Journal of Plant Sciences
2024, Vol. 12, No. 2, pp. 55-63
https://doi.org/10.11648/j.jps.20241202.11

Research Article

In vitro and *in situ* Activity of *Cymbopogon citratus* Essential Oil Against *Alternaria alternata* and *Phomopsis carica-papayae*, Causal Agents of Papaya Leaf Diseases

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Abstract

Papaya cultivation is considered to be one of the most important fruit-growing activities in Cameroon, and plays a vital role in improving the livelihoods of local producers. However, leaf diseases caused by devastating fungi are a real obstacle to the development of this crop. The aim of this study was to evaluate the antifungal potential of *Cymbopogon citratus* essential oil against *Alternaria alternata* and *Phomopsis carica-papayae*, fungal agents associated with the deterioration of papaya leaves in the Moungo region of Cameroon. The essential oil of fresh *C. citratus* leaves was extracted by hydrodistillation. Analysis of the chemical composition by GC then GC/MS revealed Geranial (45.24%), Neral (35.57%) and Myrcene (7.21%) as the majority compounds. *In vitro* antifungal tests on mycelial growth by incorporation into agar gave MICs of 900 ppm and 700 ppm against *A. alternata* and P. *carica-papayae* respectively. The MIC of 700 ppm proved fungicidal on *P. carica papayae*, while for *A. alternata* the fungicidal effect was obtained at a concentration of 900 ppm. *In situ*, the essential oil proved significantly active in inhibiting necrosis caused by the conidial complex of the two pathogens on papaya leaves, with a percentage inhibition of 85.93% at a concentration of 6000 ppm. *C. citratus* EO could therefore be used as an alternative to chemical fungicides in the fight against papaya leaf disease pathogens.

Keywords

Essential Oil, *Cymbopogon citratus*, *Carica papaya L*, Antifungal Activity, Leaf Diseases

1. Introduction

Papaya (*Carica papaya L*) is one of the most widely grown and marketed tropical fruits in the world [1]. Current world production of papaya is around 13.89 megatonnes. In Africa, production amounts to 7.369.579 tons, including 1.803.667

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Received: 20 January 2024; **Accepted:** 1 February 2024; **Published:** 7 March 2024

tons in Cameroon [2]. Papaya is important in several areas. Nutritionally, it is an important source of carbohydrates, vitamins and minerals [3]. Medicinally, papain extracted from the latex is used to treat wounds, oedema, inflammation and digestive disorders. Finally, marketing papaya is an important source of income for farmers. However, papaya production is often limited by a number of fungal diseases. Leaf spots caused by *Alternaria alternata* and *Phomopsis carica-papayae* are widespread and have become a threat to papaya production, causing significant economic damage of up to 70% [4]. Typical symptoms of the disease develop on leaves in the form of small, round, brown spots, which may become irregular in advanced stages of the disease, progressively covering a large area, causing wilting, drying and leaf drop. Similar disease symptoms on fruit predispose them to rot, creating the possibility of toxin production and loss of organoleptic properties [5].

To minimise post-harvest losses in Cameroon, growers treat papaya fruit with synthetic chemical fungicides such as Ridomyl and Benomyl. However, the use of the latter has been restricted due to their high toxicity, persistence in the environment, non-biodegradability and high carcinogenic potential. They are a threat to human health, which is why there is growing interest in a healthier, environmentally-friendly control alternative [6].

In view of the various limitations and disadvantages of chemical fungicides, the use of natural substances such as essential oils derived from plants is an interesting alternative for controlling papaya fruit pathogens. Among these plants, *Cymbopogon citratus* is widely cultivated in several regions of Cameroon for its numerous antimicrobial and insecticidal properties. Researchers have demonstrated the effectiveness of *Cymbopogon citratus* essential oil in combating a wide range of pathogens [7-9]. This essential oil could offer prospects for the biocontrol of fungi associated with papaya degradation. The present study was therefore initiated with the aim of assessing the antifungal potential of *Cymbopogon citratus* essential oil against *Alternata alternaria* and *Phomopsis carica-papayae*, pathogens associated with papaya leaf diseases.

2. Materials and Methods

2.1. Collection of Plant Material and Extraction of Essential Oil

The material consisted of fresh *Cymbopogon citratus* leaves harvested in Ndogbong from a farmer's field. The essential oil (EO) was extracted by hydrodistillation using a Clevenger-type apparatus. The essential oil separated from the aqueous phase was dried with sodium sulphate (Na_2SO_4) and stored at +4 ℃ in a dark bottle.

2.2. Determination of the Chemical Composition of the Essential Oil by GC/MS

The essential oil was analysed by Gas Chromatography (GC) and then by Gas Chromatography/Mass Spectrometry (GC/MS). A Varian CP 3380 chromatograph was used, equipped with a SPLIT injector (leakage ratio 1/100), a flame ionisation detector (FID) and a non-polar column 30 m long and 0.30 mm in diameter. The injector temperature was set at 200 ℃. The oven temperature was programmed from 50 ℃ to 200 ℃ with a gradient of 5 ℃/min. The carrier gas (nitrogen) was set at a flow rate of 1 ml/min. GC-MS was performed on a Hewlett-Packard instrument (Model 5970). The retention indices of the various constituents were calculated in relation to the retention time of a series of n-alkanes and their relative percentages calculated by electronic integration without taking into account any differences in their response coefficients. The results obtained were compared with those in table of Adams, [10].

2.3. Pathogen Isolation and Purification

Papaya leaves showing necrosis symptoms with black to brown spots were collected from a papaya field in the localities of Njombé in the Littoral region of Cameroon. The pathogens, *Alternaria alternata* and *Phomopsis carica-papayae*, were isolated, purified and tested for pathogenicity according to the method used by Kamga *et al.* [11]. Identification was carried out using the keys of Botton *et al.* [12] and Chabasse *et al.* [13].

2.4. Evaluation of the Antifungal Activity of Conidial Germination

The inhibitory effect of *C. citratus* essential oil on the germination of *A. alternata* and *P. carica-papayae* conidia was assessed using the liquid microdilution method in accordance with Clinical and Laboratory Standards Institute guidelines [14]. The suspension of conidia obtained from 10-day-old colonies was diluted with distilled water and adjusted to 10^5 conidia/mL. 100 µ of the prepared suspension was then introduced into Potato Dextrose Broth (PDB) supplemented with EO to obtain final concentrations ranging from 7.5 to 480 ppm. The PDB medium with distilled water was used as a control. The microplates were then covered and incubated at 28 ± 2 ℃. 24 h after incubation, the number of germinated conidia was assessed using a light microscope (x10) with a minimum of 100 spores counted in each treatment. Each treatment was maintained in triplicate and the experiment was repeated twice. Averaged results were converted to percentage inhibition (%I) using the formula (1):

$$\%I_{CG} = [(Gt - Gx)/Gt] \times 100 \quad (1)$$

$\%I_{CG}$ is the inhibition percentage of conidial germination;

Gt is the average number of conidia germinated in the control tubes

Gx is the average number of conidia germinated in a test tube containing EO.

2.5. Evaluation of Antifungal Activity on Mycelial Growth

The aim of assessing the *in vitro* antifungal activity of *C. citratus* essential oil was to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the EO. These tests were carried out on solid media on *A. alternata* and *P. carica-papayae*. It was carried out according to the protocol described by Lahou [15] and used by Kamsu *et al.* [16]. In Petri dishes each containing 10 ml of PDA (Potato Dextrose Agar) culture medium supplemented with antibiotics, sterilised for 15 minutes at 121 ℃ and cooled to 45 ℃. Decreasing quantities of essential oil were aseptically added to diméthylsulfoxyde (DMSO) (1/9: v/v) for a 10% EO stock solution at variable concentrations of 200, 300, 400, 500, 600, 700, 800 and 900 ppm. Then 5 mm mycelial discs of the isolated fungal strains were placed in the centre of each petri dish. Three Petri dishes were used for each treatment and the test was repeated 3 times. Petri dishes were incubated for 4 days at 28 ℃. Negative controls without EO were performed with DMSO. The mean radial growth diameters of the mycelium of each strain of fungus were then measured each day and compared with those of the negative control. Growth inhibition was determined by the mean radial mycelial growth for each fungal germ. The MIC was determined as the lowest concentration that inhibited all mycelial growth. All tests were repeated three times. The percentage inhibition of mycelial growth was calculated using the following formula (2):

$$\% I_{MG} = Dt - De / Dt \times 100 \quad (2)$$

$\%I_{MG}$ = Inhibition percentage of mycelial growth;

Dt (cm) = Average diameter of mycelial growth in the negative control box;

De (cm) = Average diameter of mycelial growth in the test box.

We tested fungicidal or fungistatic activity. This test consists of removing the mycelial disc that showed no growth at the end of incubation and reintroducing it into a new culture medium without EO. If mycelial growth is still inhibited, this is referred to as fungicidal activity, and if new growth is observed, this is referred to as fungistatic activity [17].

2.6. *In situ* Antifungal Activity Test on Papaya Leaf Necrosis

This test was carried out following a curative treatment according to the modified protocol of Davy *et al.* [6]. Papaya leaves apparently showing no symptoms of necrosis or staining were harvested, washed and disinfected. A 5 cm diameter disc soaked with 20 µl of conidial complex suspension (10 µl of *A. alternata* and 10 µl of *P. carica-papayae*) calibrated at 10^5 conidia/mL was deposited on the surface of the papaya leaves. 6 h later, a solution of *C. citratus* EO was sprayed at different concentrations ranging from 500 to 6000 ppm on the previously infected leaves. The negative control consisted of infected leaves sprayed with distilled water to replace the essential oil. After treatment, the papaya leaves are placed on blotting paper soaked in sterile water. The whole set was sealed with parafilm and incubated for 4 days at room temperature in sterile trays. Observations were made on the colour change, leaf softening and necrosis diameter. The percentage of necrosis inhibition is calculated using the following formula (3).

$$\% I_N = Nt - Ne / Nt \times 100 \quad (3)$$

$\%I_N$ = Inhibition percentage of necrosis;

Nt (cm) = Mean diameter of necrosis in negative control papaya leaves;

Ne (cm) = Average diameter of necrosis in papaya leaves treated with essential oil.

2.7. Statistical Analysis

The tests were performed in triplicate and the results expressed as mean ± standard deviation. Two analyses of variance (ANOVA) and the LSD test were performed using STAT GRAPHICS Centurion version 17.1.12 software and the significance threshold was set at p-value < 0.05 (5%).

3. Results and Discussion

3.1. Chemical Composition of the Essential Oil of *Cymbopogon citratus*

The *C. citratus* EO obtained after extraction had a light yellow colour, a density lower than that of water and a yield of 0.7%. The chemical composition of the essential oil, determined by GC and then GC-MS, is shown in Table 1 below.

Table 1. *Chemical composition by subclass of the essential oil of C. citratus.*

	KI	Compounds	% Percentage
Monoterpenes (98.08 %)			
HM			8.31
1	990	Myrcene	7.21
2	1026	Limonene	0.93
3	1036	Pinene	0.17
OM			89.25
11	1010	Linalol	0.75
12	1164	E-Geranial	0.66
13	1164	Citronellal	-
14	1182	Z-Geranial	1.04
15	1228	Geraniol	0.35
16	1241	Nerol	-
17	1242	Neral	35.57
18	1254	Chavicol	-
19	1255	Geraniol	4.43
20	1272	Geranial	45.24
21	1384	Geranyl propanoate	1.21
22	1416	Geranyl butyrate	-
Sesquiterpenes (0 %)			
Aromatic compounds (0. 52 %)			
31	1362	Eugénol	0.52
32	1365	Isoeugenol	-
Linear compounds (1.91 %)			
33	976	Vinyl amyl carbinol	-
34	985	Hept-5-en-2-one(6)-metyl	1.25
35	1228	Carvotanacetone	0,3
36	1373	Furan-2-heptyl	36
37	1384	Nerylpropionate	-
TOTAL			99.99

KI: Kovach Index; HM: Hydrogenated Monoterpens; OM: Oxygenated Monoterpens.

The essential oil of *C. citratus* is mainly composed of oxygenated monoterpenes, with geranial (45.24%), neral (35.27%) and myrcene (7.21%) as the main compounds. This composition is similar to that obtained by Degnon *et al.* [18] and Akono *et al.* [19], who obtained geranial and neral as the predominant compounds from leaves collected in Benin and Cameroon respectively. However, our composition differs from that obtained by Omolara *et al.* [20] who obtained citral (53.48%) and palmitic acid (25.64%) from fresh leaves collected in Nigeria [20]. It also differs from that of Valkov á*et al.* [21] who found citral (61.5%), geraniol (6.6%) and 1,8-cineole (6.4%) as the main compounds from *C. citratus* leaves collected in Serbia. The differences in chemical composition observed between these results may be due to parameters such as genetic differences, geographical origin, extraction method, stage of maturity, har-

vesting season, pre-treatment of the plant, climate, hydrodistillation time and length of storage of the plant material after harvesting [22]. This reinforces the fact that the chemical composition of EO also depends on the plant's chemotype and biotype [23].

3.2. Effect of *Cymbopogon citratus* Essential Oil on Conidial Germination

The minimum inhibitory concentration (MIC) is the lowest concentration that has shown complete inhibition of conidial germination. Table 2 shows the effect of *C. citratus* essential oil on the number of conidia germinated and the percentage of inhibition on *A. alternata* and *P. carica-papayae*.

The percentage inhibition of conidial germination was proportional to the concentration of essential oil. The essential oil of *C. citratus* completely inhibited the germination of *A. alternata* and *P. carica-papayae* conidia at concentrations of 240 ppm and 480 ppm respectively.

Table 2. *Effect of C. citratus essential oil on conidial germination.*

Conc. CCEO (ppm)	*Alternaria alternata*		*Phomopsis carica-papayae*	
	Ngc/100	**% I_{CG}**	**Ngc/100**	**% I_{CG}**
0	96	0.00^{μ} ±00	100	0.00^{μ} ±00
7.5	90	$3,12^{\mu}$ ±00	96	4.00^{μ} ±00
15	87	9.37^{α} ±1.21	76	24.00^{α} ±3.07
30	72	25.00^{β} ±1.47	42	58.00^{β} ±1.88
60	65	32.29^{δ} ±2.01	17	83.00^{δ} ±2.42
120	43	55.20^{η} ±1.38	8	92.00^{η} ±2.42
240	21	$78.12^{£}$ ±3.10	0	$100^{£}$ ±00
480	0	100^{π} ±00	0	$100^{£}$ ±00

Columns with different signs (μ, α, β, δ, η, £, π, ϑ) for different pathogens are significantly different according to the PLSD test at a value of $p<0.05$. CCEO: *C. citratus* essential oil; Ngc: Number of germinated conidia; % I_{CG}: Percentage of inhibition conidial germination.

3.3. Effect of *Cymbopogon citratus* Essential Oil on the Mycelial Growth of Pathogens

C. citratus essential oil significantly inhibited the mycelial growth of *A. alternata* and *P. carica-papayae* at the concentrations tested. Figure 1 below illustrates the evolution of the percentage inhibition of pathogen mycelial growth as a function of varying concentrations of *C. citratus* essential oil. *C. citratus* EO demonstrated a Minimum Inhibitory Concentration of 900 ppm on *A. alternata* and 700 ppm on. The MIC of 700 ppm proved fungicidal on *P. carica papayae*, while for *A. alternata* the fungicidal effect was obtained at a concentration of 900 ppm. Sensitivity varied from one pathogen to another. This shows that sensitivity to essential oil varies from one fungal agent to another, the most sensitive pathogen being *P. carica papayae*. It has been shown that the activity of a substance depends on the genetic nature of the target microorganism [20].

Histograms bearing different letters (a, b, c, d, e, f and g) at varying concentrations for the same pathogen are significantly different according to the PLSD test at a value of $p<0.05$.

Several previous studies have highlighted the antimicrobial potential of *C. citratus* EO. For example, Agbénébia *et al.* [24] demonstrated the efficacy of *C. citratus* EO against *A. niger*, *C. albicans*, *C. kefir*, *C. tropicalis*, *R. nigricans* and *S. cerevisiae* KVL013. Also, Kpatinvoh *et al.* [17] revealed that *C. citratus* EO strongly inhibits the growth of *Aspergillus flavus*, a cowpea contaminant, at an MIC of 0.05 µl/ml. However, Girish *et al.* [25] obtained 100% inhibition of mycelial growth of *Phomopsis azadirachtae* at a concentration of 400 ppm with the essential oil of *Ocimum basilicum*. The antimicrobial activity of EOs is most often correlated with their chemical constituents and functional groups, which may present possible synergistic interactions between majority and minority compounds. The efficacy of *C. citratus* EO is thought to be due to the action of oxygenated monoterpenes. Indeed, the work of Yan *et al.* [26] has shown that essential oils rich in oxygenated monoterpenes have deleterious effects on the mitochondrial membranes of moulds, leading to inhibition of mitochondrial energy metabolism and disruption of the cell's physiological and biochemical processes. In addition, oxygenated monoterpenes are generally antimicrobial compounds

with a broad spectrum of action, so this essential oil could be expected to have strong activity against phytopathogens [27]. The results obtained showed that *C. citratus* EO could be used as a natural agent for food preservation.

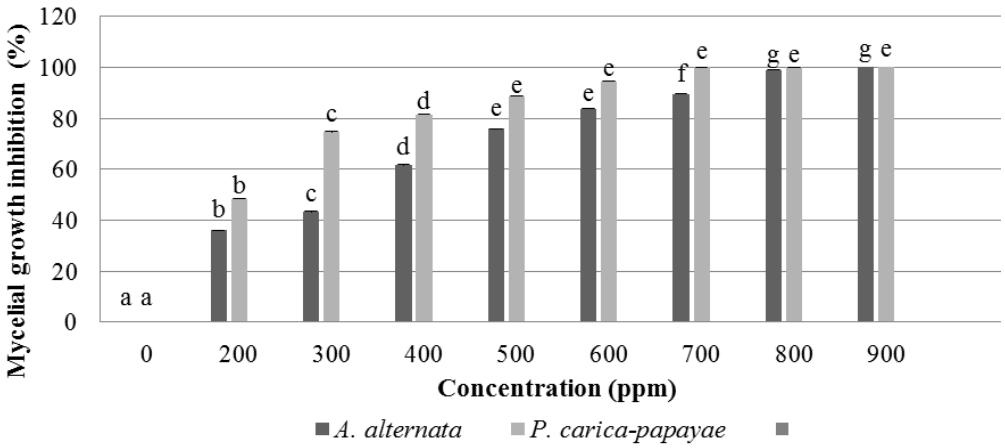

Figure 1. *Percentage inhibition of C. citratus EO on mycelial growth of A. alternata and P. carica-papayae as a function of varying EO concentrations.*

3.4. Effect of *C. citratus* Essential Oil on Papaya Leaf Necrosis

Table 3 below shows the diameter of necrosis, leaf colouration, leaf texture and the percentage of necrosis inhibition induced by the conidial complex on papaya leaves as a function of increasing concentrations of *C. citratus* essential oil. This table shows that the conidial complex of *A. alternata* and *P. carica-papayae* induces severe necrosis on papaya leaves, visible through the appearance of a brown-black coloration and a wilting/spreading of the infected region. The diameter of the necroses is inversely proportional to the concentration of *C. citratus* essential oil. Leaf spots and softening are more noticeable at low concentrations of essential oil.

Table 3. *Effect of C. citratus essential oil on papaya leaf necrosis.*

Conc. CCEO (ppm)	Diameter of necrosis (cm)	Brown-black colouring	Softening of the leaf	% Inhibition
0	6.4	+++	++	0.00^{λ} ±00
500	5.8	+++	+	9.37^{μ} ±0.77
1000	4.4	++	+	31.25^{α} ±1.02
2000	3.7	++	-	42.18^{β} ±0.68
3000	3.1	++	-	51.56^{δ} ±2.11
4000	2.6	+	-	59.37^{η} ±1.26
5000	2.1	+	-	$67.18^{£}$ ±2.05
6000	0.9	+	-	85.93^{π} ±2.05

Columns with different signs (λ, μ, α, β, δ, η, £ and π) for % inhibition values are significantly different according to the PLSD test at a value of $p<0.05$. CCOE: *C. citratus* essential oil. Legend: - Absence of brown-black coloration and softening; + Presence of brown-black coloration and softening.

Figure 2 below shows the evolution of the percentage of necrosis inhibition as a function of varying concentrations of *C. citratus* essential oil.

This figure shows that the percentages of necrosis inhibition on papaya leaves increased significantly with increasing concentrations of *C. citratus* EO. *C. citratus* EO significantly limited the development of necrosis induced by *Alternaria alternata*

and *Phomopsis carica-papayae* with an inhibition percentage of 85.93% at a concentration of 6000 ppm.

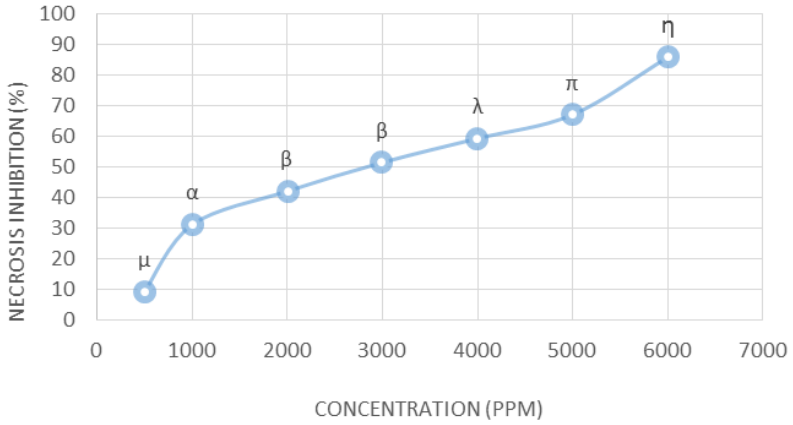

Figure 2. *Variation of the percentage of inhibition of C. citratus EO on the diameter of necroses caused by Alternaria alternata and P. carica-papayae.*

Correspondence points bearing different signs (μ, α, β, λ, π, and η) for % inhibition values are significantly different according to the PLSD test at a value of $p<0.05$.

Numerous studies have shown that the inhibitory effect of essential oils on fungal necrosis of leaf diseases is correlated with concentration. Indeed, Dalmarcia *et al.* [28] demonstrated the ability of *C. citratus* essential oil to control *Curvularia* leaf spot disease on maize leaves in Brazil. They obtained total inhibition of leaf necrosis at a concentration of 7.5 μL/mL. Also, Kahkashan and Najat [29] revealed that *Alternaria* leaf blight on tomato plants caused by *Alternaria alternata* was significantly reduced by spraying mint essential oil. The antifungal activity of essential oils on the development of leaf diseases may be linked to the profile and variability of the quantities of the bioactive components they contain. These compounds have the ability to interfere with pathogen propagation mechanisms, acting on the fungal wall to inhibit the synthesis of certain constituents such as chitin [30].

4. Conclusion

This study demonstrated the effectiveness of lemongrass essential oil in combating leaf diseases of papaya. The essential oil of *C. citratus* was mainly composed of geranial (45.24%), neral (35.27%) and myrcene (7.21%). *C. citratus* EO significantly inhibited conidial germination and mycelial growth of *A. alternata* and *P. carica papayae*. *In situ*, it limited the development of necrosis and leaf spots by up to 85.93%. The study recommends that *C. citratus* EO can be used as an effective alternative to synthetic chemical antifungals, which represent a real danger to human health and the environment. Future studies will focus on the formulation of a biopesticide based on the essential oil of *C. citratus* and evaluation of its efficacy against papaya fruit pathogens.

Abbreviations

EO: Essential Oil
MIC: Minimum Inhibitory Concentration
MFC: Minimum Fungicidal Concentration
PDB: Potato Dextrose Broth
PDA: Potato Dextrose Agar

Conflicts of Interest

The authors declare no conflict of interests.

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