African Journal of Biotechnology Vol. 7 (22), pp. 4045-4050, 19 November, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Antiradical potential and antifungal activities of essential oils of the leaves of *Citrus latifolia* against *Phaeoramularia angolensis*

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Accepted 21 March, 2008

Investigations were conducted to determine the chemical composition, antiradical and antifungal activities of the essential oil extracted from the fresh leaves of *Citrus latifolia* var. Tahiti from Cameroon against *Phaeoramularia angolensis*. The essential oil obtained by hydrodistillation was analysed by GC and GC/MS. The disc diffusion method was used to evaluate the zone of fungal growth inhibition at various concentrations of the oil while the antiradical activity of the oil was studied by the DPPH (diphenyl picryl hydrazyl) method. The chemical analysis revealed 26 components among which limonene (45.76%), geranial (13.12%) and neral (10.35%) were the main components. The antiradical activity of *C. latifolia* essential oil (SC_{50} = 9.93 g/l) was less than that of butylated hydroxyl toluene (BHT) which was used as the reference compound (SC_{50} = 7.02 x 10^{-3} g/l). After 40 days of incubation on oil – supplemented medium, the growth of *P. angolensis* was totally inhibited by 1600 mg/l of *C. latifolia* oil. Results obtained in the present study indicate the possibility of exploiting *C. latifolia* var Tahiti essential oil to combat *P. angolensis* which is responsible for heavy losses of *Citrus* fruits harvests.

Key words: *Citrus latifolia*, yield, chemical composition, *Phaeoramularia angolensis*, limonene, neral, geranial, antifungal activity.

INTRODUCTION

Citrus latifolia, usually called Citrus Tahiti*,* is a vigorous tree, largely extended, measuring 4.5 to 6 m, almost without spines, dense green foliage. The leaves are of average sizes, large lancelets, petiole in the shape of wings. The young growths are purplish. *C. latifolia* has marvellous scenting flowers. The skin of the fruit is sharp green in colour and becomes pale yellow when the fruit is ripe. It is fine, adhering to the pulp which is greenish pulpit, very acidic, juicy and almost deprived of pectin. For storage, the fruits do not require any treatment. The fresh fruits could remain in good condition for 6 to 8 weeks under refrigeration (Praloran, 1971).

Excellent for health (much vitamin C) (Economos and Clay, 1999), *C. latifolia* Tahiti is used to make lemonade. Its juice is frequently used as an alternative to vinegar sauce. It is also used as rinsing after shampooing of the hair; it can be applied to the face like refreshing lotion and can also be applied to the interior of coffee machines to eliminate unpleasant odour.

Previous works showed the antifungal properties of essential oils of the fruit pericarps of *C. latifolia* (Jazet, 2002) as well as their antiradical properties (Choi et al., 2000). We know, moreover, that the biological activity of an essential oil is related to its chemical composition, the functional groups of the majority compounds (alcohols, phenols, terpenes and ketones) and the possible synergistic effects or antagonism between the components. Therefore, the nature of the chemical structures of the

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constituents of this oil, as well as their proportions play a determining role (Pibiri, 2005). The chemical compounds with great effectiveness and broader spectrum are phenols (thymol, carvacrol and eugenol) of alcohols (α-terpineol, terpinen-4-ol, and linalool), aldehydes, and ketones and more rarely of terpenes (Cosentino and Tuberoso, 1999; Dorman and Deans, 2000)

The objective of this study is to extract, analyze the chemical composition, evaluate the antiradical properties and to test the essential oil of the leaves of *C. latifolia* and its fractions against *Phaeoramularia angolensis* with the aim of determining the active molecules.

MATERIAL AND METHODS

Plant material and extraction procedure

Fresh leaves from *C. latifolia* var Tahiti were collected from the garden of the Institute of Agronomical Research and Development of Nkolbisson, Yaounde-Cameroon in March 2006 and identified at the National Herbarium of Yaounde, where voucher specimens are deposited. The leaves were steam-distilled for about 5 h using a Clevenger apparatus. Oil recovered was dried over anhydrous sodium sulphate and stored at 4°C until used.

Oil fractionation

Essential oil obtained was fractionated split by column chromatography (DC) with silica gel G60 (63 - 200 µm). Elution was done by the hexane/ether system (Table 1) and the elutes were concentrated with the rotary evaporator.

Chemical analysis

The essential oil obtained was analysed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS).

Gas chromatography

The oil was analysed on a Varian CP-3380 GC with flame ionization detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25 µm); temperature program 50 - 200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas N_2, 1 ml/min. The linear retention indices of the components were determined relatively to the retention times of a series of *n*-alkanes and the percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

Gas chromatography spectrometry

GC/MS analyses were performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadrupole detector (GC-quadrupole MS system, model 5970). The column temperature was programmed from 70 - 200°C at 10°C/min; injector temperature was 200°C. Helium was used as the carrier gas at a flow rate of 0.6 ml/min; the mass spectrometer was operated at 70 eV.

Table 1. Protocol used for essential oil fractionation.

Fraction	Eluant	Volume (ml)
F0	Hexane/ether	100
F1	Hexane/ether	100
F2	Hexane/ether	100
F3	Hexane/ether	100
F4	Hexane/ether	100
F5	Hexane/ether	100
F6	Hexane/ether	100
F7	Hexane/ether	100
F8	Hexane/ether	100
F9	Hexane/ether	100
F10	Hexane/ether	100
F11	Hexane/ether	100
F12	Hexane/ether	100
F13	Hexane/ether	100

Identification of the components

The identification of the constituents was assigned on the basis of comparison of their retention indices and their mass spectra with those given in the literature (Adams, 2001).

Evaluation of antiradical activity

The antiradical activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995) free stable radical scavenger, which was dissolved in ethanol to give a 100 µM solution. To 2 ml of the ethanolic solution of DPPH was added 100 µl of a methanolic solution of an antioxidant reference (BHT) at different concentrations. The oil was tested using the same method. The control without antioxidant is represented by the DPPH ethanolic solution containing 100 µl of methanol. The decrease in absorption was measured at 517 nm after 30 min at room temperature. The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control. The concentration required for 50% reduction (SC_{50}) was determined graphically. All the spectrophotometric measurements were performed with a SAFAS UV-mc2 spectrophotometer, equipped with a multicell/multikinetics measuring system and with a thermostated cell-case.

Antifungal activities

The strain of *P. angolensis* CMR4 culture maintained in the culture collection of Phytopathology Laboratory of the Institute of Agronomic Research and Development of Yaounde (Cameroon) was used as test microorganism.

The antifungal activity of the essential oil of *P. angolensis* was evaluated by the agar medium assay as described by Grover and Moore (1962). The medium used was PDA (Potatoes dextrose agar). Essential oil was mixed with dimethylsulfoxyde (DMSO) in a proportion of 1/9; this was to facilitate its solubilization in the PDA medium. The mixture of essential oil/DMSO obtained was incorporated in the PDA medium in desired concentrations: initially 1000, 2000, 3000, 4000 and 5000 mg/l; concentrations lower than that having inhibited growth of the fungal was used for the search of the Minimal Inhibitory Concentration (MIC). The supplemented medium was poured into Petri dishes of 55 mm at a rate of 10 ml per dish and allowed to rest for solidification.

A mycelia disc of 6 mm in diameter taken on a 15 day old preculture of *P. angolensis* was placed directly in the centre of each dish. The dishes were incubated in an inverted position at 22°C in the dark. After 10 days, the mycelia growth was observed while measuring the diameter according to two perpendicular lines passing across the centre of the dish, with a 5 days regular interval for up to 40 days. For each concentration, three tests were carried out. Also citral was simultaneously assayed for comparison activity.

Statistical analysis

Data from three independent replicate trials were subjected to statistical analysis using SPSS Statistical package (Statsoft, 1995). Differences between means were tested using Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Essential oil composition

Essential oil was obtained by steam distillation for 5 h with a yield of 0.66%. GC and GC-MS analysis of essential oil enabled the identification of twenty-six volatile components (Table 2). In the volatile extract different group of terpenoid compounds were present, such as hydrocarbons, alcohols, aldehydes, ketones, esters and others. The monoterpenes are dominant (51.64%) and are represented mainly by limonene. Oxygenated monoterpenes are mainly represented by geranial (13.12%) and neral (10.35%). Sesquiterpenes are mainly represented by β-caryophyllene (1.49%). After the fractionation, four main fractions were obtained:

- Fractions 2+3 was largely dominated by the MTH with limonene (85.88%) as the main component.
- Fraction 9 was rich in MTO and was dominated by neryl acetate (40.06%) and geranyl acetate (30.78%).
- Fraction 11 was also rich in MTO but it consisted mainly of neral (29.18%) and geranial (45.18%).
- Fraction 13 also contained mainly MTO but was dominated by nerol (17.00%), neral (32.09%) and geraniol (33.83%).

Antiradical activities

The antiradical activity of *C. latifolia* Var Tahiti essential oil was evaluated by DPPH method and compared to the commercial antioxidant molecule (BHT) used as preservative. The results obtained are given in Table 3. Generally, it was observed that the scavenging capacity of the essential oil and BHT increases with their concentration in the reaction medium. The following results were obtained: SC_{50} (BHT) = 7.02 mg/l and SC_{50} (essential oil) = 9930 mg/l. These results indicate that *C. latifolia* essential oil is 1414 times less active than the BHT.

Antifungal activity

Results of the inhibitory activity of the crude essential oil in agar medium are presented in Figure 1. At 1600 mg/l, fungal development was completely inhibited over the 40 days of incubation. Subcultures of these treated inocula were negative indicating a lethal effect at this concentration. Mycelia growth was considerably reduced with increasing concentration of essential oil while their growth increased with incubation time. 24, 36 and 100% inhibition of mycelia growth were observed at 1000, 1500 and 1600 mg/l, respectively, after 40 days of incubation.

The MIC of citral and *C. latifolia* oil and its fractions are presented in Table 4. The overall rating for inhibition against *P. angolensis* was citral > fractions 10+11> fraction 13 > essential oil > fraction 9 > fractions 2+3.

DISCUSSION

In the present study, *C. latifolia* is rich in limonene (45.26%), geranial (13.12%) and neral (10.35). This composition is similar to previous reports (Caccioni et al., 1998). The high content of limonene in *C. latifolia* essential oil is an interesting result. In fact, this compound is among a group of chemicals known as monoterpenes. They have been shown to cause regression and prevention of recurrence of mammary tumors in rats. They also have a direct tumorstatic effect, meaning they inhibit the growth of tumors, as well has the ability to block the initiation and promotion phases of carcinogenesis (Field and Roe, 1965; Wattenberg and Coccia, 1991). Limonene can also be used as insecticide (Hooser, 1990; Ho and Fauziah, 1993).

Interest has increased considerably in finding naturally occurring antioxidant for use in foods to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenicity (Ito et al., 1983; Zheng and Wang, 2001). Several authors found that the natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods (Pryor, 1991; Lai et al., 2001). Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers. The oil of *C. latifolia* showed a lower radical scavaging capacity (RSC) than those of *Plectranthus grandis* and *Plectranthus ornatus* (Albuquerque et al., 2006), *Cinnamomum zeylanicum* (Jazet et al., 2007), *Foeniculum vulgare* subsp *piperit*um (Conforti et al., 2006) and *Clausena anisata* (Avlessi et al., 2004). This lower RSC of *C. latifolia* oil could be explained by the absence of compounds able to form stable free radical after loss of proton.

C. latifolia oil caused complete growth inhibition of *P. angolensis* at 1600 mg/l on agar plate. This concentration was also found to be lethal under this test conditions. Limonene which appears to be the main component of *C. latifolia* essential oil may not plays the major role in its antifungal activity against *P. angolensis*. In fact the fractions 2+3 which contains the higher amount of limo-

Table 2. Chemical composition of the essential oil of the leaves of *C. latifolia* var Tahiti and its fractions.

N°	Compound	IK	EO	Fractions 2+3	Fraction 9	Fractions 10+11	Fraction 13
	MTH		**51.64**	**88.44**	**12.59**	**7.27**	**2.22**
1	α-pinene	936	0.33	0.56	-	0.30	0.65
2	camphene	946	1.32	-	-	4.05	-
3	sabinene	971	0.93	0.96	-	-	-
4	myrcene	983	1.25	1.04	0.34	-	-
5	limonene	1029	**45.76**	**85.88**	**12.25**	2.19	1.57
6	Z-β-ocimene	1039	1.66	-	-	-	-
7	isocamphene	1148	0.39	-	-	0.73	-
	MTO		**43.94**	**9.83**	**81.14**	**92.72**	**97.24**
8	Linalol	1088	1.61	-	0.52	4.42	-
9	E-pinocarveol	1136	1.69	-	3.64	1.74	0.54
10	borneol	1166	0.52	0.81	-	0.44	-
11	Terpene n-4-ol	1174	0.46	-		1.03	**12.25**
12	Myrtenol	1189	0.28	-	0.86	-	-
13	citronellal	1217	0.96	2.17	1.39	-	-
14	nerol	1219	3.34	1.28	-	-	**17.00**
15	neral	1226	**10.35**	2.51	-	**29.18**	**32.09**
16	geraniol	1243	3.91	-	0.47	-	**33.83**
17	geranial	1254	**13.12**	0.88	1.87	**45.18**	0.84
18	Citronellyl acetate	1336	0.25	-	2.48	-	-
19	neryl acetate	1346	3.81	0.60	**39.54**	6.16	-
20	geranyl acetate	1363	3.64	1.58	**30.37**	4.57	0.69
	STH		**2.49**	**0**	**4.24**	**0**	**0**
21	β- elemene	1398	0.48	-	0.33	-	-
22	β-caryophyllene	1418	1.49	-	3.57	-	-
23	germacrene D	1498	0.25	-		-	-
24	β-bisabolene	1511	0.27	-	0.34	-	-
	STO		**0.88**	**0**	**0.93**	**0**	**0**
25	caryophyllene oxide	1596	0.29	-	0.93	-	-
26	farnesyl acetate	1811	0.59	-	-	-	-

nene (85.88%) was less active against the fungi than the others fractions. On the other hand, the activity of *C. latifolia* and its fractions increased proportionally with their citral content. Our results showed that *C. latifolia* fresh leaves tested on *P. angolensis* appears to be more toxic than the essential oil extracted from fruits pericarps of *C. latifolia* and tested on *P. angolensis* with the same agar dilution technique (Jazet et al., 2002). In the present study, oil obtained from fresh leaves exhibited fungistatic and fungistoxic activity at 1600 mg/l while the oil obtained from fruits pericarps exhibited fungistatic activity between 2500 – 3000 mg/l. The different activity of these two oils may be due to their different citral (neral + geranial) content which are 5.45 and 23.47% for fruits pericarps (Jazet et al., 2002) and fresh leaves respectively.

A survey of the available literature shows that the antimicrobial effect of some essential oil components of *C. latifolia* has been investigated. Mahmoud (1999) found that geraniol was effective in suppressing *A. flavus* growth at 500 mg l^{-1}. Viollon and Chaumont (1994) reported that citral, geraniol, and citronellol showed the highest antifungal activities among terpenoids. The marked action of terpenic alcohol, geraniol constituents of *C. latifolia* oil may be attributed to the polarity of the OH-group, making these compounds relatively soluble in water and the terpenoids moiety confers lipophilic properties on these molecules with the ability to penetrate the plasma membrane (de Billerbeck et al., 2001; Knobloch et al., 1989).

From these results, it can be concluded that the essential oil of *C. latifolia* var Tahiti showed very weak antioxidant activity when compared to BHT. However the antifungal activity against *P. angolensis* is interesting and indicates that *C. latifolia* oil could be used as easily accessible source of natural antifungal agent against this fungus which is responsible for heavy losses on *Citrus* fruits. For the practical use of this oil as novel fungal-control agent, further research is needed on the formu-

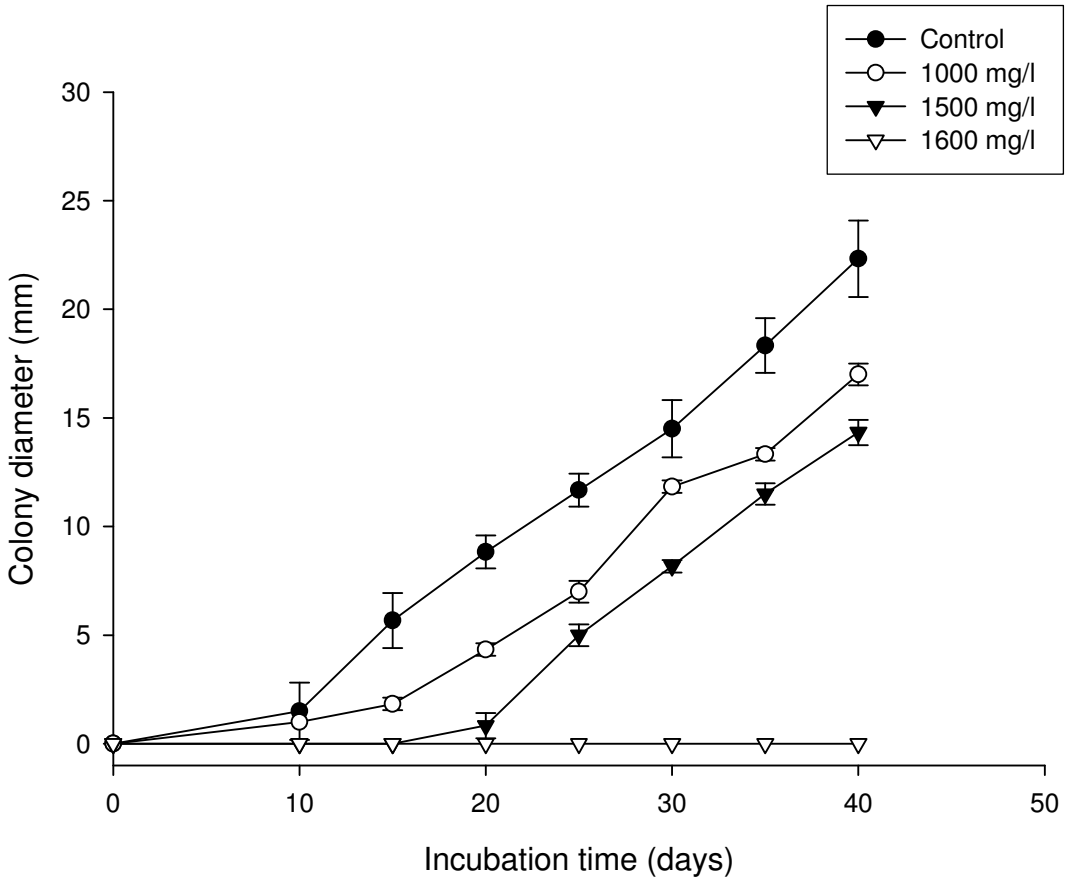

Figure 1. Effect of the essential oil of *C. latifolia* var. Tahiti on *P. angolensis*.

Table 3. Scavenging activity of *C. latifolia* essential oil on DPPH.

Concentration (mg/l)	Essential oil linhibition (%)[a]	BHT inhibition (%)[a]
0	-	0.00
2.5	-	22.35
5	-	40.23
10	-	63.97
20	-	88.58
30	-	98.14
40	-	100
50	-	-
500	-	-
1500	-	-
2500	27.29	-
5000	40.90	-
10000	49.68	-
15000	67.15	-
20000	79.35	-
25000	96.47	-
SC50 (mg/l)[b]	**9930**	**7.02**

[a]Mean values obtained from experiments performed in triplicate.
[b]Mean value determined graphically

Table 4. MIC, neral and geranial contents of *C. latifolia* Var. Tahiti and its fractions.

Oil	MIC (mg/l)	Neral (%)	Geranial (%)	Neral + Geranial (%)
Crude essential oil	1600	10.35	13.12	23.47
Fractions 2+3	2800	2.51	0.88	3.39
Fraction 9	2700	-	1.90	1.90
Fractions 10+11	800	29.18	45.18	74.36
Fraction 13	900	32,09	0,84	33.93
Citral	400	37.37	48.08	85.45

lation to improve the fungicidal potency and stability.

AKNOWLEDGEMENTS

The authors are grateful to International Foundation of Science (IFS) for his financial and material support through the F/3897-1 project, as well as COMETES project.

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