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Efficacy of Lemon Myrtle Essential Oil as a Bio-Fungicide in Inhibiting Citrus Green Mould

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Abstract: This study examined the efficacy of essential oil (EO) obtained from the Australian native plants, lemon myrtle (*Backhousia citriodora*) (LM) to inhibit *Penicillium digitatum* by *in vitro* agar diffusion and vapor assay, and in inoculated oranges. Gas chromatograph/mass spectrometry analysis revealed the main constituent of LM EO as citral. Pure citral was also included in the experiment for comparison. LM EO at 1, 2, 3, 4 and 5 µL disc-1 in the in vitro tests significantly inhibited fungal growth compared to the non-treated control. Moreover, LM EO at 4 and 5 µL disc-1 in both the assays completely inhibited fungal growth similar to its main component of citral. Inoculated oranges dipped in 1000 µL L-1 LM EO solutions for 5, 10, 15 and 30 sec showed significantly lower fungal wounds compared to control. While higher dipping times generated slight rind injury, a 5 and 10 sec dip did not cause any injury to the fruit rind. The quality assessments and sensory evaluations also revealed that the addition of LM EO did not adversely affect the quality and taste of the oranges. These findings suggest LM EO as an alternative to synthetic fungicides to inhibit wastage in citrus during storage.

Keywords: lemon myrtle; essential oil; citral; citrus fruit; rind injury; *Penicillium digitatum*; sensory test

1. Introduction

The major postharvest disease of citrus fruits is green mould caused by *P. digitatum*, which leads to considerable financial losses to the citrus industry [1]. Chemically synthesised fungicides are routinely used [2]; but there are considerable consumers' concerns on their use due to potential health and environment effects [3,4]. In addition, constant use of the chemical fungicides can also lead to development of resistance of the diseases, which result in reducing efficacy of the fungicides [5]. Therefore, application of natural substances, such as plant essential oils (EOs) can be an alternative approach to control postharvest decay [6]. Plant EOs are mostly terpenoids derived from units of isoprene (2-methyl-1,3-butadiene) with further structural diversification achieved through the inclusion of heteroatom functional groups such as alcohols, aldehydes, ketones, esters, and ethers [7]. The individual constituents of EOs show variable efficacy against postharvest pathogens [8,9,10,6]. There are also interests in EOs with high levels of aldehydes and phenols as such compounds show strong antimicrobial properties [10,11,12].

Lemon myrtle (*Backhousia citriodora*) is an Australian endemic plant, belong to the family Myrtaceae. It is very well known as medicinal plant and for lovely lemony fragrance [13,14]. The EO extracted from this plant has been used as a functional ingredient in various products, such as mouthwashes, food flavourings and herbal teas [15,16,17]. Lemon myrtle EO contains a high level of citral (3,7-dimethyl-2-7-octadienal) with two main isomeric aldehydes: neral and geranial [18,19]. The antifungal activity of citral in citrus fruits has been reported in several previous studies. For example, Rodov et al. [20] and Ben-Yehoshua and Rodov [21] reported that dipping non-inoculated Washington Navel oranges in a citral solution reduced the incident of green mould caused by *P. digitatum*. While Wuryatmo [22] applied citral as a fumigant and found it delayed onset of green mould in Navel oranges stored at low temperature but not when held at room temperature. However,

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various studies have reported phytotoxic damage to the rind following exposure to citral [23,22,24]. Notably, Southwell et al. [25,26] extracted EO from Lemon myrtle (LM) leaf and found that this EO not only contains high level of citral, but also has other bioactive compounds, such as myrcene, 6-mcthyl-5-hepten-2-one, linalool, citronellal, iso-neral, iso-geranial, neral and geranial. Wilkinson et al. [27] further tested and found LM EO was effective against 13 bacteria and 8 fungi other than *penicillium* genus. Lazar-Baker et al. [28] also reported LM EO was effective for prevention of mycelium growth and spore germination of *Monilinia fructicola*.

Although previous studies have linked LM EO with various antimicrobial activities, limited studies have examined the effect of LM EO against *P. digitatum* infection on citrus fruits. Therefore, objective of the present study was to test the impact of Australian native LM EO on inhibition of the growth of *P. digitatum* by *in vitro* agar and vapour diffusion and in inoculated oranges through dipping techniques. The efficacy of inhibition of LM EO was also compared to its main component, citral.

2. Materials and Methods

2.1. Essential Oils (EO) and Chemicals

Commercial steam distilled 100% pure lemon myrtle (LM) (*Backhousia citriodora* F. Muell.) EO was purchased from Australian Body (Crafers, South Australia) that source plant materials grown in broadly dispersed regions of Australia. LM EO were also extracted using microwave extraction technique in the science laboratory of The University of Newcastle, Ourimbah campus (NSW), Australia. The LM EO purchased from Australian body was used for all the fruit storage trials. Toluene (HPLC Plus grade), citral, limonene, linalool, standard citral, standard limonene, standard linalool and Triton-X100 were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

2.2. Penicillium digitatum Cultures and Inoculum Preparation

Cultures of *P. digitatum* (Pers.: Fr.) Sacc. was obtained from the Citrus Pathology Laboratory of NSW DPI, Ourimbah. The fungi were maintained and revived on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI). For preparing fungal culture for both in vitro experiments and infecting experimental fruits, fungal isolates were sub-cultured on PDA media in a sterile laminar flow chamber and incubated in a dark chamber at 25 °C for approximately 7 to 10 days. Plastic petri dishes (Bacto PDS 9014G) (85 mm diameter) were used for culture preparation. After 7 days, fungal incubating plates were covered by conidia of *Penicillium*. The conidia of *P. digitatum* were gathered and suspended in sterile distilled water to obtain a stock solution. The clear suspension was then diluted with sterile distilled water to a concentration of 10^4 to 10^5 spores mL^{-1}. A haemocytometer (Superior, Marienfeld, Germany) and microscope (Leitz Laborlux S, 100x/1.25, Germany) were used to determine spore concentration.

2.3. Agar Diffusion Assay

The antifungal activity of the EOs against *P. digitatum* was evaluated using the agar diffusion assay according to methods described by Rodov et al. [29], Javad Safaei Ghomi et al. [30] and CLSI [31], with slight modifications. Briefly, antifungal tests were carried out by employing 100 µL of inoculum suspension containing 104 spore mL^{-1} of fungi spread on the potato dextrose agar (PDA) medium petri dish (85 mm diameter). LM EO sample was pipetted onto a sterile 13 mm antibiotic assay paper disc (Whatman) which was previously placed on the center of the inoculated agar. Pipetting the same amounts of deionized water onto the assay paper disc was used as a control. The petri dishes then incubated at 24 °C for 3 days. The antifungal activity of the fungal growth-free zone around the paper disc was evaluated by measuring the width of the clear zone from the edge of the paper disc to the area of fungal growth and expressed in percentage as percent inhibition. For calculation, the whole width of inhibition including the diameter of the paper disc was measured, then the disc diameter was subtracted to get the actual width of inhibition. The width of the petri dish and the paper disc was 42.5 and 6.5 mm, respectively. Thus, the highest achievable inhibition

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width was calculated as 36 mm in case of total growth inhibition. The inhibition was expressed in percentage.

2.4. Vapour Assay

In order to evaluate the effects of the oils in the vapour phase, a slightly modified technique of the agar diffusion assay was also used as described by Regnier et al. [32]. Specifically, the petri dish was inverted and the paper disc was placed centrally on its lid to prevent the direct transport into agar of the test compound by diffusion from the paper disc. The paper disc was attached onto the lid to avoid the movement of the paper disc. The required amount of test EO doses were pipetted onto the paper discs stuck on the lid. The petri dishes were sealed with parafilm immediately after adding the essential oils. The inoculated petri dishes were incubated at 24 °C for 3 days. Antifungal activity was monitored visually by measuring the clear area in petri dish and recorded as inhibition area in mm and expressed as the percent inhibition of the total area.

2.5. Laboratory Extraction of EO

Lemon myrtle leaves were authenticated through the herbarium at the University of Newcastle, Australia with voucher number 10638. The leaves were collected randomly from Ourimbah, Central coast region, NSW, Australia. After collecting, the leaves were immediately transferred to the lab and extracted for EO using microwave-assisted extraction (ETHOS X, Milestone, Sorisole, Italy) for 30 minutes set at 500 watts with an oil yield of 3 - 3.5g100g^{-1}. After extraction, EO was kept in a dark coloured bottle and stored under nitrogen at -20 °C until required.

2.6. Gas Chromatography-Mass Spectrometry (GCMS) Analysis

Both the extracted and commercial EOs were analysed using GCMS (Shimadzu QP 2010SE, Canby, OR) set at the condition described by Rudback et al. [33], with a minor modification. Briefly, a stock solution of oil (35 µL mL-1) was prepared in toluene (HPLC Plus grade, Sigma-Aldrich Castle Hill, NSW) and stored at -18 °C until required. A working solution (1000 µL L^{-1}) was prepared from the stock solution and an aliquot (1 µL) was injected into the GCMS under the following conditions: helium carrier gas, column flow rate 3 mL min^{-1}, injection temperature 250 °C and split ratio 1:16. The temperature program was 80 °C for 3 min, then increased at 5 °C min^{-1} to 145 °C, then at 45 °C min^{-1} to 275 °C where it was held for 10 min. The temperature of the ion source and the interface was 250 °C. For the analysis an SH-Rxi-5Sil MS (Shimadzu) column of 30 meter in length was used. Individual constituents were identified by comparison of their mass spectra with mass libraries from The National Institute of Standards and Technology (2010) preloaded onto the GCMS.

2.7. Plant Materials

Both the Valencia and Navel orange (Citrus sinensis (L.) Osbeck) fruits according to the seasonal availability were used in experiments. Organic Navel oranges were obtained from a New South Wales Department of Primary Industries (NSW DPI) orchard at Somersby, NSW, Australia. Commercial mature Valencia oranges were sourced from a commercial citrus grower at Griffith, NSW, Australia and transported to the NSW DPI laboratories at Ourimbah, NSW, Australia. The fruits were not treated with fungicide or waxed after harvest. For all experiments, the required number of non-blemished fruits were surface sterilized with sodium hypochlorite solution (10 mL L^{-1}) and air dried for about 90 min. Each batch of fruits was then randomly distributed into the required number of treatment units with 20 fruits per unit with three to four units/replicates assigned to each treatment. Each replicate was prepared and treated separately. Most experiments utilized batches of oranges obtained at different times.

2.8. P. digitatum Inoculation and Fruit Treatment

Fruits were inoculated by wounding the flavedo with a sharpened steel rod (1 mm diameter x 2 mm length) that had been pre-immersed in the spore suspension. The fruits were then incubated at

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20 °C for 24 hr. Each fruit unit was then dipped in solution containing LM EO or citral for 5, 10, 15 and 30 seconds. Dip solutions were prepared by mixing the appropriate volume of citral and LM EO with absolute ethanol (1 mL L^{-1}) and Triton-X100 (Sigma-Aldrich, Castle Hill, NSW, Australia) (24 mg L^{-1}) to form an emulsion which was then added to 30 L water and mixed thoroughly using a blender (RPM 550, Ozito, China). Control fruits were dipped in water with and without the inclusion of ethanol and Triton-X100.

The dipped fruits were placed on trays and allowed to dry at room temperature (20-26 °C, 65-75 % RH) for one hour. Each treatment unit was then transferred into an unsealed low-density polyethylene bag that was loosely folded over the fruit to generate a high humidity but minimal change in the composition of air inside the bag and stored at 20 °C. The diameter of the infected fruit lesion on each fruit was recorded daily for 5 to 8 days. An individual fruit was considered to be decayed when a soft P. digitatum lesion diameter exceeded 4 mm.

2.9. Quality Assessment of Fruits

2.9.1. Rind Injury Assessments

To quantify the level of potential phytotoxicity of the EO treatment, rind/peel injury on the surface of fruits was visually recorded during storage using a 1-5 scoring scale where: 1 = no visible injury, 2 = 1-5% injured area (some slight damage just detectable but fruit still considered marketable), 3 = 5-20% injured area (moderate damage with fruit considered not marketable), 4 = 20–50% injured area, and 5 = >50% injury that was severe.

2.9.2. Weight Loss

Fruit weight loss was measured by weight difference (Kern & Sohn GmbH, D-72336, Balingen, Germany), where fruit weight of each treatment unit was recorded on each assessment day. The results were presented as the percentage loss of initial weight according to the following formula [34],

$$\text{Weight loss (\%)} = \frac{(\text{Initial weight (g)} - \text{Final weight (g)})}{\text{Initial weight (g)}} \times 100$$

2.10. Measurement of Fruit Firmness

A texture analyser (Lloyd Instrument Ltd, Fareham, UK) was used to determine the firmness of the fruits. The maximum force (N) was measured by compressing the fruit in the equatorial zone between two flat surfaces closing together at the rate of 1 mm min^{-1} to a depth of 2 mm. The average of two reading points from two sides of the fruit at 90º angle was recorded according to Cháfer et al. [35] with slight adjustment.

2.11. Respiration Rate

Respiration rate of stored fruits (as evolved CO_2) was measured according to Pristijono et al. [36] with slight modifications. Firstly, eight oranges from each replicate were allocated into 2L hermetic glass jars (2 fruits/jar) with a septum in the lid. Then, the jars were kept at 20 °C for five hr to accumulate respiratory gases. Finally, headspace gas sample (1 mL) was withdrawn by a syringe and transferred to an ICA40 series low-volume gas analysis system (International Controlled Atmosphere Ltd., Kent, UK). Respiration rate was expressed as $mLCO_2kg^{-1}hr^{-1}$ and calculated from the following formula,

$$\text{Respiration } (mLCO_2kg^{-1}hr^{-1}) = \frac{\%\ CO_2 \text{ X Vol. of container (mL)}}{\text{Initial produce weight (kg)} \times 100 \times \text{time (hr)}}$$

2.12. Total Soluble Solids

Total soluble solids (TSS) of the stored fruits were determined by measuring the refractive index of the fruit juice. Eight fruits from each treatment unit were manually juiced and sieved through two

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layers of cheesecloth. The juice was placed into a portable digital refractometer (Atago Co. Ltd., Tokyo) at 20 °C and the data expressed as percentage Brix.

2.13. Titratable Acidity

Titratable acidity (TA) was determined by extracting 5 mL juice as above and titrating with 0.1 M NaOH to pH 8.2 with an automatic titrator (Mettler Toledo T50, Greifensee, Switzerland). The data were expressed as percentage of citric acid.

2.14. Ethanol

To determine the ethanol content, 10 mL of juice was extracted as above and transferred into a 20 mL glass vial fitted with a crimp-top cap containing a silicone septum. The sealed juice sample was then incubated in a water bath at 30 °C for 10 minutes before analysis. Ethanol accumulation was determined by headspace analysis using a gas chromatograph (Model 580, Gow-Mac-Bethlehem, PA, USA) equipped with a flame ionisation detector (FID) and a column (Carbwax, Gow-Mac, Bethleham, PA, USA). The injector temperature was set at 190 °C, the column at 68 °C and the FID at 190 °C with gas flow rates at 30, 30 and 300 mL minute-1 for nitrogen, hydrogen and air, respectively. After incubating the juice samples in the glass vial for 10 minutes, 1 mL of the headspace gas sample was withdrawn from the vials and injected into the gas chromatograph. Accordingly, standard ethanol sample was incubated at the same time and 1 mL headspace sample injected into the system. Ethanol accumulation was calculated and expressed as $\mu L\ L^{-1}$.

2.15. Ethylene

For the assessment of ethylene production, 1 mL headspace gas sample was withdrawn 5 hr after sealing the container similar to the previously described for respiration gas. The concentration of ethylene was calculated by injecting the sample into a flame ionization gas chromatograph (Gow-Mac 580, Bridgewater NJ) fitted with a stainless steel column (2 m ×3.2 mm OD × 2.2 mm ID) packed with Porapak Q (80–100 mesh) (Altech, Sydney), with 110, 90 and 70 °C as the operating temperature of the detector, column, and the injector, respectively. Nitrogen, hydrogen and air were used as carrier and combustion gases at a flow rates of 60, 30 and 300 mL min^{-1}, respectively. The ethylene production rate was expressed as $\mu L C_2H_4\ kg^{-1}\ hr^{-1}$ and calculated from the formula as follows,

$$\text{Ethylene production } (\mu L C_2H_4\, kg^{-1}\, hr^{-1}) = \frac{C_2H_4\ (\mu L L^{-1})\ X\ \text{Vol. of container (L)}}{\text{Initial produce weight (kg) X time (hr)}}$$

2.16. Sensory Evaluation

A discrimination test also known as triangle test, was conducted to determine the difference of any perceptible sensory attributes between Valencia oranges treated by dipping with 1000 $\mu L\ L^{-1}$ lemon myrtle (LM) EO and non-treated control according to the method described by Sinkinson [37]. The panelists were provided with assessment questionnaires and were instructed to evaluate the orange. Each of the panelist were provided 3 blind coded slices of oranges, two slices were from one treatment, while one slice from different treatment. All three samples provided in a set order and instructed to select the odd sample. Crackers and water were provided to rinse the palate between tasting the orange slices.

Data interpretation was made based on the minimum number of correct answers received required for a significance level $\alpha \leq 0.05$ according to one-tailed binomial test [37,38]. The minimum number of correct answers were found in statistical table [39].

2.17. Statistical Analysis

A randomised experiment design incorporating 3 treatments with three to four replications was utilised. Multinominal Logistic Regression analysis was performed using IBM SPSS (version 25) to find out the effects of dipping times in Lemon Myrtle essential oil on the fruits during storage times.

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In case of *in vitro* tests, the evaluation carried out for 5 times to confirm the effectiveness. One-way ANOVA analysis was performed with Statistical Analysis System - version 9.4 (SAS Institute, Cary, NC, USA) and where there was a significant difference between means, the Least Significance Difference (LSD) at p = 0.05 was calculated.

3. Results

3.1. Composition of LM EO

Composition of the extracted and commercial LM EOs was analysed and the results presented in Table 1 showed that geranial and neral were the primary constituents in these EOs. Geranial accounted for 50-52%, it was followed by neral, which are approximately 35-39%, giving a total citral content of 88%. Other constituents were also found in both extracted and commercial LM EOs including, iso-geranial, benzaldehyde, iso-neral, 6-methyl-5-hepten-2-one, ethylbenzene and linalool, each constituent accounts for a range of 0.5 to 2% of total constituents.

Table 1. Composition of lemon myrtle essential oil (LM EO).

Retention Time (min)	Laboratory extracted LM EO		Commercial LM EO	
	Peak Area (%)[a]	Component	Peak Area (%)[a]	Component
3.06	1.0	Ethylbenzene	0.18	Ethylbenzene
4.90	0.6	6-methyl-5-hepten-2-one	0.31	6-methyl-5-hepten-2-one
5.03	0.6	β -Myrcene	0.25	β -Myrcene
7.70	0.5	Linalool	0.35	Linalool
9.38	1.4	Iso-neral	1.75	Iso-neral
11.60	35.3	Neral	38.50	Neral
11.90	2.0	Iso-geranial	0.31	Iso-geranial
12.43	49.5	Geranial	51.60	Geranial

[a]Values are % of total peak area of all constituents in the sample.

3.2. Efficacy of LM EO and Citral in Agar Diffusion and Vapour Assay

LM EO and citral significantly inhibited fungal growth both in the agar diffusion and vapour assay compared to the non-treated control as shown in Table 2 and 3. The inhibition of fungal growth of the LM EO was significantly higher than the control treatments. Moreover, LM EO at 4 and 5 µL disc^{-1} completely inhibited fungal growth similar to its main component of citral.

Germination and growth of *P. digitatum* were effectively inhibited when exposed to LM EO and citral in the agar diffusion assay. With the increase in concentration of the oils from 1 to 5 µL disc^{-1}, the efficacy also increased. Furthermore, the vapour assay showed higher efficacy compared to the agar diffusion assay.

Table 2. Antifungal effect of lemon myrtle (LM) essential oil (EO) and citral on the radial growth of *P. digitatum* using the agar diffusion assay after 3 days at 24 °C.

Treatments	Growth inhibition (arcsine %)				
	1	2	3	4	5 µL disc^{-1}
Control	1	1	1	1	1
LM EO	27	51	67	89	89*
Citral	34	61	69	89*	89*
LSD‡	*1.4*	*1.8*	*1.9*	*132E-8*	*132E-8*

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The data are the arcsine transform value of the percent of plate area covered without spores. Values are the means for 15 replicates (5 batches x 3 replicates). ‡ Least significant difference between mean values in each column at $p = 0.05$.* Full or 100% growth inhibition.

Table 3. Antifungal effect of lemon myrtle (LM) essential oil (EO) and citral on the radial growth of *P. digitatum* using the vapour assay after 3 days at 24 °C. .

Treatments	Growth inhibition (arcsine %)				
	1	**2**	**3**	**4**	**5 µL disc^{-1}**
Control	1	1	1	1	1
LM EO	37	60	67	89*	89*
Citral	45	62	78	89*	89*
LSD‡	*1.0*	*1.6*	*3.2*	*4.E-02*	*3.E-06*

The data are the arcsine transform value of the percent of plate area covered without spores. Values are the means for 15 replicates (5 batches x 3 replicates). ‡ Least significant difference between mean values in each column at $p = 0.05$.* Full or 100% growth inhibition.

3.3. Effect of LM EO and citral on fungal growth and rind injury of oranges

The effect of LM EO and citral against *P. digitatum* in inoculated Navel oranges was examined by dipping 20 fruits for 120 sec at 2000 - 8000 µL L^{-1} LM premixed with Triton-X100 surfactant (24 mg L^{-1}) and aqueous ethanol (1 mL L^{-1}) as stabiliser. Control fruits were dipped in water with ethanol and Triton-X100.

Citral, the principal component of the LM, at 1000 µL L^{-1} was also included for comparison. The results showed that LM EO at 2000 - 8000 µL L^{-1} significantly inhibited growth of green mould wounds after 5 days of storage at 20 °C compared to the control (Table 4). However, all concentrations of LM as well as citral caused rind injury (Table 5). Increasing the concentrations of LM significantly increased both the fungal growth inhibition and rind injury.

This study was extended with inoculated oranges by dipping 20 fruits for 120 sec at the lower concentrations of LM from 500 - 2000 µL L^{-1} and citral at 500 and 1000 µL L^{-1}.

Table 4. Fungal wounds on Navel oranges following dipping for 120 sec in a solution of lemon myrtle (LM) essential oil (EO) and citral during storage at 20 °C.

Treatments	Decay (lesion diameter, mm)		
	3	**4**	**5 days**
Control	14	29	44
LM EO (µL L^{-1})			
2000	0	4	9
4000	0	2	5
6000	0	1	4
8000	0	1	3
Citral (µL L^{-1})			
1000	0	4	8
*LSD*ǂ	*1.8*	*3.2*	*5.2*

Values are the mean of 3 units of 20 fruits (1 batch x 3 replicates). ǂ Least significant difference between mean values in each column at $p = 0.05$.

Table 5. Rind injury symptoms on Navel oranges dipped for 120 sec in solutions containing lemon myrtle (LM) essential oil (EO) and citral during storage at 20 °C.

Treatments	Rind injury score				
	1	**2**	**3**	**4**	**5 days**
Control	1.0	1.0	1.0	1.0	1.0
LM EO (µL L^{-1})					

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2000	1.9	1.9	2.1	2.7	2.7
4000	2.2	2.2	2.4	3.1	3.1
6000	2.5	2.5	2.7	3.2	3.2
8000	2.8	2.8	3.0	3.4	3.4
Citral (µL L^{-1})					
1000	1.4	1.4	1.6	1.7	1.7
LSD‡	*0.2*	*0.2*	*0.2*	*0.2*	*0.2*

Values are the mean of 3 units of 20 fruits (1 batch x 3 replicates). ‡ Least significant difference between mean values in each column at $p = 0.05$. Scoring: 1 = non damage at all, 2 = 1 - 5% damage i.e., some slight damage detectable (just), but still OK, 3 = 5 - 20% damage – moderate damage – not saleable, 4 = 20 - 50% damage, 5 = > 50% damage – severe.

The results in Table 6 show that LM EO and citral significantly inhibited the growth of green mould wounds during storage compared to the control. The most effective concentrations of LM after five days were between 1000 and 2000 µL L^{-1}, with no significant difference among the LM concentrations, therefore the optimum effectiveness was achieved at 1000 µL L^{-1}.

Table 6. Fungal wastage of Navel oranges following dipping for 120 sec in a solution of lemon myrtle (LM) essential oil (EO) and citral during storage at 20 °C.

Treatments	Decay (lesion diameter, mm)		
	3	**4**	**5 days**
Control	20	37	57
LM EO (µL L^{-1})			
500	2	14	27
1000	1	5	13
1250	1	5	13
1500	1	3	12
2000	1	4	12
Citral (µL L^{-1})			
500	3	10	26
1000	2	7	19
LSD‡	*1.9*	*3.5*	*5.4*

Values are the mean of 6 units of 20 fruits (2 batches x 3 replicates). ‡ Least significant difference between mean values in each column at $p = 0.05$.

The 120 sec dip of all concentrations (500 - 2000 µL L^{-1}) of LM EO and citral caused rind injury as shown in Table 7. Injury was present at the first examination at day one after dipping but did not increase in severity during storage. The level of injury was mild for fruits dipped in 500 µL L^{-1} LM EO but the rind injury found more severe at concentrations ≥ 1000 µL L^{-1}.

Further examination was conducted with LM EO at 1000 µL L^{-1} with the aim of determining the lowest dipping time that generates an optimal balance between maximising fungal growth inhibition with minimal rind injury.

Table 7. Rind injury symptoms on Navel oranges dipped for 120 sec in solutions containing lemon myrtle (LM) essential oil (EO) and citral during storage at 20 °C.

Treatments	Rind injury score				
	1	**2**	**3**	**4**	**5 days**
Control	1.0	1.0	1.0	1.0	1.0
LM EO (µL L^{-1})					
500	1.3	1.3	1.3	1.3	1.4
1000	3.2	3.2	3.2	3.2	3.3
1250	3.2	3.2	3.2	3.2	3.4

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1500	3.2	3.2	3.2	3.3	3.5
2000	3.3	3.3	3.3	3.3	3.6
Citral (μL L^{-1})					
500	1.3	1.4	1.4	1.4	1.5
1000	3.4	3.4	3.5	3.5	3.6
LSD‡	*0.2*	*0.2*	*0.2*	*0.2*	*0.2*

Values are the mean of 6 units of 20 fruits (2 batches x 3 replicates). ‡ Least significant difference between mean values in each column at $p = 0.05$. Scoring: 1 = non damage at all, 2 = 1 - 5% damage i.e., some slight damage detectable (just), but still OK, 3 = 5 - 20% damage – moderate damage – not saleable, 4 = 20 - 50% damage, 5 = > 50% damage – severe.

Inoculated Valencia oranges were examined to determine green mould inhibition after dipping 20 fruits in 1000 μL L^{-1} LM EO for 5, 10, 15 and 30 seconds. Control fruits were dipped in water with ethanol and Triton-X100. An additional control water dip that did not contain the wetting agent and stabilizer was also added to confirm that Triton-X100 and ethanol had no effect on mould growth. The results in Figure 1 show firstly that there was no significant difference {$\chi 2$ (1) = 0.69, $P = 0.41$} between the two control treatments. The results in Figure 1 also show that there is significant difference {LRT $\chi 2$ (4) = 56.01, P = 0.00} between the inoculated fruits of control and treated with 1000 μL L^{-1} lemon myrtle EO by dipping during the storage times. 40% rotting of the fruits occurred in the control fruits after 3 days of storage and it turned to 100% after 5 days. On the contrary, dipping the inoculated oranges in 1000 μL L^{-1} lemon myrtle EO for 5, 10, 15 and 30 seconds showed a significantly lower percent of rots over control fruits throughout the storage time. On the 3rd and 4th day of storage on an average less than 2 and 20 percent of the inoculated fruits were rotted, respectively. Interestingly, the effect of dipping times in 1000 μL L^{-1} lemon myrtle EO on the inoculated oranges is equivalent according to the likelihood ratio test in the Multinominal Logistic Regression model {LRT $\chi 2$ (3) = 0.00, P = 1.00}.

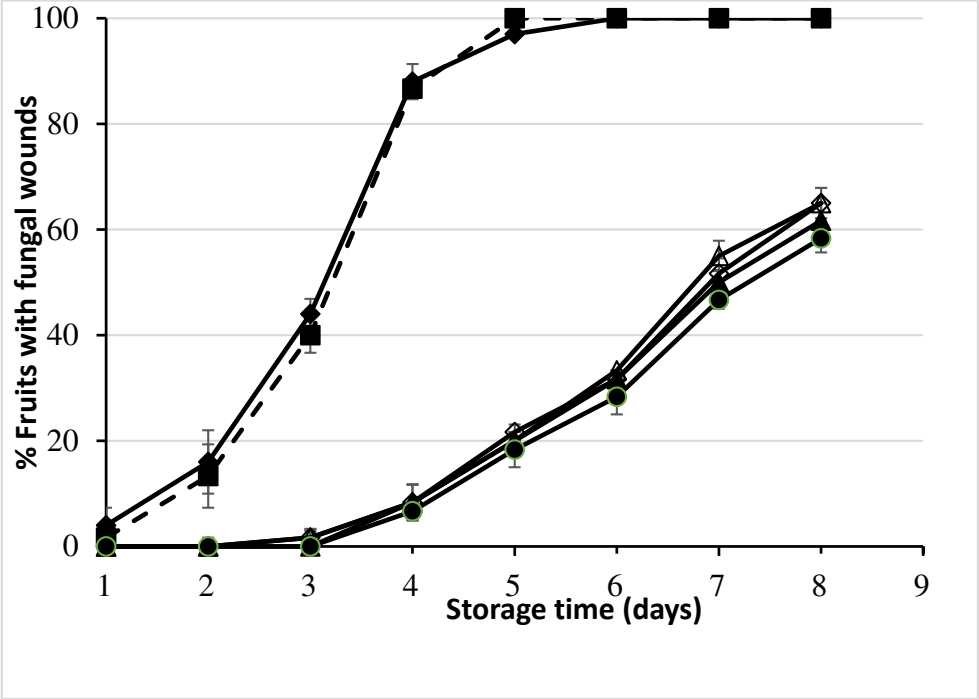

Figure 1. Fungal wounds in Valencia oranges during storage at 20 °C following dipping in 1000 μL L^{-1} lemon myrtle essential oil (LM EO) solution for different times. Control 1 (water) 30 sec (♦); control 2 (water + wetting agent + ethanol) 30 sec (■); LM EO dip 5 sec (Δ); 10 sec (◊); 15 sec (▲); 30 sec (●). Values are the mean of 6 units of 20 fruit (2 batches x 3 replicates). The effect of dipping times in LM

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EO is equivalent according to the likelihood ratio test in the Multinominal Logistic Regression model {LRT $\chi 2$ (3) = 0.00, P = 1.00}.

The data in Table 8 show that there was rind injury on some fruits dipped in LM EO but was not present on any control fruit. Rind injury after 15 and 30 sec dips occurred on a few fruits with a mean score of 1.2 - 1.3 (Table 7); but no injury was observed on fruits dipped in LM EO for 5 and 10 sec.

Table 8. Rind injury symptoms on Navel oranges during storage at 20 °C following dipping in lemon myrtle (LM) essential oil (EO) at 1000 µL L^{-1} for different times.

Dip time (sec)	Rind injury score				
	1	2	3	4	5 days
(Control 1) 30	1.0	1.0	1.0	1.0	1.0
(Control 2) 30	1.0	1.0	1.0	1.0	1.0
5	1.0	1.0	1.0	1.0	1.0
10	1.0	1.0	1.0	1.0	1.0
15	1.1	1.2	1.2	1.2	1.2
30	1.2	1.3	1.3	1.3	1.3
LSD‡	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*

Values are the mean of 6 units of 20 fruits (2 batches x 3 replicates). ‡ Least significant difference between mean values in each column at p = 0.05. Control 1 (water dip) 30 sec; control 2 (water + wetting agent + ethanol dip) 30 sec. Scoring: 1 = non damage at all, 2 = 1 - 5% damage i.e., some slight damage detectable (just), but still OK, 3 = 5 - 20% damage – moderate damage – not saleable, 4 = 20 - 50% damage, 5 = > 50% damage – severe.

3.4. Quality Assessment Study of LM EO Treated Valencia Oranges

Valencia oranges were treated with 1000µL L^{-1}LM EO and citral solution by dipping method for 30 sec, followed by storage at 20 °C and 65% RH for four weeks. The effects of dipping on weight loss, firmness, respiration rate, total soluble solids (TSS), titratable acidity (TA), ethanol and ethylene production are presented in Tables 9 and 10. The results show that there was no significant effect of LM EO or citral on the weight loss, firmness, respiration rate, TSS, TA, ethanol and ethylene production compared to control fruits.

A discrimination triangle test was conducted to determine if a perceptible sensory difference existed between oranges dipped in1000 µL L^{-1} LM EO premixed with TritonX-100 (24 mgL^{-1}) and aqueous ethanol (1 mL L^{-1}) for 30 sec and control fruits dipped in water.

Out of the 21 panellists, only five were able to correctly identify which samples were treated with LM EO and untreated fruits. This number is less than the 12 required for a significant difference between treatments at p=0.05 [35]. Therefore, it was concluded that the addition of LM EO did not adversely affect the taste of the oranges.

Table 9. Effects of dipping oranges in 1000 µL L^{-1} lemon myrtle (LM) essential oil (EO) and citral on weight loss, firmness and respiration rate during storage at 20 °C.

Quality Parameters/ Dipping Treatments (1000 µL L^{-1})	Weeks				
	1	2	3	4	Mean
Weight loss (%)					
Time - 0	0				
Control	2.7	3.9	4.8	5.4	4.2
LM EO	2.4	3.7	4.8	5.4	4.1
Citral	2.4	3.6	4.8	5.6	4.1
LSD‡					*2.1*
Firmness (N)					
Time - 0	33.5				

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Control	27.9	24.5	23.47	22.8	27.9
LM EO	26.2	25.7	23.9	22.5	26.2
Citral	25.9	25.1	23.4	22.5	25.9
LSD‡					*0.5*
Respiration ($mLCO_2kg^{-1}hr^{-1}$)					
Time - 0	8.1				
Control	8.6	10.5	11.3	12.6	10.8
LM EO	8.6	10.3	11.4	12.5	10.7
Citral	8.8	10.5	11.4	12.5	10.8
LSD‡					0.7

Values are the mean of 4 units of 20 fruits (1 batch x 4 replicates). ‡ Least significant difference between mean values in column at $p = 0.05$.

Table 10. Effects of dipping oranges in 1000 µL L^{-1} lemon myrtle (LM) essential oil (EO) and citral on total soluble solids (TSS), titratable acidity (TA) and ethanol accumulation during storage at 20 °C.

Quality Parameters/ Dipping Treatments (1000 µL L^{-1})	**Weeks**				
	1	**2**	**3**	**4**	**Mean**
TSS (%)					
Time - 0	10.0				
Control	10.1	10.3	10.6	11.0	10.5
LM EO	10.4	10.5	10.8	11.0	10.6
Citral	10.1	10.6	10.8	11.3	10.7
LSD‡					*0.3*
TA (% citric acid)					
Time - 0	1.3				
Control	1.2	1.1	1.1	0.9	1.1
LM EO	1.2	1.2	1.1	1.0	1.1
Citral	1.2	1.1	1.0	0.9	1.1
LSD‡					*0.1*
Ethanol accumulation (µL L^{-1})					
Time - 0	1.1				
Control	1.3	1.4	1.6	1.7	1.5
LM EO	1.2	1.4	1.6	1.7	1.5
Citral	1.3	1.5	1.7	1.7	1.5
LSD‡					*0.3*
Ethylene production ($\mu LC_2H_4\ kg^{-1}\ hr^{-1}$)					
Time - 0	1.1E-05				
Control	1.1E-05	1.4E-05	1.3E-05	1.4E-05	1.3E-05
LM EO	1.0E-05	1.4E-05	1.4E-05	1.6E-05	1.4E-05
Citral	1.1E-05	1.4E-05	1.4E-05	1.6E-05	1.4E-05
LSD‡					*2.8E-06*

Values are the mean of 4 units of 20 fruits (1 batch x 4 replicates). ‡ Least significant difference between mean values in column at $p = 0.05$.

4. Discussions

The most of the compositions of the extracted LM EOs in the current study are consistent with the findings of Southwell et al. [25], Kurekci et al. [40] and Buchbauer et al. [41]. The LM EO, which is predominantly rich in citral, was found to effectively inhibit the germination and growth of *P. digitatum* in the agar diffusion assay and with the increase in concentration of the oils from 1 to 5 µL $disc^{-1}$, the efficacy also increased. The complete inhibition of the fungal growth at 4 and 5 µL $disc^{-1}$

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indicates strong biological activity of the oil on the pathogen. The antifungal efficacy of LM EO and citral in this study is align with the previous findings by Sultanbawa [39], Wilkinson et al. [27] and Lazar-Baker et al. [28] who reported LM EO as an effective antimicrobial agent in *in vitro* against 13 bacteria, one yeast and eight fungi other than *Penicillium* spp. There was greater antifungal efficacy of citral and LM EO in the vapour assay than in the agar diffusion assay which was considered due to the vapour providing better penetration into the agar media than through the diffusion assay. This is supported by the findings of Rodov et al. [29] who reported the antifungal efficacy of citral against *P. digitatum* in vapour assay as almost double in comparison to agar diffusion assay.

The inhibitory action of citral against *P. digitatum* could be explained based on its molecular structure. Citral is an aliphatic aldehyde that contains double bonds conjugated to its carbonyl group. Kurita, *et al.* [42] reported the fungal inhibition activity is closely related to the energy of its lowest empty molecular orbital for which citral is capable of forming charge transfer complex, for example with a good electron donor such as tryptophan. Citral thus can form charge transfer complexes with electron donors of fungus cell and results in fungal growth inhibition [43,40,42]. The *in vitro* findings of the current study indicate that of the high citral content of LM EO may results in it having *in vivo* efficacy.

Navel oranges dipped in different concentrations of LM EO and citral for 120 sec significantly inhibited the growth of green mould compared to the control. The optimum effectiveness was achieved by 1000 μL L^{-1} LM EO. However, the initial dipping time of 120 sec for all concentrations of LM EO and citral resulted in rind injury. Screening LM EO for lower dipping times showed a significant difference in fungal inhibition due to dip time with the magnitude of reduction being 120 sec > 30 sec≈ 15 sec ≈ 10 sec ≈ 5 sec. There was no rind injury on fruits dipped for 5 and 10 sec, while minor rind injury was observed on fruits dipped for 15 and 30 sec. A greater level of rind injury was observed on fruits dipped for 120 sec. The LM EO was selected for evaluation due to its high level of citral but the application of 1000 μL L^{-1} LM EO in inoculated Navel oranges was significantly more effective in inhibiting mould growth than 100% citral after 5 days of storage. However, the greater effect of LM EO did not seem to be related to any difference in geranial and neral proportions in citral and LM EO. The greater inhibitory effect of LM EO would then seem to be due to any other minor compound(s) in the LM EO which has greater antifungal activity than the citral constituents or their combinations.

The obvious advantage of using EOs extracted from plants over synthetic fungicides is that they can be considered to be derived from 'natural' sources. This would have marketing value for consumers who are suspicious of synthetic chemicals being added to foods and they should also be acceptable for use on organic citrus fruits. Essential oils in this study are hypothesised to inhibit the growth of *P. digitatum* by deforming the mitochondrial morphology, being involved in arresting the respiratory metabolism lead to decreasing the activities of tricarboxylic acid cycle (TCA) -related enzymes and changing the TCA metabolic abilities.

From the results obtained in this study where dipping in LM EO gave good inhibition of *P. digitatum* in Navel and Valencia oranges, it is worthy of further investigation for ability to control other mould wounds of citrus fruits, and maybe other fungi on other fruits. While the dipping of oranges in the LM EO did not have any adverse effect on the internal quality and sensory attributes, a major barrier to the use of LM EO for fresh fruits marketing will be the potential for generation of rind injury. The rind injury would seem to be due to the action of citral, which has been shown to disrupt fruit membrane structure and is assumed to be the mechanism whereby it disrupts the growth of *P. digitatum* [23,44]. The ability of citral to damage rind cells is supported by the findings of Knight [24] and Wuryatmo [45] who reported rind injury was more severe on fruits in direct contact of citral. In the current study, fruits dipped in 100% citral exhibited more rind injury than in LM EO which comprises only 85% citral.

The reduction of rind injury achieved by dipping fruits for shorter times was probably due to less citral accumulating on the rind. For commercial dipping practices, immersion of fruits for 30 sec would seem to be the lowest possible throughput time to avoid rind injury possibility. However, while dipping in LM EO for 30 sec was able to inhibit development of green mould, it was

accompanied by a low level of rind injury on some fruits. Dipping fruits in LM EO for 5 and 10 sec did not generate rind injury, while this dipping time was not effective to inhibit mould growth as compared to 120 sec. In order to overcome rind injury and maintaining longer exposure times, it would seem to be worthwhile to investigate different application methods of LM EO combined with edible films, coatings and/or nanoencapsulation. Rodov et al. [29] observed that citral dissolved in 25% (v/v) ethanol suppressed *P. digitatum* decay in 'Eureka' lemons without visible rind damage.

5. Conclusions

Both extracted and commercial LM EOs contain high levels of citral with a content of 88%. Other constituents were also found in these LM EOs including, iso-geranial, benzaldehyde, iso-neral, 6-methyl-5-hepten-2-one, ethylbenzene and linalool, each constituent accounts for a range of 0.5 to 2% of total constituents. LM EO was effective in inhibition of mould *in vitro* and in oranges. In the *in vitro* tests the crude oil was used without any dilution in the current study. So, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by microdilution was not performed. The level of mould inhibition of LM EO was even greater than citral, the major and well-known antimicribial compound. It indicates LM EO might have some as yet unknown minor components, that might exhibit potent antimicrobial activity. Therefore, future studies are suggested to examine the MIC and MBC of LM EO and the individual and synergistic effects of other minor components in LM EO on prevention and treatment of bacterial or other fungal growth in oranges and other fresh produce.

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