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Research Article

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In-Vitro Assessment of Thyme Oil (Thymus Vulgaris) as Antifungal Agent Against Phyllosticta Citricarpa

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ABSTRACT

The spread of Citrus black spot (CBS) is a major concern in the citrus industry because the disease threatens fruit marketability and citrus tree health. Furthermore, there is a public concern about the safety and side effects of synthetic fungicides currently being used to control citrus black spot. Synthetic fungicides are reported to have carcinogenic effects on humans and are also toxic to the environment, however this depends on the structure of the active ingredient and the dose present. Furthermore, microorganisms tend to develop resistance to most synthetic fungicides. This problem has prompted research into the identification of new ways with broad activity in treatment of microbial disease in plants such as the use of essential oils.

The purpose of the study was to investigate the use of Thyme oil (Thymus vulgaris) as an alternative antifungal against Citrus Black Spot (CBS). Thyme oil was characterised using GC/MS. Thymol (32.1%) and ρ-Cymene (20.4%), were identified, as major compounds. Furthermore, the minimum inhibitory concentration (MIC) of essential oils against the test organism was 25 (µg/ml). The antifungal activity of Thyme oil, Thyme hydrosol and antifungal anti-mitochondrials was tested in vitro against Phyllosticta citricarpa using the agar diffusion bioassay. Comparative antifungal activity was observed between antifungal anti-mitochondrial, Thyme oil and hydrosol as indicated by an inhibition zone, minimal growth, and maximum growth zone towards the edge of the plate. We propose that Thyme oil, like antifungal anti-mitochondrials, inhibits fungal growth by targeting structures with increased mitochondrial activity. This was further confirmed by Scanning Electron Microscopy (SEM) and XTT colorimetric assay. This indicates that Thyme oil and hydrosol can be used as potential alternative antifungal agents against Citrus Black Spot (CBS).

Keywords: Citrus Black Spot, Thymus Vulgaris, Phyllosticta Citricarpa, Anti-Mitochondrion Activity, Antifungal Activity, Hydrosol, Minimum Inhibition Concentration

Kindly note that much of this work has been reported previously in my Postgraduate thesis cited below. An Investigation of Alternative Antifungals against Phyllosticta citricarpa Kiely and Guignardia mangiferae (Msc dissertation, Bloemfontein: Central University of Technology, Free State)), and as such is not considered a prior publication.

Introduction

Citrus Black Spot (CBS) is a widespread problem for citrus production globally. It is a disease caused by the fungus Phyllosticta citricarpa which affects the rind of citrus fruits without resulting in internal decay [1–6]. Foliar and fruit blemishes affecting the rind of the fruit bringing about cosmetic lesions have been observed in infected fruits [7]. Heavy infections near the pedicel of the developing fruit may lead to premature fruit drop [4]. The above-mentioned factors have resulted in worldwide losses in the field, during storage, in transit and commercial decay, resulting in a decrease of the harvest and income in the citrus fruit industry [1,8,9]. Reported losses can amount up to 25% of the total production in developed countries, whilst in developing countries losses often exceed 50%, due to lack of adequate storage facilities [10,11]. Losses may be substantial especially in developing countries because affected fruits are no longer suited for the fresh fruit market [12,4]. Fruits that are heavily infected with P. citricarpa are used for juice production, which yields a much lower income per ton produced, highlighting a need for effective treatment [13]. Treatment programs to control CBS are extremely costly, but if

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left untreated the entire crop could be lost due to CBS infections [13]. In most citrus production areas where CBS is prevalent, production will be impossible without an effective CBS control program [13]. However, when permitted, synthetic fungicides are used as primary means of control. It is estimated that over 23 million kg of synthetic fungicides are used annually worldwide [14].

Synthetic fungicides inhibit fungal growth by targeting reproductive structures (ascospores and conidia) known to be important in the fungal life cycle. These structures play an important role in the growth and dispersal of fungi that habitually cause huge damages in agriculture resulting in critical losses of yield, quality, and profit [15,7]. However, over the years, the use of synthetic fungicides has increased concern due to their toxicity, negative effects on the environment and development of resistance [1, 19-19,]. Regular use of fungicides results in environmental pollution risk particularly if residues are retained in soil or transferred into water. This has a negative effect on soil organisms and carries a potential risk to long-term fertility of the soil. Compounds that could improve pathogen control, yet minimize environmental risk are considered extremely valuable to resolve observed challenges. Consequently, both farmers and researchers have started to consider the use of alternative methods to control fungal diseases [20].

Showed that anti-inflammatory compounds such as salicylic acid (SA) possess antifungal properties. A yeast bio-assay was developed to expose antifungal properties of this compound using Eremothecium ashbyi as test organism. Similar treatments were also done by using Mucor circinelloides and Aspergillus fumigatus respectively. After exposing fungi to anti-inflammatory compounds, asci formation and sporangia were affected. These authors further showed that asci and sporangia contain increased mitochondrion activity when compared to hyphae. It is observed that anti-inflammatory compounds selectively target structures with increased mitochondrion activity. Although alternative methods are investigated and used to control postharvest decay during storage, natural plant products such as essential oils (EO's) and hydrosol, a by-product of essential oil production, are gaining recognition and the attention of researchers globally due to their biodegradable, eco-friendly, economical and safety properties. The EO's reported in various studies have shown to exhibit antifungal properties possibly by targeting reproductive structures such as ascospores and conidia for both in vitro and in vivo in different fresh produce. However, there is limited information regarding the effect of essential oils against P. citricarpa. The current study investigated the inhibitory effects of essential oils on the reproductive structures of P. citricarpa in vitro. We propose that EO's can be ideal candidates for use as alternative fungicides as well against Citrus Black Spot (CBS).

Materials and Methods

Strains used and Cultivation Method

The P. citricarpa that was used in the study was supplied by the national collection of fungi Agriculture Research Council-Plant Protection Research Institute. (ARC-PPRI) in Pretoria South Africa. P. citricarpa was cultured in a Petri dish on yeast malt (YM) agar at 25 ° C until the spore-releasing were observed.

Mitochondrial Mapping for Phyllosticta Citricarpa

Mitochondrial mapping was performed to determine which structure of P. citricarpa has increased mitochondrial activity.. Simply put, fungal cells from P. citricarpa were scraped from the yeast malt slab. Fungal cells were washed separately with phosphate buffered saline (PBS) in a 2 mL plastic tube to remove agar and debris. They were then treated with 31 µl Rhodamine 123 (Rh123) for 1 hour in the dark at room temperature and then the cells were washed again with PBS to remove excess pigment. Finally, the cells were fixed to the slide and observed using a confocal laser scanning microscope (Axioplan, Zeiss, Go¨ttingen, Germany) coupled to a Colorview Soft Imaging System (Mu¨nster, Germany)]. Rh123 is a cationic lipophilic mitochondrial pigment used to selectively image mitochondrial function (Δψm). This is due to the highly specific attraction of this cationic fluorescent dye to the relatively high negative potential across the mitochondrial membrane of living cells. For this dye, the high Δψm is indicated by yellow-green fluorescence (collected at 450 nm). On the other hand, low Δψm is indicated by red fluorescence at 625 nm

Essential oil Characterization using Gas Chromatography Mass Spectrometry (GC-MS)

Gas Chromatography Mass Spectrometry (GC-MS) was performed to characterize Thyme oil (Thymus vulgaris). Briefly, the essential oil was dissolved in hexane (10% hexane) and injected in a Finnigan Focus Gas Chromatograph (GC) which was operated under the following conditions: the injector temperature was set at 230˚C. The GC was equipped with an AB-1MS (30m x 0.25mm id 0.25µm) capillary column. Helium was used as carrier gas at a constant flow of 1mL min-1 (at a split ratio of 50:1). The temperature programme was set at 40˚C for four minutes and then raised at 5˚C min-1 to 200˚C and then held at 200˚C for 1 minute and then raised at 5˚C to 220˚C where it was held for 10 min. Mass analysis of the oils was done using a Finnigan Focus DSQ mass spectrometer. The ion source temperature was set at 250˚C with an ionization voltage of 70 eV and mass scan range of 50-650 amu. Individual GC peaks and mass spectra were identified by searching commercial libraries and this was followed by expert matching of MS data.

Agar Diffusion Method for Phyllosticta Citricarpa

The bio-assay (using P. Citricarpa as test organism) was based on the agar diffusion method as described by where activity of essential oils: Thyme oil (Thymus vulgaris), hydrosol and a known antifungal anti-mitochondrial compound which was used as a positive control (salicylic acid (SA); and Ethanol (negative control) were measured along a concentration gradient across the agar plate (i.e. from position of compound addition) by observing the growth inhibition-zone. To ensure precise comparison of all tested compounds (EO's, hydrosol and anti-mitochondrial compound) the bioassays were carried out according to the method of Ncango et al. (2010). Briefly P. Citricarpa was suspended in sterilized distilled water (dH20) and 0.2ml was streaked out on YM (0.5% m/v agar) to produce a homogenous lawn across the surface of the agar. Subsequently, a well (0.5cm in diameter and depth) was constructed at the centre of the Petri dish and 46µl of essential oil (that is, 2ml in 100ml 96% ethanol) was added separately to each plate containing the organism. The same procedure was carried out for known

antifungal anti-mitochondrial compounds 46µl (that is 2g salicylic acid added to 100ml of ethanol) was added, and 96% ethanol was added alone as a control. Similar amounts of Thyme oil hydrosol alone were tested for antifungal activity using the bioassay only for P. Citricarpa. All plates were incubated at 25°C until different textured growth zones were observed.

Broth Microdilution Assay (Minimum Inhibitory Concentration Determination)

The antifungal activity of thyme (Thymus vulgaris) and ethanol (negative control) against the fungus P. Citricarpa was investigated using the microdilution method developed Tonial et al., (2017) with a slight modification. To determine the minimum inhibitory concentration (MIC), microdilution was performed in 96-well plates by adding 90 µL of Potato Dextrose Broth (PDB), 10 µL of the fraction being tested (Thyme oil and ethanol respectively), and 50 µL of P. Citricarpa conidial suspension in saline solution (6 × 105 conidia ml−1) to each well. The plates were incubated at 25◦C for 7 days. Where after 40 µl of 4 mg/ml Iodonitrotetrazolium salt solution was added to each well and further incubated for another day. Growth was indicated by a change of colour to pink or violet after a day of incubation (Light pink (+") denotes minor growth, Pink (++") denotes medium growth, and intense pink (+++) strong growth). The absence of fungal growth in the well was considered a positive result (denoted by (-)), with the broth remaining colorless. The assay was performed in triplicate. Serial dilutions of the evaluated fraction, and control were performed to determine the MIC. The evaluated concentrations of thyme oil (stock solution of 2.0 mg.mL−1) were 100, 50, 25, 12.5, 6.25 and 3.13 µg.mL−1. Furthermore, descriptive statistics and bar charts were used to analyses the captured data. The error bars were calculated in excel using standard deviation to establish the variation or distinctions between the mitochondrion activity in the two different growth zones.

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to evaluate morphological changes that occurred due to activity of thyme (Thymus vulgaris) and ethanol (negative control) on the fungus P. Citricarpa in terms of shape and structure. Preparation of cells for analysis using SEM was carried out according to. Briefly, cells (from different textured zones) of P. Citricarpa were fixed using 3% v/v of a sodium phosphate buffered glutaraldehyde solution at pH 7.0 and a similarly buffered solution (1% m/v) of osmium tetroxide for 1h. Subsequently, the material was dehydrated in a graded series of ethanol solution (30%, 50%, 70%, 90%, and 100% for 30 min per solution). Next, the ethanol-dehydrated material was critical-point dried, mounted, and coated with gold using a sputter coater (Biorad, London, UK) to make it electrically conductive. This preparation was then examined using a SEM (Joel 6400 WINSEM). Micrographs were taken to investigate the general pattern of the fungi for specific morphological features.

Quantitative Measurement of Metabolic State

The XTT (a tetrazolium salt) colorimetric assay was used to determine the activity of mitochondrion dehydrogenases, an indicator of metabolic activity, this was done according to. Cells of P. Citricarpa were scraped off from different textured zones (representing asexual and maximum growth zone) on agar diffusion plates (bioassay). Five millilitres of PBS were used to suspend 1g of cells from each respective zone. Following this, 2.5ml XTT [2.5g XTT in 1L Ringer's lactate solution] and 400µl menadione were then added. Cells were then incubated at 37°C for 3h in the dark. A 96-well, flat bottom polystyrene microtiter plate was used and 150µl of the formazan product was transferred to each well and the formazan product in the supernatant spectrophotometrically measured in terms of optical density at 492nm using Spectramax ME2 (Molecular Devices).

Results and Discussions

Mitochondrial Mapping

In the current study mitochondrial activity ($\Delta\psi$ m) in P. Citricarpa was mapped using Rhodamine 123. This is a cationic lipophilic mitochondrion stain used to map mitochondrion function selectively. This is attributed to the highly specific attraction of this cationic fluorescing dye to the high negative electric potential across the mitochondrion membrane in living cells. With this dye, a high $\Delta\psi$ m is signified by a yellow-green fluorescence (collected at 450nm), while a low $\Delta\psi$ m is signified by a red fluorescence collected at 625nm. Figure 1 shows conidia on a conidiophore of P. Citricarpa being released. The conidia (C) released and those still attached to the conidiophore (Figure 1 b and c) appeared to contain high mitochondrion activity when compared to hyphal structures (not shown in the figure) and conidiophores. On Figure 1 (b and c) the conidia structures contained high mitochondrial activity visible after being superimposed on corresponding light micrograph and only immunofluorescence respectively. These are represented by yellow-green fluorescence (collected at 450nm).

To substantiate this observation, the conidiophores (CP) as observed in Fig1c had low mitochondrion activity identifiable, signified by red fluorescence collected at 625nm. Generally, fungal sexual structures (ascospores and conidia) are characterized by elevated mitochondrial activity when compared to hyphae. Results in the current study showed that Rh 123 selectively stains the reproductive structures i.e., conidia of P. Citricarpa (Figure.1). Based on these results it is evident that conidia of P. Citricarpa possess increased mitochondrion activity when compared to hyphae. It would be of value to explore the use of antifungal anti-mitochondrial compounds including essential oils in combating P. Citricarpa since the fungus also depends on these structures for development and dispersal.

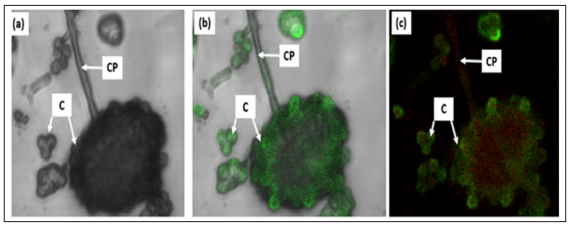

Figure 1: Confocal Laser Scanning Micrographs of P. Citricarpa Stained with Rhodamine (Rh) 123.

- Light micrographs showing a cluster of conidia.
- Immunofluorescence micrograph superimposed on corresponding light micrograph showing a cluster of conidia.
- Only immunofluorescence micrograph showing a cluster of conidia.

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

Essential oil Composition

Literature indicates that the antimicrobial properties of essential oils depend mainly on their composition, it was thus essential to characterize Thyme oil and elucidate its mode of action against P. citricarpa. The chemical composition of Thyme oil (Thymus vulgaris) was thus analysed using Gas Chromatography Mass Spectrometry (GC-MS) and the results are shown on Table 1 in terms of the identity and the percentage content of the individual components that were identified.

In this study ten compounds were identified representing 71% of the total oil composition of the test sample. Thyme oil (Thymus vulgaris) consists mostly of terpenes as major compounds. Terpenes and their derivatives play a major role in the antimicrobial activities of essential oils. These include Thymol (32.1%) and ρ-Cymene (20.4%) as main compounds, while there were other minor compounds detected in ranges between 0.5-5.1%. The minor components identified included α-Pinene (5.1%), Terpinene (3.0%) and other minor compounds (not reflected on the table). Similar results observed in a study conducted in Brazil indicated that Thyme oil consists of high amounts of ρ-Cymene (18.6%) and its monoterpene phenol derivative thymol (44.7%). Even though, the composition of the Thyme oil characterized in this study differed slightly from results observed by Porte and Godoy (2008), Thymol and ρ-Cymene were detected as major compounds in both studies. In another study where Thyme oil (Thymus vulgaris) was characterized, the composition differed from what was observed in the current study as thymol was (8.7%) while ρ-Cymene was a minor component at (0.1%). These variations can occur because of the difference in cultivation area and the method used to extract the oils, as such the variation can affect the effectiveness of essential oils.

Table 1: Identified Constituents and Percentage Composition of Thyme (Thymus Vulgaris) Essential oil Analysed using Chromatography Mass Spectrometry (GC-MS)

Composition of Thymus vulgaris	
Compounds	Thymus vulgaris (%)
Borneol	0.5
Camphene	1.3
Caryophyllene	1.2
Caryophyllene oxide	1.4
Hexane, 3-methyl	4.5
Pinocarvone	1.5
Terpinene	3.0
Thymol	32.1
α-Pinene	5.1
ρ-Cymene	20.4
Total Identified	71.0
n.i	29.0

n.i.: not identified.

Agar Diffusion Method for Phyllosticta Citricarpa

Generally, EO's have long been considered to possess anti-inflammatory properties and antimicrobial activity, with activity against fungi. Studies have reported that EO's can target structures with increased mitochondrial activity. Other Studies by have indicated that structures with increased mitochondrial activity such as ascospores, conidia, asci, sporangia and phialides play an important role in the fungal life cycle and development. If these structures are inhibited, they will probably limit the spread of the fungi since they can act as the prime source of infection. However, before such a claim could be tested, it was paramount to first determine antifungal anti-mitochondrial activity of EO's oils, together with their hydrosol and antifungal anti-mitochondrial compounds (positive controls) using the agar diffusion bioassay for P. citricarpa.

Bio-assay analysis has become more important in effectively controlling the quality of biopharmaceutical development and manufacturing. The general approach of most bioassays is to perform a dilution assay, which measures the biological responses at certain doses. Table 2 and Figure 2 (A-D) indicates bio-assay results of P. citricarpa growth. When Thymus vulgaris, hydrosol and salicylic acid (positive control) were applied to the bio-assay of P. citricarpa, three zones were observed: an inhibition zone (i), asexual zone (a) and maximum growth zone (m), except for ethanol, where only maximum growth zone (m) was observed (Figure 2 D). Thymus vulgaris produced the largest inhibition zone of 55mm in diameter as observed in Table 3 and Figure 2 (A). Both Thyme oil (Thymus vulgaris) hydrosol and salicylic acid (positive control) produced similar inhibition zones in size of 10mm in diameter as indicated in Table 2 and Figure 2 (B and C) respectively. When ethanol was tested as a negative control no inhibition zone was observed, however only a maximum growth zone was observed with no inhibition of fungal growth as indicated in Table 2 and Figure 2 (D).

The current study was done based on the findings of that proposed that salicylic acid exhibits antifungal properties. The current study not only proposed to assess antifungal properties of tested compounds, but it also sought to elucidate the mode of antifungal action of all tested compounds. Studies have shown that compounds that inhibit fungal growth by first targeting development structures with increased mitochondrial activity could serve as an efficient strategy to reduce the spread of fungi. The results of antifungal activity of the known antifungal anti-mitochondrial compounds, essential oils (EO's) and hydrosol, against P. citricarpa obtained from the agar diffusion bio-assay are shown in Table 2 and Figure 2. Thyme oil (Thymus vulgaris) exhibited antimicrobial activity against this fungal pathogen, which resulted in significant inhibition of the fungus P. citricarpa when compared to other tested compounds, with the inhibition zone of 55mm. Furthermore, Thyme oil (Thymus vulgaris) hydrosol used in the current study showed antifungal effects against the test organism with an inhibition zone of 10mm. These results indicate that hydrosol can be used as an antifungal against CBS, with the advantage over essential oils of being water soluble, cheaper, and consisting of traces EO's. Salicylic acid (SA), (positive control) produced similar inhibition zone as hydrosol (10mm). These observations are in line with the

findings by that salicylic acid possess' antifungal properties. Results from other studies have indicated similar findings that known antifungal anti-mitochondrial compounds have potential antifungal activities against pathogenic fungi. These compounds are believed to act by first targeting structures with increased mitochondrial activity such as conidia and ascospores. Furthermore, other researchers believe that this interaction results in changes in prostaglandin production, membrane potential, and reduction of extracellular polysaccharide leading to the cell death. In agreement with previous studies showing that known antifungal anti-mitochondrial compounds can directly impede growth in several fungal species, the present study documents that antifungal anti-mitochondrial compounds have potential inhibitory effects against P. citricarpa. However, ethanol which was used as a negative control indicated no significant inhibition zone in diameter; it is therefore concluded that ethanol alone had no effect against P. citricarpa the causative agent of CBS. These results agree with studies done by respectively where ethanol alone did not show any inhibitory effect against the different tested fungal organism. Consequently, MICs (minimum inhibitory concentration) of the thyme oil was determined thereof.

Table 2: Bio-Assays of P. Citricarpa Showing Effects of Different Known Antifungal Anti-Mitochondrial, Essential oils; Hydrosol Compounds and Ethanol Measured in mm

Compounds tested, EO's and Hydrosol	Inhibition zone(mm)
Thyme (Thymus vulgaris) oil	55
Thyme (Thymus vulgaris) oil Hydrosol	10
Salicylic acid (SA) (Positive Control)	10
Ethanol (Negative Control)	0

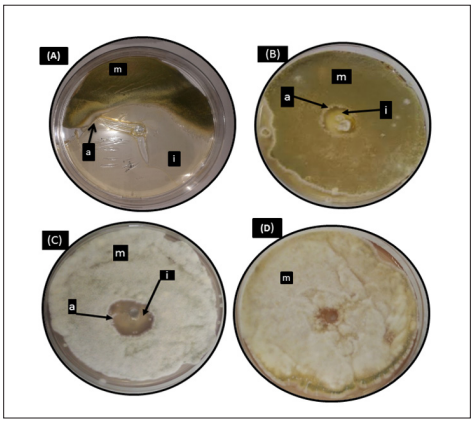

Figure 2: Bio-Assays of P. Citricarpa Showing Effects of Thyme oil (Thymus Vulgaris), Hydrosol, Known Antifungal Anti-Mitochondrial Compound Salicylic acid (Positive Control) and Ethanol (Negative Control).

- Thymus Vulgaris
- Hydrosol
- Salicylic Acid [SA]
- Ethanol (Control).

i,- Inhibition zone; a- asexual zone; m- maximum growth zone.

Broth Microdilution Assay (Minimum Inhibitory Concentration Determination)

There is an increasing demand for accurate knowledge of the minimum inhibitory concentrations (MIC) of essential oils to enable a balance between the sensory acceptability and antifungal efficacy. MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after incubation for a period of time. This was achieved by using broth micro dilution in-vitro done in triplicate (100–3.13 µg/ml) using the test oil. The analyses of inhibition of thyme oil against P. citricarpa showed that at 100–25 (µg/ml) there was a complete inhibition of the fungi (-). Therefore 25 (µg/ml) given in (Table 3) was the MIC value observed. This is the lowest concentration of the thyme oil capable of inhibiting the growth of P. citricarpa as it was the last tube in the dilution series which exhibited no growth. While at 6.25 µg/ml dilution the P. citricarpa had minimum growth (++), and at 3.13 µg/ml dilution the P. citricarpa indicated maximum growth, thereby showing that no inhibition of the P. citricarpa by thymus oil at 6.25 µg/ml and 3.13 µg/ml respectively. However, ethanol used as the control did not have MIC value against the test organism, indicated by a strong growth (+++) of the fungi, thereby concluding that ethanol does not have any effect on growth of P. citricarpa. Detailed microscopic analysis of P. citricarpa treated with Thyme oil (Thymus vulgaris) and ethanol respectively were then conducted using scanning electron microscopy in order to determine the effects of the Thyme oil (Thymus vulgaris) and ethanol on the conidia and thereby confirm the mode of action of the tested compounds.

Table 3: The minimum inhibitory concentration (MIC) of thyme oil against Phyllosticta citricarpa isolate.

Dilution of thyme oil and control (µg/ml):	≥100	≥50	≥25	≥12.5	≥6.25	≥3.13
Diluted Thyme oil:	–	–	–	+	++	+++
Ethanol (Control):	+++	+++	+++	+++	+++	+++

Data are reported as "+++" indicates strong growth of fungi, "++" medium growth, "+" minor growth and "–" indicates inhibition of growth of fungi (sensitive to thyme oil).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to examine the morphological changes of P. citricarpa conidia structures (size and shape) after being treated with Thyme oil (Thymus vulgaris) and ethanol respectively. Scanning electron microscopy (SEM) revealed that conidia structures were completely inhibited after treatment with thyme oil (Thymus vulgaris) when analysing the area towards inhibition zone (from the bio-assay results) (Figure 3 A); however, the P. citricarpa treated with ethanol (negative control) alone did not inhibit the fungal growth and was represented by a high amount of conidia (c) (Figure 3 B). Previous studies have shown that P. citricarpa does not form perithecia with ascospores on the agar media, although it is able to produce them in the environment on fallen leaves. As expected, this was also observed in the current study. P. citricarpa produced conidia from conidiophores. Scanning electron microscopy (SEM) was used to investigate whether Thyme oil (Thymus vulgaris) inhibits fungal growth by first targeting structures with increased mitochondrial

activity. The area towards the inhibition zone (from the bio-assay results) was assessed and Thyme oil (Thymus vulgaris) inhibited the development of conidia structures with increased mitochondrial activity completely (Figure 3 A). This indicates possible antifungal anti-mitochondrial properties of these oils. Additionally, the oils affected the morphology of hyphae (Fig 3 A), hyphae appeared granular and wrinkled. Based on these findings it can be presumed that Thyme oil can inhibit parts of the P. citricarpa life cycle by first targeting conidia structures. Furthermore, it is believed that this occurs because Thyme oil consist of high amounts of terpenes which play an important role in the plant defence mechanism against pathogenic fungi. However, Ethanol (Figure 3 B) used as the negative control did not have any antifungal anti-mitochondrial properties against the test organism, indicated by a high amount of conidia thereby concluding that ethanol does not have any effect on conidia structures of P. citricarpa. However, P. citricarpa in figure 3 B had deflated conidiophore, these might be due to effects of ethanol which resulted in dehydration during the sample preparation stages. Results in the current study also showed that spore-releasing-structures such as conidia with increased mitochondrion activity are more sensitive to mitochondrial inhibitors including EO's compared to vegetative cells and hyphae analysed using SEM. This may be of value in combating fungi that depend mainly on these structures for dispersal.

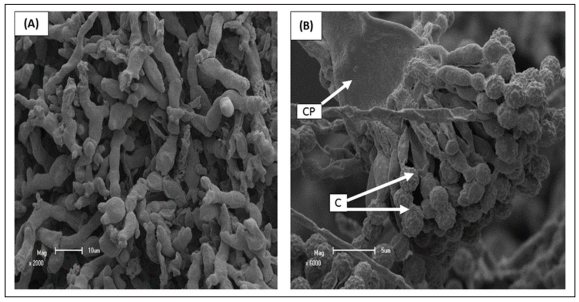

Figure 3: Detailed Scanning Electron Microscopy (SEM) Analysis.

- P. citricarpa Cell treated with Thyme oil (Thymus vulgaris),
- Ethanol (control).
- Conidia; CP, conidiophores.

Cells with Thyme oil (Thymus vulgaris) were scraped from minimal growth zone in bioassay (agar diffusion method) plate, while the cells treated with ethanol were scraped towards the high concentration gradient of ethanol.

Quantitative Measurement of Metabolic State and Statistical Analyses

Scanning Electron Microscopy indicated that conidia of P. citricarpa shown to possess increased mitochondrial activity are affected by the Thyme oil (Thymus vulgaris). It was therefore important to determine the effect of the antimicrobial compounds on the activity of mitochondrion dehydrogenases, an indicator of metabolic activity. This was done using XTT (a tetrazolium salt) colorimetric assay. The mitochondrial dehydrogenase activity of P. citricarpa, at different growth zones (asexual and maximum growth zone) was assessed. Here, we believe Tetrazolium Salt (XTT) was cleaved by various mitochondrial dehydrogenase enzymes to produce a coloured formazan product, which indicates fungal metabolic activity. As expected, the maximum growth zone contained increased mitochondrial dehydrogenase activity when compared to asexual zone. P. citricarpa cells treated with Thyme oil showed significantly higher ($p < 0.001$) mitochondrion activity in the maximum growth zone (0.96 ± 0.01 measured at 492nm) compared to the asexual zone (0.46 ± 0.01 measured at 492 nm) (Figure 4). These findings reveal that the mitochondrial dehydrogenase activity increased significantly from the asexual growth zone to the maximum growth zone as the organism moved away from the inhibition zone. The bar chart shows that the minimum mitochondrion activity was 0.46 which was in the asexual zone whilst the maximum mitochondrion activity was 0.96 which was in the maximum growth zone. These values give an average mitochondrion activity of 0.71 measured at 492nm, a range of 0.5 and a standard deviation of 0.35. The range and the standard deviation confirm that there are differences in the measurement of mitochondrion activity in the different growth zones. This is further confirmed by the error bars which are non-overlapping indicating that the mitochondrion activity was different in the two zones. There are greater variations of increased mitochondrion activity in the maximum growth zone as shown by a wider error bar unlike in the asexual zone where the error bar is shorter or less wide indicating restricted mitochondrion activity due the concentrated presence of Thyme oil. This is an indication of the effect of essentials oils on structures with elevated mitochondrial activities when compared to vegetative cells and hyphae. This study further supports previous studies which have shown that spore-releasing-structures such as yeast asci, sporangia and phialides with increased mitochondrion activity are more sensitive to mitochondrial inhibitors when compared to vegetative cells and hyphae. Structures with elevated mitochondrial activity play an important role in the life cycle of P. citricarpa especially for the dispersal of the organism.

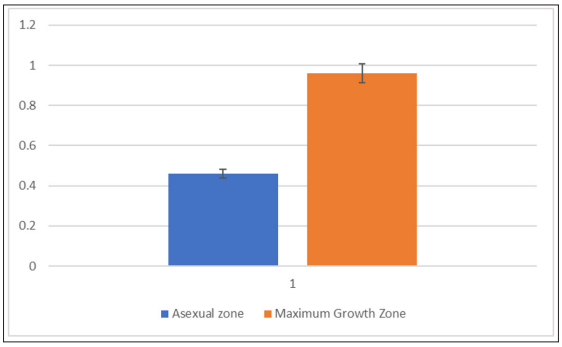

Figure 4: Results of the XTT Assay Studies Performed on Different Growth Zone (Asexual and Maximum Growth Zone) of P. Citricarpa Treated with Thyme oil (Thymus Vulgaris)

Conclusions

This study indicated that essential oils possess antifungal activity and can be exploited as an ideal treatment for future citrus disease management programs eliminating fungal spread. Treatment with thyme oil inhibited P. citricarpa in vitro, and we believe this can improve the quality of citrus fruits, however in vivo studies still need to be done, furthermore, future studies will explore the possibility of essential oils fractionation prior to the exposure to the targeted microorganisms. This will identify specific molecules that exhibit the highest efficacy. The study also showed that cheaper alternative methods such as using hydrosol can be used as antifungals against CBS, with the advantage over essential oils of being water soluble and consisting of traces EO's. It is concluded that EO's also target conidia structures responsible for the life

cycle of P.citricarpa, probably by decreasing energy production necessary for normal development and conidia dispersal. This provides a dual function to these compounds, that is, anti-mitochondrial as well as antifungal. Furthermore, suppression of conidia production could make a major contribution to limiting the spread of the pathogen by lowering the conidia load on citrus fruits. Moreover, EO's consist of complex composition; therefore, fungal resistance might be minimal [20-59].

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Data summary
The author confirm all the protocols have been provided within the article.

Conflicts of Interest
The author declare no conflict of interest.

Author Contributions
B.T Magunga. Principal investigator

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