

In-vitro effects of garlic extracts on pathogenic fungi *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*

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The antifungal activity of garlic extracts applied directly and through volatile release was tested against the growth of postharvest pathogens *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*. Mycelial growth of *B. cinerea* and *P. expansum* was inhibited by aqueous and ethanol dilutions on garlic extract amended media (direct method) in a dose-response manner. The aqueous dilution was more effective than the ethanol dilution. Both dilutions inhibited mycelial growth of *N. alba* to a similar extent but no trend in data was noted across the concentration range. Calculated EC₅₀ values indicated that 13.36% and 8.09% aqueous dilutions could be used to inhibit growth of *B. cinerea* and *P. expansum*, respectively; however, values generated for *N. alba* either bordered on or exceeded the concentration range. The volatile vapour application of garlic was able to inhibit mycelial growth and spore germination of all pathogens at concentrations as low as 20%. Gas chromatography–mass spectrometry analysis showed that 85.95% of compounds present in the garlic sample belonged to a sulphur or sulphur-derived group. Allicin, the active component of garlic, was not found; however, breakdown products of allicin were present in high amounts. Overall, the antifungal activity of garlic extracts for the control of *B. cinerea* and *P. expansum* was confirmed. Further investigations into the antifungal effect of garlic extracts on *N. alba* is required, although garlic volatiles seem to be effective. This report is the first of antifungal activity of garlic extracts against *N. alba* – the causal agent of bull's eye rot, one of the major diseases of apples.

Introduction

Several pathogenic fungi, such as *Botrytis cinerea* Pers., *Penicillium expansum* (Link) Thom. and *Neofabraea alba* (E.J. Guthrie), are major infectious agents of apples, especially in the postharvest stage. Pathogenic fungi are controlled primarily through the use of synthetic fungicides; however, restrictions are being placed on the use of chemicals because of the perceived negative effects that pesticides may have on human health and the environment. Increasing regulations on the use of synthetic fungicides, build-up of chemical residues on the fruit and the emergence of pathogen resistance to the most frequently used fungicides, validate the search for novel biological control strategies.¹

In recent years, a number of plant extracts, their essential oils and their volatile components have been reported to have strong antifungal activity.² In the agricultural sector, plant extracts, essential oils and their components are gaining increasing interest as a result of their volatility, reasonably safe status, eco-friendly and biodegradable properties, and wide consumer acceptance.³ Some extracts and essential oils of 'medicinal' plants have been found to be effective against fungal and bacterial pathogens.⁴ The fungicidal activity of essential oils from citrus, eucalyptus and thymus has been demonstrated. For example, in-vitro studies have shown that the oil of eucalyptus inhibits mycelial growth of important soilborne and postharvest disease pathogens such as *Pythium* spp., *Rhizoctonia solani*^{5,6} and *Collectotrichum gloeosporioides*⁶. Nosrati et al.⁷ proposed that spearmint essential oil could be used in the control and management of *Fusarium oxysporum* f. sp. *radicis-cucumerinum* which is the causal organism of stem- and crown rot of greenhouse cucumber. Pawar and Thaker⁸ found that the essential oils of lemongrass, clove, cinnamon bark, cinnamon leaf, cassia, fennel, basil and evening primrose had an antifungal effect on *Alternaria porri* and *Fusarium oxysporum* f. sp. *cicer*.

Garlic (*Allium sativum* L.) has been used for centuries for culinary purposes and its medicinal properties in traditional and conventional medicine are well documented.^{9,10} The wide range of antifungal and antibacterial activities of garlic has been largely attributed to the presence of high concentrations of sulphur-containing compounds.^{9,11}

Cavallito and Bailey¹² were responsible for the discovery of an oxygenated sulphur compound called allicin (diallyl thiosulphinat), which they considered to be responsible for the aroma and flavour of garlic. Since then, several researchers have attributed the antimicrobial action of garlic to allicin, which is present as the main active component.¹¹⁻¹⁴ The formation of allicin is followed by its rapid decomposition into sulphur-derived compounds such as diallyl disulphide, diallyl sulphide, diallyl trisulphide, sulphur dioxide, allyl propyl disulphide and diallyl tetrasulphide.^{15,16} This fact has led some researchers to suspect that the antimicrobial activity may be a result of the action of a combination of sulphur and sulphur-related compounds.^{14,17}

The antifungal effect of garlic on plant pathogens has been shown by Russel and Mussa¹⁸ for the control of *Fusarium oxysporum* f.sp. *phaseoli*. Investigations have also shown inhibitory effects of garlic against *Penicillium digitatum*.^{19,20} Whilst many studies have highlighted the antimicrobial action of garlic on pathogens, little research has been done relating to postharvest plant pathogens,¹⁹ and specifically the postharvest pathogens of apples.

In light of the above, in the present study we aimed to evaluate the antifungal efficacy of garlic extracts on the in-vitro mycelial growth and conidial germination of *B. cinerea*, *P. expansum* and *N. alba*. Gas chromatography–mass spectrometry (GC-MS) was employed to attain information on the chemical constituents of the garlic sample, in an effort to highlight the compounds potentially involved in the antimicrobial effect of garlic on pathogens.

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Materials and methods

Pathogen isolation

Three pathogens – *B. cinerea* (B62-SUN), isolated from pears, and *P. expansum* (P1110-SUN) and *N. alba* (DOK7-SUN), both isolated from infected apples – were used. All three pathogens were obtained from the fungal collection of Stellenbosch University's Department of Plant Pathology. All the isolates were tested for pathogenicity on apples and pure isolates were prepared. Both *B. cinerea* and *P. expansum* were cultured on potato dextrose agar (PDA, pH 5.6, Merck, Johannesburg, South Africa) at 25 °C for 3 days for mycelial plugs and 7 days for the production of conidia. *Neofabraea alba* was cultured on acidified PDA (pH 3.5, Merck, Johannesburg, South Africa) for 1 month at 25 °C. Conidia were harvested by flooding the medium surface with sterile distilled water together with Tween 80 (0.05% w/v), and gently agitating the plate to dislodge spores. The final inoculum concentration was adjusted to 10⁴ conidia/mL for each pathogen.

Preparation of garlic extract

Fresh garlic (*Allium sativum* L.) cloves were purchased from a retail store (Woolworths, Stellenbosch, South Africa). The garlic cloves were peeled and surface sterilised using ethanol (99.9% v/v). The garlic cloves were allowed to air dry before 800 g was weighed out and crushed in a blender. Ethanol (1 L) was added to the crushed garlic and the mixture was then placed into a glass container and incubated overnight at room temperature (20–25 °C). The extract was then filtered through a Büchner funnel using Whatman qualitative filter paper (No. 4). The filtrate was then subjected to a rotary evaporator (at 60–80 °C) to remove the ethanol. The filtrate was evaporated down to a final volume of 150 mL, yielding an extract with a semisolid consistency. This extract was considered to be the 100% concentrate and stored at 4 °C until subsequent use. The 100% extract was diluted down to make up the required concentrations used for efficacy testing.

Effect of garlic extract on mycelial growth

The effect of garlic extract on mycelial growth of *B. cinerea*, *P. expansum* and *N. alba* was determined following the poisoned food technique of Shahi et al.²¹, with slight modification. A concentration range (80–2.5% w/v) of garlic extract was prepared by adding the requisite amount of the extract to ethanol or sterile distilled water up to a volume of 2.8 mL, which was then added to 140 mL of PDA medium (pH 5.6), and 20-mL aliquots of the amended PDA were poured into 90-mm Petri plates. Control sets consisted of unamended PDA.

Mycelial discs of 3 mm diameter cut out from the periphery of 3-day-old cultures (*B. cinerea* and *P. expansum*) and 7-day-old culture (*N. alba*) were aseptically transferred, mycelium side down, onto the surface of the agar. Petri plates were incubated at 25 °C for 3 days for *B. cinerea* and *P. expansum* and 7–21 days for *N. alba*. Radial mycelial growth was measured using digital calipers. Percentage of mycelial growth inhibition (MGI) was calculated as follows: $MGI (\%) = (dc - dt) \times 100 / dc$, where *dc* is the mycelial growth diameter in control sets and *dt* is the mycelial growth diameter in treatment sets.

The nature of antifungal activity – fungistatic (temporary inhibition) or fungicidal (permanent inhibition) – of the garlic extract was determined by transferring the inhibited fungal discs from the above-mentioned method onto unamended PDA and observing growth. Three replicates were used for each of the three pathogens and for each concentration tested and the whole experiment was repeated once.

Effect of garlic extract on conidial germination

To determine the effect of garlic extract on conidial germination of *B. cinerea* and *P. expansum*, 100 µL of fungal conidia suspensions (10⁴ conidia/mL) were pipetted onto the centre of garlic-amended PDA plates. Inoculated plates were incubated at 25 °C for 3 days. The control plates consisted of the pathogen on unamended PDA. Three replicates were used for each pathogen for each concentration. Plates were evaluated for germination (+) and non-germination (-) of conidia. The experiment was repeated once.

Effect of garlic volatiles on mycelial growth and conidial germination

A phytatray chamber assay was used to determine the effect of the volatile vapour of garlic extracts on all three pathogens in vitro. A glass Petri dish containing 5 mL of garlic extract, diluted with water to concentrations of 0%, 20%, 30% or 40% (wt/v), was fixed to the base of a disposable phytatray (Zibo, Cape Town, South Africa). Sterilised distilled water was used as the control. Four 65-mm PDA Petri plates inoculated with the respective fungi were fixed to the sides of the phytatray. Each chamber contained two plates inoculated with 3-mm mycelial plugs cut from the leading edge of an actively growing culture and placed mycelial side down onto the PDA, as well as two plates inoculated with 100 µL of a 10⁴ conidia/mL conidial suspension by means of a spread plate method. The lid was closed and the chamber was then incubated at 20 °C (at 95% RH) and -0.5 °C (at 95% RH) for a total of 3 days for *B. cinerea* and *P. expansum* and 7 days for *N. alba*, before evaluation. Plates incubated at -0.5 °C were further incubated at 20 °C for 3 (*B. cinerea* and *P. expansum*) or 7 (*N. alba*) days. A total of three replicates with five phytatray chambers was used for each concentration. Plates inoculated with mycelial plugs were evaluated by measuring mycelial growth of the fungi using digital calipers and mycelial inhibition was calculated as described previously. Fungal spore plates were evaluated for germination (+) and non-germination (-) and subsequently converted to a percentage for statistical analysis.

GC-MS analysis of garlic extract

Approximately 1 mL of garlic crude extract was transferred to 20-mL solid phase microextraction vials for analysis. The vials were allowed to equilibrate for 2 min in the heating chamber of the CTC autosampler maintained at 30 °C. The volatile compounds were extracted by exposure of a 50/30 m divinylbenzene-carboxen-polydimethylsiloxane coated fibre (Supelco™, Port Edward, South Africa) on the headspace of the samples. Following extraction, desorption of the volatile compounds from the fibre coating was carried out for 10 min in the injection port of the GC-MS operated in splitless mode. The temperature of the injection port was maintained at 240 °C. Separation of the volatile compounds was performed on an Agilent 6890 N (Agilent, Palo Alto, CA, USA) gas chromatograph coupled with an Agilent 5975 MS (Agilent, Palo Alto, CA, USA) mass selective detector. Chromatographic separation was performed on a DB-FFAP (60-m length, 250-µm inner diameter and 0.5-µm film thickness) capillary column from Agilent technologies. Analyses were carried out using helium as a carrier gas with a flow rate of 1.9 mL/min operated in constant flow mode. The injector temperature was maintained at 240 °C.

The oven temperature was as follows: 70 °C for 1 min and then ramped up to 225 °C at 5 °C/min and held for 3 min. The mass selective detector was operated in full-scan mode and the ion source and quadropole were maintained at 230 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C and total run time was approximately 46 min. Authentic standards were unavailable so compounds were tentatively identified by comparison with mass spectral libraries (NIST05 and Wiley 275.L). For quantification, the automatically calculated relative abundances were used and are expressed as a percentage. The sample was run twice, each time with three replicates.

Statistical analysis

In all cases, the experimental design was completely randomised. Conidial germination data were binary (present or absent), summed across the Petri dishes and expressed as a percentage. Mycelial growth was measured as a diameter (mm) and converted to percentage inhibition, which was analysed by an appropriate analysis of variance (ANOVA). The treatment means were compared using a Student's *t*-test with least significant difference at 5% ($p=0.05$).²² A logarithmic growth curve was fitted to the concentration range to calculate the concentration at which 50% inhibition was achieved (EC₅₀ values). The percentage inhibition and EC₅₀ values were submitted to an appropriate ANOVA to compare treatments. Analysis was performed using SAS version 9.2 statistical

software.²³ All three pathogens were analysed separately and no comparison was made between them.

Results

Effect of garlic extract on mycelial growth

A clear dose-response effect was obtained. The aqueous (sterile distilled water) and ethanol diluted extracts showed complete inhibition (100%) of *B. cinerea* at the higher concentrations (80% and 60%), with a fungicidal effect noted at both concentrations (Table 1) for both diluents tested. Aqueous diluted extract at a concentration of 40% showed 92.08% inhibition of *B. cinerea*. At a concentration of 80%, the aqueous and ethanol diluted extracts inhibited *P. expansum* by 96.21% and 99.21%, respectively. Ethanol diluted extracts seemed to be more effective against *N. alba* with 80% extract showing 79.63% inhibition. Overall, comparison between the diluents used indicated that the aqueous diluted extract provides significantly better results than the ethanol diluted extract (Table 1).

The effective concentrations at which 50% pathogen inhibition (EC_{50}) resulted from the use of garlic extracts were calculated. *B. cinerea* could be controlled using 20.59% of an aqueous diluted extract or 13.36% of an ethanol diluted extract. For *P. expansum*, a 19.95% ethanol diluted extract or 8.09% aqueous diluted extract could be used to retard pathogen growth. For *N. alba*, results indicated that 50% control could not be achieved unless the extract was at a concentration of no less than 79.51% (Table 2).

Effect of garlic extracts on conidial germination

Aqueous and ethanol diluted extracts were tested for their antifungal activity against conidial viability of the pathogens *B. cinerea* and

P. expansum at concentration ranges from 0% to 80%. *B. cinerea* and *P. expansum* germinated when exposed to concentrations of 0–10% aqueous diluted extract (Table 3); however, germination of both pathogens was completely inhibited at the higher concentration ranges of 20–80% aqueous extract. For both pathogens exposed to the ethanol diluted extracts, conidial germination was completely inhibited at all concentrations except the lowest concentration of 2.5% (Table 3).

Effect of garlic volatiles on mycelial growth and conidial germination

The volatile vapours of aqueous diluted extracts were strongly active against mycelial growth of all the fungi, especially at concentrations of 30% and 40%. In all cases it was noted that the percentage mycelial inhibition increased with an increase in garlic concentration (Table 4).

Conidial germination of all fungi tested was almost completely inhibited by volatiles of garlic extracts (Table 4), irrespective of the concentration of the extract.

Mycelial growth for plates incubated at -0.5 °C showed complete inhibition against all pathogens tested, irrespective of the garlic extract concentration (Table 5), in comparison with the control, because of the low incubation temperature.

When the phytatrays from -0.5 °C were incubated further at 20 °C, concentrations of 20–40% were strongly active against mycelial growth of all fungi tested. The combination of garlic volatiles with low temperature (-0.5 °C) resulted in a stronger antifungal activity, especially against *P. expansum* and *N. alba*, than that exhibited for phytatrays that were only incubated at 20 °C, as is suggested by the difference between control and treatment sets.

Table 1: Inhibitory effect of aqueous (sterile distilled water) and ethanol diluted garlic extracts on mycelial growth of *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*

Garlic extracts	Concentrations (% w/v)	Inhibition (%)		
		<i>B. cinerea</i>	<i>P. expansum</i>	<i>N. alba</i>
Aqueous extracts	0	0.00 ^a	0.00 ^h	0.00 ^e
	2.5	11.50 ^e	28.82 ^f	5.95 ^{de}
	5	12.91 ^e	29.48 ^f	6.03 ^{de}
	10	36.36 ^d	60.03 ^d	30.08 ^{bc}
	20	52.05 ^c	70.60 ^c	6.43 ^{de}
	40	92.08 ^a	83.82 ^b	34.29 ^{bc}
	60	100.0 ^{af}	95.68 ^a	40.15 ^b
	80	100.0 ^{af}	96.21 ^a	25.05 ^{bc}
Ethanol extracts	0	0.00 ^a	0.00 ^h	0.00 ^e
	2.5	0.86 ^a	5.10 ^a	21.53 ^{cd}
	5	0.44 ^a	11.59 ^a	1.94 ^e
	10	1.62 ^a	10.92 ^a	4.31 ^e
	20	42.17 ^c	41.17 ^e	6.68 ^{de}
	40	69.20 ^b	72.88 ^c	7.26 ^{de}
	60	100.0 ^{af}	85.40 ^b	39.26 ^b
	80	100.0 ^{af}	99.21 ^a	79.63 ^a

Values represent means of measurements made on three independent plates per treatment. In each column, values followed by the same letter do not differ significantly ($p < 0.05$), as determined by a Student's *t*-test.

^fIndicates fungicidal effect (permanent inhibition).

Table 2: The effective concentrations of aqueous and ethanol diluted garlic extracts that resulted in 50% inhibition (EC_{50}) of mycelial growth of *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba* in vitro

Pathogen	EC_{50} value†	
	Aqueous extract	Ethanol extract
<i>Botrytis cinerea</i>	13.36 ^a	20.59 ^b
<i>Penicillium expansum</i>	8.09 ^a	19.95 ^b
<i>Neofabraea alba</i>	81.39 ^b	79.51 ^a

† EC_{50} values were determined on garlic amended potato dextrose agar growth medium. Values represent means of measurements made on three plates per pathogen. Mean values followed by the same letter(s) represent data that are not significantly different ($p < 0.05$), as determined by a Student's t-test.

Table 3: Inhibitory effects of aqueous and ethanol diluted garlic extracts against conidial germination of *Botrytis cinerea* and *Penicillium expansum*

Concentrations (%)	Inhibitory effect of garlic extracts			
	<i>Botrytis cinerea</i>		<i>Penicillium expansum</i>	
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
0	+	+	+	+
2.5	+	+	+	+
5	+	-	+	-
10	+	-	+	-
20	-	-	-	-
40	-	-	-	-
60	-	-	-	-
80	-	-	-	-

Plates were evaluated for germination (+) and non-germination (-) of conidia. The experiment was repeated once.

Table 4: Inhibitory volatile action of aqueous extracts against mycelial growth and conidial germination of *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba* in phytatrays incubated at 20 °C

Concentration (%)	Inhibition (%)					
	Mycelial growth			Conidial germination		
	<i>B. cinerea</i>	<i>P. expansum</i>	<i>N. alba</i>	<i>B. cinerea</i>	<i>P. expansum</i>	<i>N. alba</i>
0	0.00 ^e	0.00 ^f	0.00 ^d	0.00 ^c	0.00 ^b	0.00 ^b
20	61.55 ^b	75.41 ^d	100.0 ^a	100.0 ^a	96.67 ^a	100.0 ^a
30	97.14 ^a	89.60 ^b	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a
40	99.35 ^a	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a

Values followed by the same letter, down the column, do not differ significantly ($p < 0.05$) according to a Student's t-test. The pathogens were not compared against each other (i.e. across rows).

Similar results were observed for conidial germination trays incubated at -0.5 °C (data not presented). The data showed that conidial germination of all three fungi was completely inhibited at -0.5 °C by concentrations of 20–40% when plates were further incubated. This outcome is in agreement with the results obtained for mycelial growth.

GC-MS analysis of garlic extract

From the GC-MS analysis of crude garlic extract, 43 volatile compounds were detected. Of this 43, 25 compounds were identified to be sulphur or sulphur-derived compounds, 2 compounds belonged to the alcohol group and one compound was an ester; the remaining compounds were not identified.

Sulphur and sulphur-derived compounds made up 85.95% of the entire sample concentration. The relative abundances (%) of all sulphur compounds identified in this study are presented in Table 6. Allyl methyl sulphide (7.93%), allyl methyl disulphide (7.86%), allyl methyl trisulphide (13.85%), diallyl disulphide (24.10%) and dimethyl trisulphide (11.36%) were present in the highest percentages within the sample. The chromatograph (Figure 1) highlights compound abundance

relative to the retention time, in correspondence with samples listed in Table 6. The percentage relative abundances (%) presented in this study were calculated automatically using the peaks obtained from the chromatograph. Similar results were obtained for the second sample run, with the exception of the detection of three additional compounds: 3-vinyl-1,2-dithiacyclohex-4-ene, 3-vinyl-1,2-dithiacyclohex-5-ene and 1-oxa-4,6-diazacyclooctane-5-thione.

Discussion

Garlic extracts had a significant effect on the growth of the pathogens tested in this study. This finding is in agreement with earlier reports on the antifungal properties of garlic. The effect of garlic extracts on postharvest pathogens was determined by direct exposure as well as through volatile action.

Studies have shown that the method by which a plant extract is prepared will influence the type of activity (antifungal or other) it has.^{24,25} Different extraction and dilution solvents will affect the extraction of different chemical compounds and the physiological properties within a plant, and therefore different extracts may contain different compounds, or the same compounds in varying quantities.^{26,27}

Table 5: Inhibitory volatile action of aqueous diluted garlic extracts against mycelial growth of *Botrytis cinerea*, *Penicillium expansum* and *Neofabraea alba*, incubated at -0.5 °C with further incubation at 20 °C

Concentration (%)	Inhibition (%)					
	<i>B. cinerea</i> [†]		<i>P. expansum</i> [†]		<i>N. alba</i> [†]	
	-0.5 °C	20 °C	-0.5 °C	20 °C	-0.5 °C	20 °C
0	0.00 ^e	0.00 ^e	0.00 ^e	0.00 ^e	100.0 ^a	0.00 ^e
20	100.0 ^a	69.41 ^b	100.0 ^a	97.76 ^{bc}	100.0 ^a	100.0 ^a
30	100.0 ^a	97.62 ^a	100.0 ^a	99.14 ^{ab}	100.0 ^a	100.0 ^a
40	100.0 ^a	97.12 ^a	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a

[†]Trays incubated at respective temperatures for 3 days.

[‡]Trays incubated at respective temperatures for 7 days.

Values followed by the same letter, down the column, do not differ significantly ($p < 0.05$), as determined by a Student's t-test. The pathogens were not compared against each other (i.e. across rows).

Table 6: Percentage composition of sulphur and sulphur-derived compounds in garlic extract

Retention time (min)	Name of compound [†]	Molecular formula	Molecular weight (g/mol)	Percentage peak [‡] (%)
3.36	Methanethiol	CH ₄ S	48.10	0.96
7.20	Dimethyl disulphide	C ₂ H ₆ S ₂	94.18	3.16
9.05	Allyl methyl sulphide	C ₄ H ₈ S	88.15	7.93
13.44	Allyl methyl disulphide	C ₄ H ₈ S ₂	120.21	11.23
17.45	Dimethyl trisulphide	C ₂ H ₆ S ₃	126.24	11.36
18.82	Allyl propyl disulphide	C ₆ H ₁₂ S ₂	148.26	0.06
21.14	Diallyl disulphide	C ₆ H ₁₀ S ₂	146.25	24.10
25.63	Allyl methyl trisulphide	C ₄ H ₈ S ₃	152.27	13.85
30.49	3,4-Dihydro-3-vinyl-1,2-dithiin	C ₆ H ₈ S ₂	144.23	4.66
31.87	Diallyl trisulphide	C ₆ H ₁₀ S ₃	178.31	5.07

[†]Compound identification based on mass spectrum and retention index matching reference samples from the mass spectral libraries (NIST05 and Wiley 275.L).

[‡]Percentage reflected is the average of triplicate analysis expressed as the relative percentage of analyte in the garlic sample.

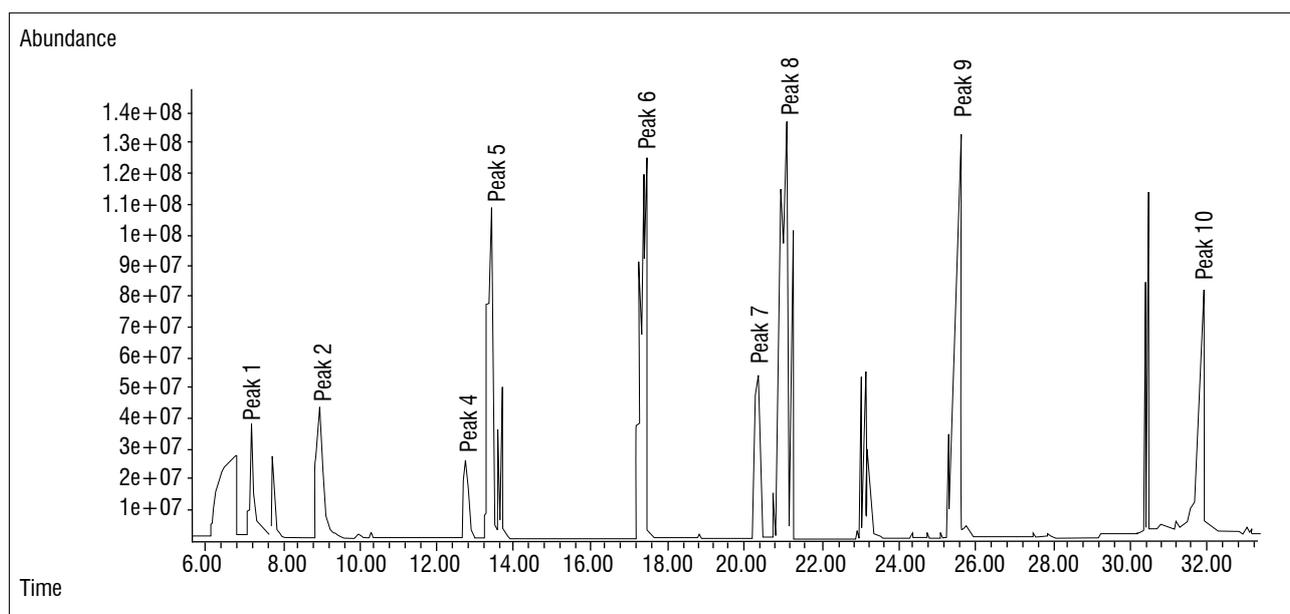


Figure 1: Gas chromatography–mass spectrometry (full scan) chromatogram showing the peaks corresponding to compounds present in the garlic extract in relation to retention time: peak 1, dimethyl disulphide; peak 2, diallyl sulphide; peaks 3 and 4, allyl methyl sulphide; peak 5, allyl methyl disulphide; peak 6, dimethyl trisulphide; peak 7, 1-oxa-4,6-diazacyclooctane-5-thione; peak 8, diallyl disulphide; peak 9, allyl methyl trisulphide; peak 10, diallyl trisulphide.

Aqueous dilutions of the garlic extract showed better activity than the ethanol dilutions of the extract. Previous studies both support²⁸ and contradict²⁹ this finding; however, all studies do support the fact that aqueous extract preparations do result in antimicrobial activity. When comparing aqueous preparations of extracts to preparations using other organic solvents tested against *B. cinerea*, a parallel may be drawn between results from the present study and those achieved by Senhaji et al.²⁷, in that the present study revealed that complete (100%) mycelial inhibition could be achieved by using a 60% aqueous diluted extract (Table 1). Timothy et al.³⁰ noticed a dose-dependent antifungal activity of leaf extracts of *Cassia alata* when tested against clinical isolates of pathogenic fungi, including a species of *Penicillium*. The same trend was noted in this study, with *B. cinerea* and *P. expansum* each eliciting a decrease in colony diameter with increasing concentration of garlic extract.

For the pathogen *N. alba*, percentage inhibition of mycelial growth was significantly different across the concentration range for both extract dilutions tested. The ethanol dilutions were more effective at reducing mycelial growth of the pathogen; however, results pertaining to the antifungal effect of garlic extracts on *N. alba* were inconclusive.

Plant extracts of *Allium* and *Capsicum* have been shown to completely inhibit spore germination of *B. cinerea*.³¹ When garlic extracts were tested in this instance to determine the effect on conidial viability of *B. cinerea* and *P. expansum*, across the chosen concentration range, both pathogens behaved in an identical manner with regard to exposure to the aqueous and ethanol diluted extracts. Exposure to the ethanol extract yielded a greater inhibitory effect on pathogen conidial viability.

In recent years, studies have been carried out concerning the application of essential oils as antimicrobial agents,³² with the majority of reports focused on the antifungal activity of essential oils and plant extracts exposed directly to fungus. However, few studies concerned the antifungal activity of volatiles of plant essential oils and extracts.³³

An investigation into the effectiveness of garlic extracts for the control of pathogenic bacteria and fungi has demonstrated that allicin (the putative active ingredient of garlic) supplied via the vapour phase was effective in reducing *Phytophthora infestans* in vitro.³⁴ In the present in-vitro study, the volatiles released from extracts were effective in limiting mycelial growth and conidial germination of all the pathogens tested.

The individual pathogens responded variably to the garlic extracts, with *N. alba* being the most sensitive to the volatile vapours.

At 20 °C, conidial germination and mycelial growth of all three pathogens were effectively inhibited by garlic volatiles across the concentration range, with pathogen inhibition increasing as the concentration of garlic extract volatiles increased (Table 4). Volatile substances released from essential oils derived from *Ocimum sanctum*, *Prunus persica* and *Zingiber officinale* were reported to have a similar effect on the control of *B. cinerea* on grapes.³⁵ A recent study showed that the vapours of thyme, peppermint and citronella oils caused a gradual inhibition of the growth of *P. expansum* and other postharvest pathogens.³⁶

When the pathogens were incubated at -0.5 °C, the low temperature affected their growth, as indicated by the reduced growth of the control sets of *B. cinerea* and *P. expansum*. However, for both these pathogens, complete inhibition (100%) was noted on all plates exposed to the garlic treatments. The further incubation at 20 °C indicated that mycelial growth was inhibited, while conidial germination remained 100% inhibited (data not presented) for all three pathogens even at 20 °C. This finding suggests a synergistic relationship between the low temperature and the garlic extracts. Under standard conditions, incubation at -0.5 °C is expected to suppress growth of pathogens; however, the garlic extracts enhanced pathogen inhibition and allowed for added control of pathogens, as can be seen when comparing the control sets to the treated sets (Table 5).

No comparison was made between the pathogens in this study but the results indicate that while all three pathogens were sensitive to the garlic extracts, each individual pathogen reacted differently to the extracts. When exposed to garlic volatiles, *N. alba* was most sensitive to the extracts at both temperatures tested, followed by *P. expansum* and *B. cinerea*.

GC-MS has been previously employed to profile chemical compounds in garlic and other plant essential oils. Available literature on garlic composition reveals compounds common across the various studies, but also isolated detection of compounds, suggesting that even though this method is quite sensitive to its purpose, variation in compound detection will occur between sample sets. This variation is probably for a variety of reasons which include the type of analysis carried out, the conditions surrounding the study and also the garlic sample itself – with sample preparation and cultivar type also playing a role in the compounds that would be amplified.¹⁰

Over 35 different compounds have been identified in garlic,¹⁰ with the sulphur-containing compounds the main focus of research studies conducted on garlic and related species. The full profile analysis in this study rendered a total of 43 compounds highlighted within the garlic sample and further investigation found that the total amount of sulphur-containing compounds made up approximately 85.95% of the sample tested. Allicin (diallyl thiosulphinate) could not be directly detected in this study; however, it has been reported that allicin decomposes to diallyl disulphide, diallyl trisulphide and sulphur dioxide¹⁵ and these compounds, together with other volatiles typically present in crushed garlic, were found in relatively high amounts in this study. Furthermore, exposure time between extract preparation and analysis could be integral to detecting allicin, because of its rapid decay rate.

The major sulphides that have been identified in garlic include diallyl sulphide, allylmethyl, dimethyl- and mono-tohexasulphides together with small amounts of allyl 1-propyl and methyl 1-propyl, and di-, tri- and tetrasulphides,¹⁵ although different studies^{9,10,37} including the present study, have reported different amounts of these compounds. Khadri et al.⁹ reported that the two major compounds present in a garlic sample tested were methyl allyl trisulphide (34.61%) and diallyl disulphide (31.65%). Both of these compounds were found in the sample tested in this study, but at lower concentrations of 13.85% and 24.10%, respectively. According to the authors, no other reports of allyl methyl trisulphide had been made previously and they concluded that the cultivar used represented a new chemotype typical of eastern Algeria⁹; however, this cannot be the case as the garlic used in the present study was not sourced from that geographical region. The compounds 3-vinyl-1,2-dithiacyclohex-4-ene and 3-vinyl-1,2-dithiacyclohex-5-ene have been reported as the compounds responsible for allinase activity.³⁸ Another compound detected, which is worth mentioning, is 1-oxa-4,6-diazacyclooctane-5-thione, which was found to be present in 'rosy garlic' (*Allium roseum* L.) and was reported in the study to not have been recorded in the literature.³⁹

As a result of various compounds highlighted in different studies on components of garlic, Amagase¹⁵ speculated that, while garlic is recognised for the abundance of sulphur compounds present, perhaps compounds other than allicin could contribute to the various antimicrobial activities. The present study supports this hypothesis as allicin was not found in the sample tested; however, other sulphur and sulphur-derived compounds were found in high amounts. The possibility exists that a complex of compounds, rather than one individual compound, is responsible for the antifungal activity noted by garlic samples throughout this study. It is recommended that if individual compounds can be sourced then each individual compound should be subjected to an antimicrobial screening to determine whether or not it makes any contribution to the antimicrobial action of garlic extracts.

In conclusion, we have shown that garlic extracts can have a significant effect on preventing the growth of *B. cinerea* and *P. expansum*. However, growth of *N. alba* was not significantly suppressed by the garlic. The solvent used for dilution concentrations (water or ethanol) had an influence on the antifungal activity of the garlic extract. Aqueous dilutions of the extract had greater antifungal activity than ethanol diluted extracts, possibly because the longer the extracts were exposed to ethanol, the more the antifungal activity was reduced.

When tested in the vapour phase, garlic extracts were able to control growth of *B. cinerea*, *P. expansum* and *N. alba*. Our findings confirm those of fellow researchers who stated that application in the vapour phase is preferred because of increased volatile activity and the ability to use lower concentrations.⁴⁰ In the present study, concentrations used in the volatile experiment were at a lower garlic concentration than the amended media experiments. Furthermore, increased antifungal activity was noted. This finding is significant as it gives a possible lead into using a garlic preparation as a fumigant to control pathogens that may be present in the air and on regular surfaces in a pack house or containers. Also, where garlic extracts are combined with effective storage conditions, this application could be adopted into a closed packaging system.

In conclusion, volatile vapour of garlic extracts showed more potent antifungal activity against conidial germination than against mycelial growth of the test fungi. Volatile vapours of garlic extracts were more effective than the direct method, as efficacy in volatile assays was at concentrations of 20–40% compared with concentrations of 60–80% for the direct method.

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Authors' contributions

C.K.D. is a master's student and performed all the laboratory experiments and wrote the manuscript; C.L. supervised the study; and F.V. initiated the project and supervised the project at the Agricultural Research Council where most of the experiments were performed.

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