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Item Type	Article
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DOI	https://doi.org/10.1021/acsagscitech.1c00232?
Publisher	ACS Publications
Rights	Attribution-NonCommercial-ShareAlike 4.0 International
Download date	2026-03-11 22:49:00
Item License	http://creativecommons.org/licenses/by-nc-sa/4.0/
Link to Item	https://hdl.handle.net/20.500.14519/1549

Inhibition of Kumquat Postharvest Fungi through Vapor Contact with Spearmint Essential Oil and Carvone

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 Cite This: *ACS Agric. Sci. Technol.* 2022, 2, 330–339 Read Online

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ABSTRACT: The antimicrobial activities of spearmint essential oil and six of its constituents were evaluated in vitro and in vivo analysis using direct contact and vapor exposure assays. Qualitative analysis of spearmint essential oil revealed that the main constituents of the essential oil are carvone (62.0%) and limonene (24.6%). Limonene (45%) was found to be the major constituent of the essential oil vapor phase, when sampled using direct headspace and Tenax TA tubes. Spearmint essential oil, carvone, and 1,8-cineole exhibited the highest antifungal activity against *Penicillium digitatum* and *Geotrichum citri-aurantii* (Ferraris), with the vapor phase application resulting in more than 50% growth inhibition at 1429 mg/L for 1,8-cineole and complete inhibition occurring at 714 mg/L for carvone and spearmint. The treatment of kumquats with spearmint essential oil and carvone vapors resulted in an average reduction in weight loss and decay rate of approximately 30% and 40%, respectively, when the fruits were stored at 25 °C.

KEYWORDS: gas chromatography, spearmint essential oil (*Mentha spicata*), headspace, Tenax TA, SPME, *Penicillium digitatum*, *Geotrichum citri-aurantii* (Ferraris) Butler

1. INTRODUCTION

The South African citrus industry is the third largest horticultural industry in the country in terms of gross value, making it a key contributor to the economy.¹ In 2015, it was estimated that as many as 100 000 permanent and numerous seasonal farm workers were employed by the industry.² Kumquat (*Citrus japonica* Thunb) belongs to the Rutaceae family.³ The fruit is round or oval-shaped, with a smooth orange-yellow, sweet, fragrant, and edible rind that clings tightly to the fruit. Although kumquat originated from southeastern China and Japan,⁴ it is cultivated in the Southern Hemisphere in countries including South Africa, Brazil, and Australia. South Africa is an important supplier of kumquat to Europe and the United Kingdom^a.

Penicillium digitatum (green mold), *Penicillium italicum* (blue mold), and *Geotrichum citri-aurantii* (sour rot) are the most common and economically detrimental pathogens that affect citrus fruit, with kumquat not spared.⁵ These opportunistic wound-pathogens reduce the shelf life of the fruit through decay.^{6,7} Cold storage and hot water dips are some of the postharvest treatments used to control pathogens and preserve the fruit.⁴ However, these treatments are unable to completely prevent postharvest pathogen development. The application of synthetic fungicides is prohibited on kumquats, since the peel is eaten with the fruit. Therefore, alternative interventions are needed for the prevention of postharvest decay of these fruits.

Essential oils with strong antifungal activity have been explored as alternative fungicides for crop protection,^{8–10} since their complex chemical composition prevents the development of pathogen resistance. Spearmint essential oil has been reported to be active against a wide range of fungi and bacteria.^{11,12} Carvone-rich essential oils, including spearmint

oil, demonstrated their efficacy when applied to citrus as an ecofriendly alternative to synthetic fungicides.¹³ Numerous studies have investigated the use of essential oils to control postharvest pathogens using direct contact assays.^{14–16} However, sensory modification arising from the direct application of the essential oils should be taken into consideration because of the specific organoleptic properties of the essential oils. The application of essential oils in the vapor phase offers a number of advantages including low residue levels, good penetration into wounds and a reduced risk of phytotoxicity, since the concentrations used are lower than in the liquid phase.^{17,18} Furthermore, studies have shown that some oils are more effective when applied as fumigants rather than in solution.¹⁹

Most studies conducted on the application of essential oils in the vapor phase rely on the liquid essential oil composition as an indication of the observed antimicrobial activity.^{16,17,20} However, changes in the vapor composition might significantly affect the vapor phase antimicrobial properties of the essential oil. The concentrations of active components in the vapor phase are affected by the sample volume temperature and relative volatility of the analytes. When the vapor composition of a complex liquid sample is examined, according to Dalton's Law, each compound in the vapor phase contributes to the total vapor pressure within the container or enclosed space.

Received: September 30, 2021

Revised: February 14, 2022

Accepted: February 14, 2022

Published: March 1, 2022



Other factors that affect the concentration/peak area of a sample in the headspace include temperature and equilibration time.²¹ Therefore, any changes in the experimental conditions may affect the distribution coefficient of the analytes. There are also some difficulties in the quantitative headspace analysis when using an internal standard, since the introduction of another volatile compound modifies the partial pressures of the individual components in the sample, thus adding another dynamic to the equilibrium. Therefore, when performing headspace analysis, optimization of extraction methods and equilibration time is essential to ensure reproducible representative samples.²²

This study was aimed at evaluating the feasibility of using spearmint essential oil vapors for the treatment of postharvest pathogens prevalent in kumquats. Given the proposed application of spearmint essential oil, an understanding of the composition of the essential oil in both liquid and vapor phases is imperative to ascertain the contribution of the different components present to the antimicrobial activity of the oil. With this aim, the antifungal properties of vapor phase spearmint oil and its major components were compared to the direct contact (liquid phase) application of the essential oil and of the major compounds. The vapor phase composition of spearmint essential oil was studied by means of various headspace sampling techniques to establish the most appropriate technique for determining the composition of the spearmint oil vapors in a packhouse.

2. MATERIALS AND METHODS

2.1. Quantification of Liquid-Phase Components in Spearmint Essential Oil. A 20.0% (v/v) sample solution of the spearmint essential oil (Holistic Emporium, Edenvale, South Africa) was prepared in hexane (AR grade, Aston Manor, Johannesburg, South Africa). Analysis was carried out using a gas chromatograph Agilent 6890 N gas chromatograph (GC) (Agilent Technologies, Illinois, USA), equipped with a 5973 Agilent mass spectrometer (MS) and a flame ionization detector (FID) operated at 250 °C, as described by Combrinck et al.²³ Mass spectra were obtained over the range m/z 35–550 in electron impact mode (70 eV). Helium (99%, Afrox South Africa) was used as carrier gas at a constant flow rate of 1.2 mL/min. A 1.0 μ L sample aliquot was injected at 24.79 psi using a split ratio of 200:1 and an inlet temperature of 250 °C. Separations were performed using an HP-Innowax polyethylene glycol column (60 m \times 250 μ m internal diameter \times 0.25 μ m film thickness) using the following temperature gradient: oven temperature was initially held at 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. Identification of the sample components was confirmed by the NIST, Mass Finder and Flavor spectral libraries (\geq 80% library match). Authentic standards (\geq 97% pure) of the following compounds were used for the confirmation of the identities of the compounds: α -pinene, camphene, β -pinene, α -phellandrene, terpinolene, limonene, 1,8-cineole, dihydrocarvone, and carvone (Sigma-Aldrich, Darmstadt, Germany).

2.2. In Vitro Determination of the Antifungal Properties of Spearmint Oil and Its Major Components. **2.2.1. Postharvest Pathogens.** Isolates of *P. digitatum* (green mold) and *G. citri-aurantii* (sour rot) fungal cultures isolated from infected fruit were verified and supplied by Citrus Research International (CRI)(Nelspruit, South African). The cultures were subcultured onto Potato Dextrose Agar (PDA) (Biolab, Wadeville, South Africa) and incubated for 8 days at 25 °C. The original cultures were then stored at 4 °C.

2.2.2. Toxic Medium Assay. Spearmint essential oil and six individual terpenes (α -pinene, camphene, β -pinene, α -limonene, 1,8-cineole, and carvone), which were major components of the essential oil, were weighed separately into Eppendorf tubes and 400 μ L of the emulsifier Tween20 (Sigma-Aldrich, Aston Manor, Johannesburg,

South Africa) was added to each of the tubes.²⁴ The essential oil or pure terpenes were then thoroughly mixed with sterile lukewarm potato dextrose agar (PDA) to final concentrations of 1000, 2000, 3000, and 5000 mg/L. The PDA was poured into Petri dishes (100 \times 21 mm; ThermoFisher Scientific) and allowed to set. The fungal spore suspensions were obtained by scraping the plate surface with a sterile L-shaped glass rod and 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, and the inoculum concentration was then adjusted to 1×10^6 and 1×10^8 spores/mL for green mold and sour rot in 1/4-strength Ringer's solution, respectively, corresponding to an optical density (OD) of 0.14 at a wavelength of 425 nm. The growth medium was then inoculated in the center with 10 μ L of a spore suspension of either *P. digitatum* or *G. citri-aurantii*. Guazatine (250 μ L/L) and Imazalil (500 μ L/L) (Makhteshim-Agan (Pty), Ltd., South Africa) served as positive controls, while the growth control contained only Tween20. Each test sample was replicated five times and the entire assay was repeated for validation purposes. The treated cultures were incubated at 25 °C for 7 days. Growth inhibition was calculated as the percentage of radial mycelial growth of the cultures exposed to the treatments, relative to the growth of those on the control plates.²⁴ To distinguish between fungicidal and fungistatic activity, mycelial plugs that did not grow were transferred to fresh PDA, and the observed radial fungal growth was measured after a total of 5 days incubation at 25 °C.

2.2.3. Volatile Exposure Assay. The vapor exposure tests were done according to the method described by ref 25. The test samples comprised spearmint oil and the pure compounds present in the oil. Approximately 4 h prior to exposure to the samples, the two test organisms were plug-inoculated onto 20 mL of PDA set in Petri dishes. Droplets of undiluted test samples (10, 50, or 100 μ L) were placed onto glass coverslips fixed to the lids with glycerol. Each treatment was replicated five times and sunflower oil served as a control. The Petri-dishes were inverted and sealed with Parafilm (American Natural, Chicago) to ensure that none of the vapors escaped. The test was replicated for validation purposes. All treated cultures were incubated for 7 days at 25 °C. The fungal growth was recorded, thereafter, as the percentage of radial mycelial growth. A distinction between the fungicidal and fungistatic activity of the test samples against the target organism was made as explained (section 2.2.2).

2.4. In Vivo Analysis of Spearmint Oil or Carvone Applied As Fumigants on Kumquats. Freshly picked untreated, export quality kumquat fruit were obtained in the 2018 season from a packhouse in Levubu (Limpopo, South Africa). The fruit were evenly sized and displayed no visible signs of damage. The standard commercial treatment of kumquats, involving a rinse in a hot water bath at 37 °C, was applied. No commercial fungicides were included in these treatments. The treatments applied to the fruit are summarized in Table 1. Each treatment group consisted of 20

Table 1. Treatment Groups of Spearmint Essential Oil or Carvone Used in the in Vivo Trial, with Hot Water Rinse at 37 °C Being the Standard Commercial Treatment^a

treatment	application	specification
control (C)	none	hot water rinse at 37 °C
spearmint 1000 μ L/L (SML)	liquid	standard commercial treatment + sprayed with essential oil–tween–water mixture
spearmint (SMV)	vapor	standard commercial treatment + 500 μ L spearmint oil in 100 μ m polyethylene plastic bag (40 \times 40 mm)
carvone (CRV)	vapor	standard commercial treatment + 500 μ L carvone in 100 μ m polyethylene plastic bag (40 \times 40 mm)

^a n = 20 cartons of fruit per treatment group contain \pm 200 fruit per box.

boxes (19 × 29 × 8 cm); consisting of ±200 fruit, which were weighed prior to treatment of the fruit. The liquid treatment comprised concentrated spearmint essential oil mixed with Tween20 and diluted with deionized water to a final essential oil concentration of 1000 μL/L. The vapor treatments consisted of either spearmint essential oil or carvone, which were placed in polyethylene sleeves (40 × 40 mm), of which four were distributed evenly around each box and placed on top of the fruit. Two different conditions were selected for storage: storage at 25 °C for a period of 21 days, or storage at 4 °C for 14 days (in line with maximum transportation period via ship), followed by storage at room temperature for a 7-day period. The percentage weight loss of the fruit was monitored by weighing the boxes. Fruit decay was visually assessed and reported as % of fruit presenting any stages of decay. To determine the percentage decay, the number of decayed fruits were counted and expressed as a percentage of the total number in each box. The average was calculated for each treatment group.

2.5. Qualitative Determination of the Vapor Phase Components of Spearmint Oil Using Various Headspace Techniques. Qualitative determination of the headspace composition of spearmint essential oil was attained using gas chromatography–time-of-flight–mass spectrometry (GC-ToF-MS), following the vapor extraction using direct headspace and polydimethylsiloxane solid phase microextraction (PDMS SPME) fiber (HS-SPME). Vapor phase spearmint essential oil samples extracted with Tenax TA (Tube type: Tenax TA, 1/4-in. OD × 3 1/2-in. size) tubes were analyzed using GC-MS coupled to a thermal desorption unit.

2.5.1. Direct Headspace Analysis. Direct headspace analysis of the vapor phase constituents of spearmint oil was carried out using a LECO Pegasus 4 GCxGC system (Michigan, USA) operated in one dimension. The system comprised a Multi-Purpose Sampler (Gerstel); an Agilent 7890 gas chromatograph with cryogenic thermal modulator and a secondary oven coupled to a Time-of-Flight (ToF) mass spectrometer. The offset temperature of the secondary oven was set at +5 °C, with the modulation period set to zero (unmodulated). The detector was operated at an ionization energy of 70 eV, in the 35–550 Da range. Separations were carried out using an Rxi-5Sil MS capillary column (30 m × 250 μm internal diameter × 0.25 μm film thickness, Restek), with helium (99%, Afrox South Africa) as the carrier gas, at a constant flow of 1.5 mL/min. The inlet was operated in splitless mode at a temperature of 250 °C. The oven temperature was programmed to an initial temperature of 40 °C (held for 2 min), then raised to 240 °C, at 7 °C/min, and held at this temperature for 2 min.

Optimum equilibration time was determined by adding exactly 1.0 μL of the concentrated spearmint essential oil into seven 20 mL headspace vials, which were sealed with 18 mm polytetrafluoroethylene (PTFE) screw caps (Sigma-Aldrich, Germany). The samples were left to equilibrate on the instrument's headspace sampling platform for 10, 20, 30, 40, 60, 100, or 120 min. After each time period, the vapor phase of one sample (1.0 mL) was drawn up using the Multipurpose autosampler, fitted with a 2.5 mL gastight syringe, and analyzed using GC-ToF-MS. Following the establishment of the optimum equilibration time, the samples were prepared as described above, equilibrated for 30 min at 25 °C (temperature used to simulate the commercial shelf storage temperature) and analyzed.

2.5.2. Thermal Desorption Using Tenax TA Tubes. An airtight bottle was utilized for sampling of spearmint oil volatiles. The cap of a 100 mL Schott bottle (United Scientific (Pty) Ltd., South Africa) was modified to enable Tenax TA tubes to be inserted to withdraw the headspace. The accurate volume of the Schott bottles and sampling syringe (C-BIO01 BioVOC Breath Sampler, Markers, England) was determined by filling the weighed sampling bottle and syringe with distilled water. The filled bottle and syringe were again weighed and the mass of water determined by difference. The temperature of the water was taken and the exact volume was determined using the density of water at that temperature. Prior to the qualitative analysis, a 5.00 μL volume of the concentrated spearmint essential oil was placed in each of seven sampling bottles at 25 °C for the determination of the optimum equilibration time. The bottles were sealed with the

designed lid and left to equilibrate for 10, 20, 30, 40, 60, 100, and 120 min, respectively. After standing for the required time, a 25.0 mL of the headspace was withdrawn through the Tenax TA tubes using a calibrated fixed volume syringe. The samples were desorbed from the Tenax TA tubes using a thermal desorption unit (TDU; Unity, Markes International) connected to a GC-MS. All the samples were prepurged for 2 min at 15 mL/min with helium and subsequently desorbed by heating the tubes for 10 min at 280 °C. The volatiles were cryofocused (split ratio of 2:1) onto a trap at −10 °C. Thereafter, the trap was heated to 300 °C at 100 °C/s. The qualitative analysis of the released volatiles was achieved using GC-MS conditions as described in section 2.1. The desorbed volatiles were separated using a 5%-phenyl-methylpolysiloxane (DB5) capillary column (60 m × 250 μm × 0.25 μm, Agilent Technologies). The oven temperature program was as follows: 50–80 °C at 10 °C/min, then raised to 130 °C at 6.0 °C/min, held for 5 min, and finally increased to 240 °C at 20 °C/min, modification to the method was made to decrease the total run time. The detector was operated in electron impact mode at an ionization energy of 70 eV, over the range 30–550 Da, with a scanning speed of 2.76 scan/s at 230 °C.

2.5.3. Headspace Solid-Phase Microextraction. The HS-SPME method was optimized by evaluation of the exposure time of the fiber to the sample headspace. Prior to sampling, the SPME fiber (100 μm PDMS); (Supelco, Munich, Germany) was conditioned in the GC injector port according to the manufacturer's instructions. The samples were prepared as before (section 2.5.1) and equilibrated for 30 min at 25 °C. The time required for complete saturation of the SPME fiber was investigated by varying the exposure time: 5, 10, 15, 30, 45, and 60 min intervals were tested. A 1 min analyte desorption time was used for desorption of the volatiles from the SPME fiber. The vapor analysis was conducted using the same instrument and conditions as specified in section 2.5.1.

2.6. Data Analysis. The percentage composition of the vapor phase of spearmint essential oil components was calculated using the peak area normalization method.²⁶ Data obtained from the direct headspace and SPME analysis was processed using LECO ChromaToF software (version 4.5). Identification of the compounds determined by all three techniques (thermal desorption using Tenax TA tubes, direct headspace and SPME analysis) was done using the NIST 5 spectral library and the identities of these compounds were verified by comparison with authentic reference standards (Sigma-Aldrich, Germany). Data obtained in this study was subjected to analysis of variance (ANOVA), and the mean values were compared using the Fisher's least Duncan's Multiple Range test at 95.0% confidence level.

3. RESULTS AND DISCUSSION

3.1. Spearmint Essential Oil Composition. Fifteen compounds, representing 97.9% of the total oil composition, were identified in the spearmint essential oil analyzed using GC-FID/MS (Table 2). The major essential oil constituents were carvone (62.0%) and limonene (24.6%). Having carvone as the principal constituent of spearmint essential oil is beneficial for this study, since the strong antifungal activity of the oil has been attributed to carvone.²⁷

3.2. Biological Activity. **3.2.1. Antifungal Activity of Spearmint Essential Oil and Six of Its Components in the Liquid Phase Determined with Toxic Medium Assay.** Inhibition percentages of the samples against green mold and sour rot (Table 3) confirmed the efficacy of spearmint essential oil and its major constituent (carvone) against both pathogens. The IC₅₀ values, which is the concentration that resulted in a 50% of the mycelium growth was calculated by probit analysis. Imazalil and Guazatine inhibited the growth of green mold and sour rot, respectively, at a concentration of 250 mg/L. The inhibitory character of both spearmint essential oil and carvone is evident at concentrations as low as 1000 mg/L toward green

Table 2. Essential Oil Composition of *Mentha spicata* Essential Oil in the Liquid Phase As Determined by GC-FID and Identified Using GC-MS ($n = 3$)

compound name ^a	percentage peak area ^c	retention index
α -pinene	1.1 \pm 0.2	938
camphene	0.8 \pm 0.1	943
β -pinene	1.4 \pm 0.1	949
myrcene	0.6 \pm 0.3	958
α -phellandrene	0.3 \pm 0.1	969
terpinolene	0.2 \pm 0.1	998
limonene	24.6 \pm 0.3	1018
1,8-cineole	4.6 \pm 0.2	1059
menthol	0.5 \pm 0.1	1079
cyclohexanone ^b	0.5 \pm 0.1	1116
dihydrocarvone	0.7 \pm 0.1	1179
carvone	62.0 \pm 0.3	1190
copaene ^b	0.3 \pm 0.1	1293
β -bourbonene ^b	0.4 \pm 0.1	1339
caryophyllene ^b	0.5 \pm 0.1	1414
total	97.9	

^aCompounds listed in order of elution from an HP-Innowax column.

^bCompounds identification based on retention indices. ^cValues are mean \pm standard deviation

mold. Similarly, spearmint essential oil and carvone also displayed the highest inhibitory effect toward sour rot as compared to the other compounds (Table 3). The application of 5000 mg/L of 1,8-cineole caused 73.1% inhibition in the growth of green mold mycelia, indicating substantially lower activity than carvone. The effectiveness of spearmint essential oil when used in direct contact against *Penicillium* sp. has been well documented.^{28,29}

At a 1000 mg/L concentration of limonene, the mycelial growth of sour rot was enhanced and not repressed. However, upon increasing the limonene concentration, slight inhibition of the pathogen was observed. Similarly, limonene was found to have stimulatory effect on the growth of *P. digitatum* and *P. italicum* growth, but had no effect on *P. expansum* and *B. cinerea* (which are noncitrus pathogens).³⁰ The results observed in this study, indicate the induced growth enhancement of the pathogens occurred after exposure to a lower limonene concentration (1000 mg/L), which might be a consequence of an adaptive defense by the fungi toward limonene since it is the major component of citrus peel.³¹

The presence of α -pinene, camphene and β -pinene resulted in slight inhibition of the target pathogens, confirming a limited individual activity from these compounds, these compounds also had the lowest IC₅₀ values. Numerous *in vitro* analysis involving monoterpenes such as α -pinene, β -pinene, p-cymene, β -myrcene, limonene, and γ -terpinene indicate that such terpenes display little or no antimicrobial activities when tested against bacteria^{32,33} and fungi.^{34,35} Carvone and 1,8-cineole are monoterpenoids, a class of compounds known to be active against a broad spectrum of microorganisms (fungi, yeasts and bacteria).³⁶ In addition to these terpenoids being major compounds of the oil, it is also possible that an additive or even synergistic effect results from all the components when present as a mixture in the essential oil, even though the other compounds are not highly active on their own. Therefore, a more in depth understanding of the additive and synergistic activity of the minor components present in the essential oil is required. The mycelial growth

inhibition of both pathogens was found to be dependent on concentration for all the terpenes and spearmint essential oil. The toxicity of all the active test substances was fungistatic rather than fungicidal, suggesting that sustained exposure to the essential oil, for example in vapor form, could be beneficial for pathogen control.

3.2.2. Antifungal Activity of Spearmint Essential Oil and Six of Its Components in the Vapor Phase. The antifungal activity of spearmint essential oil and its major components from the vapor phase were tested at dosage levels of 143, 714 and 1 429 μ L/L air space; calculated as the amount of essential oil (10, 50, or 100 μ L)/ free airspace (L) of the Petri dish. The effects of the vapor phase application of the substances tested are illustrated in Figure 1. Exposure to 710 μ L/L of spearmint essential oil and carvone vapors resulted in complete inhibition of green mold (Figure 1A), validating the use of essential oils as fumigants. Vapor application of 1429 μ L/L of 1,8-cineole resulted in an approximately 50% inhibition of green mold, with α -pinene, camphene and β -pinene vapors having little effect on the growth of the pathogen (\pm 20% inhibition). However, the activity of these terpenes in the vapor phase was considerably higher than that observed in the liquid phase/direct contact assays. Once again, limonene enhanced the growth of green mold, with an increase of about 20% of the mycelial growth as compared to the control. The efficacy of spearmint essential oil and carvone vapors against sour rot at these low concentrations is of interest, since the use of guazatine (the only permitted synthetic fungicide that is currently effective against sour rot of citrus) has been banned in many countries.³⁷

The degree of antimicrobial activity of an essential oil when determined *in vitro* is said to depend on the solubility and infusibility of that essential oil when it is added to a medium.^{38,39} Thus, the distribution of the essential oil into the aqueous medium depends on the hydrophobic and hydrophilic properties of the essential oil.⁴⁰ However, in the vapor phase, the activity only depends on the relative volatility of the components. A difference in the essential oil efficacy can therefore be expected when applied in the liquid relative to the vapor phase. The increased effectiveness of the spearmint essential oil components in the vapor phase is thus associated with the vapor pressure of the compounds. A higher vapor pressure of an individual component results in a higher headspace concentration of that component. Therefore, the observed increase in the stimulatory effect of limonene might be attributed to a high vapor pressure (1.98 mmHg at 25 °C), which results in an increased limonene headspace concentration. The results obtained in this study provide evidence that spearmint essential oil and its components are more effective antifungal agents when applied in the vapor phase as compared to liquid phase application. Therefore, both spearmint essential oil and carvone hold potential as natural fumigants in combating citrus postharvest diseases.

3.2.3. In Vivo Efficacy of Spearmint Oil and Carvone Applied As Vapor. A preliminary determination of the efficacy of the essential oil in both the liquid and vapor phase, was conducted using kumquats, at both room temperature and under cold storage. The use of spearmint essential oils and other natural products in low concentrations has no limitations since they are classified as "Generally Recognized as Safe" (GRAS) by the Food and Drug Administration (FDA).^{16,41} The *in vivo* analysis was conducted using pure carvone and spearmint essential oil over a 21-day period and the mass loss

Table 3. Percentage Mycelial Growth Inhibition (\pm Standard Deviation around the Mean Percentage Inhibition) and IC_{50} Values of *Penicillium digitatum* and *Geotrichum citri-aurantii* Obtained Using the Toxic Medium Assay^a

test sample	concentration (mg/L)	<i>Penicillium digitatum</i>		<i>Geotrichum citri-aurantii</i>	
		pathogen inhibition (%)	IC_{50} (mg/L)	pathogen inhibition (%)	IC_{50} (mg/L)
imazalil	250	100 \pm 0.0		NI	
	500	100 \pm 0.0		NI	
guazatine	250	NI		100 \pm 0	
	500	NI		100 \pm 0	
α -pinene	1000	9.37 \pm 2.04	1.69 $\times 10^4$	1.52 \pm 0.81	1.34 $\times 10^4$
	2000	9.79 \pm 1.91		6.80 \pm 2.42	
	3000	16.6 \pm 0.46		13.3 \pm 5.1	
	5000	17.3 \pm 0.69		16.5 \pm 3.3	
camphene	1000	1.16 \pm 0.18	1.20 $\times 10^4$	10.0 \pm 3.6	1.37 $\times 10^4$
	2000	12.1 \pm 2.9		14.8 \pm 1.4	
	3000	13.8 \pm 4.0		17.0 \pm 4.4	
	5000	18.3 \pm 0.7		19.9 \pm 3.2	
β -pinene	1000	5.54 \pm 2.0	1.28 $\times 10^4$	9.41 \pm 3.63	1.61 $\times 10^4$
	2000	12.7 \pm 6.5		10.4 \pm 3.7	
	3000	13.3 \pm 2.0		13.1 \pm 3.0	
	5000	19.2 \pm 7.6		16.1 \pm 3.4	
limonene	1000	0.990 \pm 0.80	1.38 $\times 10^4$	-4.11 \pm 0.89	1.43 $\times 10^4$
	2000	3.80 \pm 0.50		4.69 \pm 0.27	
	3000	12.9 \pm 5.0		7.16 \pm 1.74	
	5000	14.1 \pm 4.2		10.1 \pm 1.6	
1,8- cineole	1000	22.8 \pm 5.7	3.29 $\times 10^3$	34.5 \pm 1.7	3.74 $\times 10^3$
	2000	33.9 \pm 5.6		38.7 \pm 1.8	
	3000	44.2 \pm 3.0		47.2 \pm 0.9	
	5000	73.1 \pm 3.5		56.3 \pm 3.7	
carvone	1000	100 \pm 0	5.86 $\times 10^2$	67.6 \pm 2.9	8.41 $\times 10^2$
	2000	100 \pm 0		100 \pm 0	
	3000	100 \pm 0		100 \pm 0	
	5000	100 \pm 0		100 \pm 0	
spearmint	1000	44.5 \pm 5.0	1.10 $\times 10^3$	48.8 \pm 4.8	1.46 $\times 10^3$
	2000	100 \pm 0		74.9 \pm 3.7	
	3000	100 \pm 0		100 \pm 0	
	5000	100 \pm 0		100 \pm 0	

^aColony radius was measured after 7 days at 25 °C ($n = 5$). NI = no inhibition; (–) negative value indicates increased growth as compared to the control; IC_{50} = the concentration in mg per L that inhibited 50% of mycelium growth) calculated by a probit analysis.

of the fruits over the storage period was determined (Figure 2A). From the results, it was evident that the storage of kumquats at 4 °C reduces the rate of mass loss. There was no significant difference between the percentage mass loss of the control fruit and the essential oil-treated fruit at this temperature ($p > 0.05$). However, a pronounced reduction in mass was observed for the control fruits that were kept at 25 °C over the 21-day trial period. Exactly 50% of the total mass of the control fruit was lost, while fruits treated with either spearmint essential oil or carvone had a mass loss of only around 20% ($p > 0.05$) for both the liquid and vapor application. It is speculated that essential oil vapors form a coating on the fruit surface, thus modifying gas permeation and resulting in a reduction in respiration rate and water loss.¹⁸ However, this is just a hypothesis, and the actual mechanism of the prevention of weight loss by vapors remains unknown. There was no significant difference in the mass loss of the fruits treated with either the liquid or vapor phase. Previous studies revealed a reduction in weight loss of cherries, grapes and strawberries after treatment with eugenol, thymol, menthol, or eucalyptus essential oil vapors.^{42–45}

Figure 2B indicates that all the applications of spearmint essential oil or carvone, in both the vapor and liquid phase,

significantly inhibited fruit decay when stored at both 25 and 4 °C over a 21-day period ($p < 0.05$). Kumquats treated with carvone vapors presented with the least decay (22%), followed by fruit treated with spearmint oil vapors (28%). Fruit treated with the liquid spearmint essential oil had a percentage decay of 32%, while 69% of the control fruit were spoiled when stored at room temperature for 21 days. This provides further evidence that carvone has a stronger antifungal activity as compared to spearmint essential oil. Similar to what was observed in the in vitro assays, the vapor applications were more efficient in decay prevention as compared to the liquid application. This trend of spearmint essential oil or carvone vapors was the same for fruits stored at room temperature and at cold storage, with a higher percentage decay observed for fruit kept at room temperature, as expected. Most studies agree that essential oil volatiles result in substantial morphological changes to the fungal hyphae, most prominently a reduction in their diameter and wall thickness.^{16,46} This vapor essential oil–hyphae interaction may result in plasma membrane disruption, a decrease in lipid and saturated fatty acid content, nutrient leakage from the hyphae, and plasma membrane disruption. The use of polyethylene, in combination with essential oil or carvone vapors, gave an indication of the in vivo efficacy of the

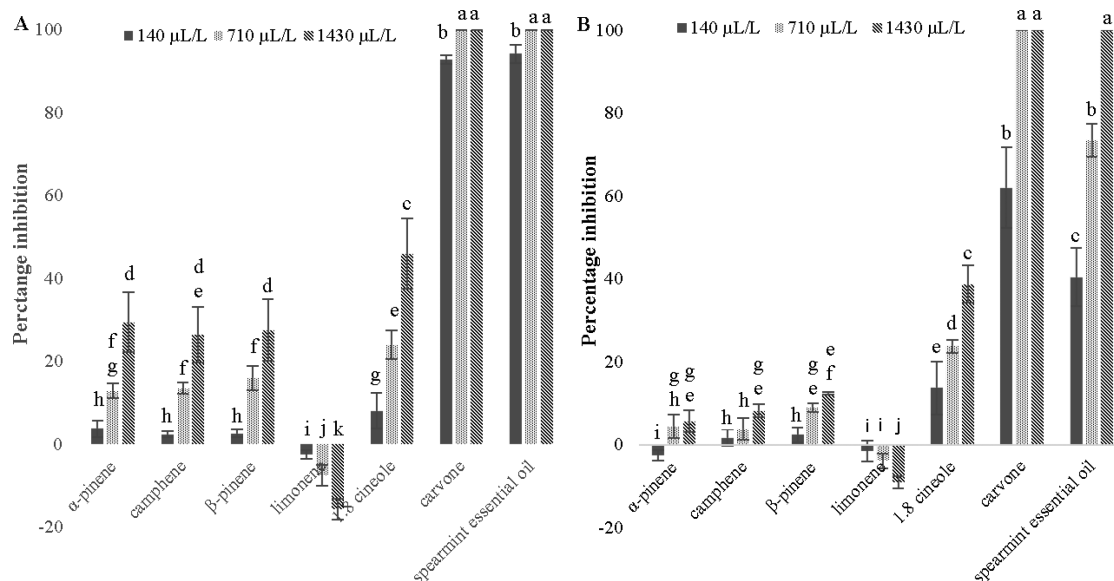


Figure 1. Effect of vapor phase application of different concentration spearmint oil and its major components on the mycelial growth, as determined using the volatile assay, where A = *Penicillium digitatum* and B = *Geotrichum citri-aurantii*. Colony radii were measured after 7 days at 25 °C, where $n = 5$, error bar indicates the standard deviation from the mean percentage inhibition. Means with at least one identical letter do not significantly differ at $p > 0.05$, according to Duncan's multiple range test.

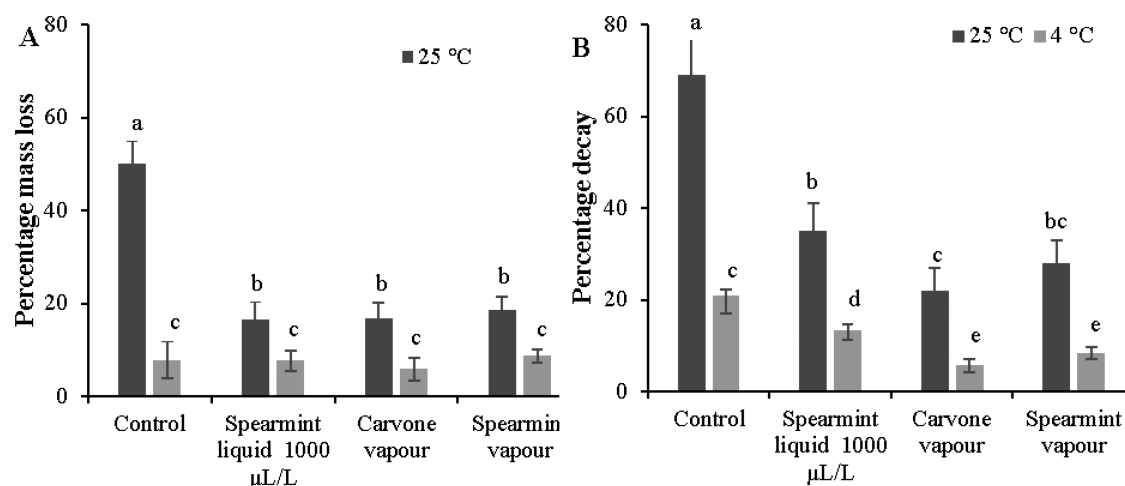


Figure 2. Effect of spearmint essential oil or carvone in the vapor or liquid phase on kumquats stored under typical packhouse and transportation conditions after a 21-day treatment period; the error bars represent the standard deviation around the mean percentage decay ($n = 10$ boxes of fruit), where A = % mass loss and B = % decay. Means with at least one identical letter do not significantly differ at $p > 0.05$, according to Duncan's multiple range test.

vapor applications, showing improved resistance of kumquats toward fungal attack as a result of fumigation using spearmint essential oil or carvone.

3.3. Vapor Composition of Spearmint Essential Oil.

3.3.1. Optimization of Equilibration Time for Carvone Content in Spearmint Essential Oil Vapor. Carvone was selected as the target compound, since it is the major component of spearmint essential oil and it is responsible for the antifungal activity of the essential oil. Prior to any headspace sampling, the optimum equilibration time for carvone present in the spearmint essential oil sample was determined using three headspace extraction techniques. The influence of the equilibration time on the determined carvone content when sampled using the different techniques is illustrated in Figure 3. The samples taken by SPME were analyzed only once to obtain an indication of the extraction

trend of the fibers for comparison purposes, since it will not be feasible to sample using the fibers in the packhouses. Headspace sampling using SPME fibers offers the advantage of sampling mobility, which is essential in this study, since sampling will be done in the packhouse. However, a single SPME fiber is needed for each sample, which will result in the sampling process being cost intensive. Results obtained in this study indicated that the optimum carvone equilibration time for direct headspace and Tenax TA sampling is 30 min, since no significant change in the carvone peak area was observed for both extraction methods after this period (Figure 3). Therefore, all the extractions performed using both direct headspace and Tenax TA were achieved following a 30 min sample equilibration at 25 °C. The concentration of the analyte present on a SPME fiber depends on a three-way equilibrium that exists between the sample, its vapors and the fiber.⁴⁷

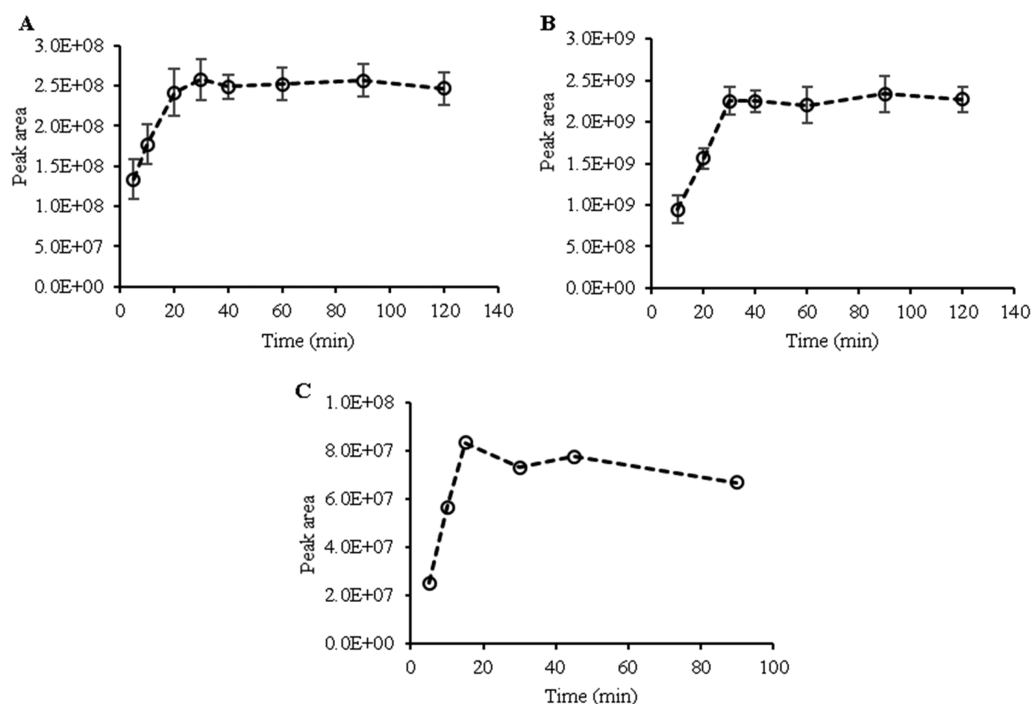


Figure 3. Peak area of carvone attained from a spearmint essential oil sample equilibrated at different time intervals at 25 °C, extracted using (a) Tenax TA ($n = 3$) and analyzed using thermal desorption GC-MS, (b) direct headspace ($n = 3$), and (c) PDMS SPME fibers, and analyzed using GC-ToF-MS

Therefore, the optimum equilibration time for the SPME was determined as the maximum fiber exposure time required for complete saturation of the fiber at 25 °C, following a 30 min equilibration time of the sample at the same temperature. The optimum fiber exposure time was found to be 15 min (Figure 3). Fluctuations in the carvone peak area was observed for exposure times longer than 15 min. A variation in the peak areas of specific volatile compounds of saturated alkenes when exposed to PDMS SPME fibers for different time periods at room temperature was also established.⁴⁸

As observed earlier, the composition of spearmint essential oil in the liquid phase mainly comprises monoterpenes and monoterpenoids, which are highly volatile compounds. Thus, the application of high temperatures is not necessary for the volatilization of these compounds. Larger standard deviations were observed for the Tenax TA sampling, as compared to direct headspace, which is an automated sampling technique. The manual sampling applied with Tenax TA tubes is more prone to uncertainties.

3.3.2. Qualitative Analysis of Spearmint Essential Oil Vapors. Ten compounds were identified using direct headspace extraction, while 12 compounds were identified when using PDMS SPME fiber and only eight compounds were extracted with Tenax TA tubes (Table 4). The most representative composition of the actual spearmint essential oil vapor is obtained using direct headspace analysis, because a portion of the total amount of the analyte is collected and analyzed, without the use of sorbents, which are usually more selective toward some compounds than to others.⁴⁹ Limonene was found to be the main constituent for both direct headspace and Tenax TA extractions. Unlike the latter two headspace extraction techniques, using SPME fibers resulted in a more selective adsorption of carvone, with a relative peak area of 57%.

Table 4. Spearmint Essential Oil Vapor Composition Sampled Using Direct Headspace, Tenax TA Tubes (Thermal Desorption), or Solid-Phase Microextraction Fibers (PDMS)

compound ^a	relative percentage peak area (%)		
	direct headspace	H/S-TD Tenax TA	H/S-SPME PDMS
α -pinene ^b	11	10	0.42
camphene ^b		0.081	0.013
<i>cis</i> -sabinene	0.96	8.8	0.36
β -pinene ^b	1.0	7.2	
β -myrcene		0.39	
α -phellandrene ^b			0.15
terpinolene			
limonene ^b	45	39	40
1,8-cineole ^b	7.1	6.9	0.22
terpineol	0.054		
3-octanol			0.43
menthol	0.15		0.69
dihydrocarvone ^b	0.62		
<i>p</i> -menthan-1-ol	0.99		
carvoel			0.34
carvone ^b	33	27	57
α -bourbonene			0.62
<i>cis</i> -verbenol			0.26

^aCompounds listed in order of elution from the column.

^bCompounds identified using authentic standards.

An increase in the α -pinene, limonene, and 1,8-cineole content was evident in the vapor composition sampled using direct headspace and Tenax TA. The gas phase concentration of any analyte depends on the partition coefficient (ratio of concentrations of a compound in a mixture of two immiscible solvents/phases at equilibrium), which is highly dependent on

boiling point. α -Pinene, limonene, and 1,8-cineole all have lower boiling points, thus they are more volatile compared to carvone (Table 4). It was, therefore, expected that the percentages of these compounds would increase in the vapor phase, as compared to what was observed in the liquid essential oil composition analysis. Similarly, the liquid and vapor phase composition of bergamot essential oil revealed a drastic decrease of the linalool and linalyl acetate vapor content, while the limonene and β -pinene content increased significantly as compared to what was observed in the essential oil composition.⁵⁰

Compounds, such as *l*-menthone, terpineol, dihydrocarvone, and *p*-menthan-1-ol, were solely detected from the direct headspace extraction. These compounds are some of the typical compounds found in spearmint essential oils.^{51,52} Following the distillation process, essential oil constituents are particularly prone to oxidative damage, chemical transformations, or polymerization reactions, with the aging processes resulting in the loss of quality.⁵³ Similar to the results obtained in this study, a significant difference between headspace compositions of *Lippia alba* essential oil volatiles were obtained using direct headspace, dynamic headspace and SPME extraction.⁵⁴ Solid-phase microextraction fibers have a number of binding sites with special affinities to certain compounds.⁵⁵ Sample throughput is also reduced when using SPME, due to the equilibration time. Two separate equilibration times are required; the first being the sample equilibration, which is then followed by equilibration of the headspace with the fiber. The use of SPME fibers for packhouse vapor sampling would be impractical, since there would be a large number of samples. Therefore, the use of the SPME fiber in this study was to give an indication of the extraction trend of the fiber and for comparative purposes.

A strong correlation exists between the major components of an essential oil and its antimicrobial property, the volatility of these component will, thus, affect the activity of the essential oil when applied in the vapor phase.⁵⁶ A lower relative carvone content is present in the spearmint essential oil vapor phase as compared to the essential oil. Thus, it is likely that the activity of the carvone is modified by other minor components, resulting in the observed strong inhibitory effect of spearmint essential oil vapor, regardless of the lower carvone and higher limonene content in the essential oil vapor. Studies done on the antibacterial activities of 14 essential oils applied in the gaseous phase indicated that the minimum inhibition dose values were highly dependent on the evaporation rate of the essential oil.⁵⁷ Similarly, the antimicrobial activity of *Eucalyptus globulus* increased due to the increase of the monoterpene hydrocarbon content in the vapor phase as compared to the liquid phase.⁵⁸ Therefore, the presence of compounds with low volatility might result in a moderate to low antimicrobial activity. The effective quantification of carvone present in spearmint oil was achieved using Tenax TA extraction. These sampling tubes can be effectively used for the quantification of the carvone vapor content present in spearmint essential oil, allowing for direct monitoring of the terpenoid when it is applied in biological tests and in packhouses. Further studies should be aimed at finding efficient vapor release material to ensure controlled and sustained release of the active compounds/essential oils.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank NRF (grant number: 111201) and the Postharvest Innovation Fund (PHI), a public-private partnership between the Department of Science and Innovation (DSI), and the Fresh Produce Exporters' Forum (FPEF) of South Africa, for funding this project. The authors would also like to thank farmers, Steven Beghin and Sean, who supplied the kumquat.

ADDITIONAL NOTE

^a<https://www.freshplaza.com/article/2196709/south-africa-2018-kumquat-season-gets-underway/>

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