from the packhouse near Levubu (Limpopo, South Africa) was found to be contaminated mainly with green mould, with about 11% of the fruit lost, after 6-7 days of transport. The application of cinnamon leaf and spearmint EOs was found to reduce decay substantially. Spearmint oil, due to its pleasant and light odour, holds the biggest promise as an alternative fungicide.

Finally, this study has highlighted the need for conducting trials of this nature under packhouse conditions. Many reasons substantiating this statement were brought to light during this study. Firstly, the *in vitro* results were not reflected by the *in vivo* results. Lemongrass EO, which had demonstrated a good antifungal activity *in vitro*, was outperformed by the other two oils *in vivo*. The opposite was observed with spearmint oil. Secondly, the *in vitro* trials were unable to predict the effects of the treatment on the physiology of the fruit. For example, the application of wax supplemented with EO to the rind decay resulted in a high incidence of decay. Du Plooy *et al*. (2009) reported that the application of wax amended with EO had reduced disease incidence in a commercial trial using Tomango oranges. However, the application of the same treatment to kumquat increased the disease incidence, possibly due to differences in the physiology of the fruit. This was attributed to the large surface area to volume ratio of the tiny fruit when compared to the large Tomango oranges. In addition, the EOs were unable to control decay in artificially inoculated fruit due to its drastic effects on the fruit. Natural infection provides a more sensible approach and allowed the outcome of applying EOs to be compared to the normal treatment. Thirdly, the prevailing conditions in a laboratory used for *in vivo* trials to those in a packhouse are very different. There is a great deal of air movement in a packhouse, and the temperature is not regulated. This may lead to the increased evaporation of the EOs due to their highly volatile nature. Application in a “real-life” situation will provide more realistic and robust results than *in vivo* trials in a temperature-controlled laboratory. The same argument applies to the transporting of fruit, the conditions of which are very different to those in a laboratory or cold storage room.

5.3 RECOMMENDATIONS FOR FUTURE WORK

From this study, the following recommendations can be made for further research:

- More work should be undertaken to assess the inhibitory and film-form capability of the mixture EO/chitosan at a lower concentration as previously applied on citrus (Chien *et al*., 2007; El Guilli *et al*., 2016) and on other fruits (Guerra *et al*., 2016).
- Since it was recommended that the concentration of wax should be monitored (Hassan *et al*., 2014), and in this study, the application of apple wax in combination with EO resulted in high percentage of decay incidence of kumquat, more experiments should be conducted to establish the concentration of the wax in combination with EOs that would be effective to enhance quality and prolonged shelf life of kumquat.
- The use of oil-mediated should be further investigated for pathogen control on citrus since they demonstrated potential as antifungal agents.
- The most important recommendation emanating from this study is that studies of this nature must be carried out in a packhouse from the start, and the fruit transported as usual, with evaluations done at the point of arrival. This will provide

a more realistic and implementable solution to the quest for natural and effective fungicides.

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