Full Length Research Paper

Correlation between chemical composition and antifungal properties of essential oils of *Callistemon rigidus* and *Callistemon citrinus* of Cameroon against *Phaeoramularia angolensis*

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Leaf essential oils of *Callistemon rigidus* and *Callistemon citrinus* obtained by steam-distillation were assigned for their antifungal activity against *Phaeoramularia angolensis*. The oils of *C. rigidus* and *C. citrinus* were dominated by the presence of 1,8-cineole (79.1% and 73.8% respectively). Fractions 6 and 5 isolated from *C. rigidus* and *C. citrinus* respectively were rich in1,8-cineole (100% and 91.4% respectively). The determination of the antifungal activity of the essential oils was done by the agar medium assay. Total inhibition was obtained with the concentrations of 6500 ppm and 6000 ppm for *C. rigidus* and *C. citrinus* essential oils respectively. Fraction 12 of *C. citrinus* rich in α-terpineol and terpinen-4-ol (88.7% and 10% respectively) was the most active showing total inhibition against *Phaeoramularia angolensis* at 1500 ppm. The two essential oils used showed fungicidal properties against *P. angolensis* and could constitute an alternative to synthetic fungicides usually used for citrus fruit crops.

Key words: Essential oil, *Callistemon citrinus, Callistemon rigidus,* essential oil composition, *Phaeoramularia angolensis*, antifungal activity.

INTRODUCTION

Citrus fruits belong to a very interesting food group due to their high vitamins, fibre and rock salt contents. The consumption of these fruits is a primordial guarantee to good health. Indeed, vitamins are antioxidants. These vitamins and rock salt provide for a good functioning of the human system by their intervention in many metabolic pathways and in the stimulation of many syntheses (nervous balance, fight against tiredness and defence of the organic system). The fibres make it possible to control intestinal transit time, control hunger and protect from cancer caused by foreign bodies etc. Citrus fruits may thus not only solve many medical problems, but they may also contribute to the revival of the economies of developing countries (fresh foodstuff, fruit juice, essential oils and aromas etc) (Economos and Clay, 1999).

In tropical Africa particularly in Cameroon, efforts to develop this crop are hindered by many obstacles

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amongst which are parasitic diseases. Indeed, for a few decades, a pathogenic fungus, *Phaeoramularia angolensis* has inflicted heavy losses on the production of citrus fruits harvests leading to over 80% loss of total harvest in certain areas of the country (Kuate, 1997). Analysis of the use of some chemical agents against *P. angolensis* showed inhibitory effects on products containing copper (Seif and Hillocks, 1993). These chemical fights remain however difficult to manage, not only because of numerous possible resistances but also because of the harmful effects on man and the environment (Rakitsky et al., 2000; Suwalsky et al., 2000; Hurst and Sheahan, 2003; Blasco et al., 2005; Ortelli et al., 2005; Jawich, 2006). These problems have for some time caused research to be directed towards natural substances.

Essential oils have proven to be an alternative because of their potentials as pesticides (Nguefack et al., 1998; Tagne et al., 2000; Jazet Dongmo et al., 2002). Thus, the aim of this work is to evaluate the antifungal activity of essential oils of some Cameroonian *Callistemon* species, and to identify their active ingredients that can fight against the mycelial growth of *P. angolensis.*

MATERIALS AND METHODS

Plant material

The plant materials used in this work were made up of fresh leaves of *C. rigidus* collected from a wild population near the University of Ngaoundere, Cameroon and fresh leaves of *C. citrinus* collected from a wild population at Bonamoussadi-Douala, Cameroon.

These plants were collected in April 2006 and identified at the National Herbarium of Cameroon, where voucher specimens are deposited with the following voucher number: 18564/SRF/CAM and 25801/SRF/CAM for *C. rigidus* and *C. citrinus* respectively.

Fungal material

The fungal material consists of a crop stock of CMR4 of *P. angolensis* offered by the Laboratory of Phytopathology of the Insti-tute of Agricultural Research for Development (I.R.A.D) Nkolbisson, Yaounde-Cameroon.

Extraction of essential oils

The leaves were steam-distilled for 5 h using a Clevenger apparatus. Oil recovered was dried over anhydrous sodium sulphate and stored at 4°C until use.

Fractionation

Essential oils obtained were fractionated by column chromate-graphy with silica gel G60 (63-200 μm). Elution was done by the hexane/ether system and the elutes were concentrated with the rotary evaporator.

Chemical analyses

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

Gas Chromatography: Essential oil (10 μl) was dissolved in pentane (100 μl) and 2 μl of the solution was injected into a Varian CP-3380 GC fitted with flame ionization detector (FID) and integrator C-R6A-chromatopac (Shimadzu Co, Japan). The column used was SUPELCOWAX fused silica (film thickness: 0.25 μm, Supelco USA, 60 m x 0.25 mm). Column temperature was pro-grammed at 50 to 200°C with a rate of 5°C/min, injector tempe-rature 200°C, detector temperature 200°C and carrier gas N_2, 1 ml/min. The linear retention indices of the components were determined relative 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.

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 (Jennings and Shibamoto, 1980; Joulain, 1998; Adams, 2001).

Antifungal activities

Fungal strain

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

Evaluation of the antifungal activity

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 EO/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 pre-culture of *P. angolensis* was placed directly at 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 with the diameter measured along two perpendicular lines passing across the centre of the dish, with a 5 day regular interval for up to 40 days. For each concentration, three tests were carried out. Also 1,8-cineole was simultaneously assayed in order to compare their activities.

Table 1. Constituents (%) of essential oil of *Callistemon rigidus* and its fractions.

Compounds	IR*	Essential oil	Fraction D	Fraction E	Fraction F	Fraction J
Linear compounds		0.3	0.2	-	-	-
γ-butyrolactone	869	0.2	0.1	-	-	-
Isobutyl isobutyrate	901	0.1	0.1	-	-	-
MTH		14.5	12.7	5.3	-	-
α-pinene	936	12.9	11.4	5.3	-	-
Sabinene	975	0.1	0.1	-	-	-
β-pinene	977	0.8	0.7	-	-	-
Myrcene	982	0.2	0.2	-	-	-
α-phellandrene	1002	0.4	0.2	-	-	-
γ-terpinene	1054	0.1	0.1	-	-	-
MTO		84.4	86.3	94.7	100	100
1,8-cineole	1030	79.1	81.8	94.7	100	100
Linalool	1088	0.3	0.3	-	-	-
Trans-pinocarveol	1138	0.3	0.3	-	-	-
Borneol	1166	0.1	0.1	-	-	-
Terpinen-4-ol	1173	0.3	0.2	-	-	-
α-terpineol	1184	4.1	3.4	-	-	-
Nerol	1219	0.1	0.1	-	-	-
Geranial	1249	0.1	0.1	-	-	-
STH		0.5	0.3	-	-	-
(Z)-β-farnesene	1456	0.1	0.1	-	-	-
Germacrene D	1499	0.1	0.1	-	-	-
β-bisabolene	1510	0.1	-	-	-	-
δ-cadinene	1537	0.1	0.1	-	-	-
α-cadinene	1585	0.1	-	-	-	-
STO		0.1	-	-	-	-
α-humulene oxide	1621	0.1	-	-	-	-
Total % of identified compounds		99.8	99.5	100	100	100

*Retention indices on HP-1.

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 the Duncan Multiple Range Test.

RESULTS AND DISCUSSION

Chemical composition of essential oils

The chemical compositions of the various fractions used for the antifungal tests are presented in Tables 1 and 2.

Table 1 reveals that oxygenated monoterpens were the most predominant components of essential of *C. rigidus*. In this regards the major components identified were 1,8-cineole, α-pinene (12.9%) and the α-terpineol (4.1%) representing 79.1, 12.9 and 4.1%, respectively. In addition the corresponding fractions were equally dominated by oxygenated monoterpens particularly1,8-cineole which

was observed in fraction F and K at the level of 100%.

Table 2 shows the essential oil composition of *C. citrinus* and its fractions. There were three major compounds in the oil: 1.8-cineole (73.8%), α-pinene (16.3%) and α-terpineol (4.8%). 1.8-cineole and α-pinene were equally the major components in fractions D and E but were absent in fractions L and M.

Evaluation of the effect of essential oils and their fractions on *P. angolensis*

Results of the inhibitory activity of the essential oils in agar medium are presented in Figures 1 and 2 and in Table 3. At 6500 mg/l and 6000 mg/l, fungal development was completely inhibited by essential oils of *C. rigidus* and *C. citrinus* respectively over the 40 days of incubation. Subcultures of these treated inocula were negative, confirming a lethal effect at these concentrations. These results show that *C. citrinus* essential oil was more active

Table 2. Constituents (%) of essential oil of *C. citrinus* and its fractions.

Compounds	IR*	Essential oil	Fraction D	Fraction E	Fraction K	Fraction L
Linear compounds		0.6	2.6	0.5	-	-
Isobutyle isobutyrate	900	0.4	-	-	-	-
(3Z)-hexenyl acetate	1001	Tr	-	-	-	-
(3^E)-hexenyl acetate	1003	0.2	2.6	0.5	-	-
MTH		17.7	37.0	8.1	-	-
A-pinene	937	16.3	28.5	4.2	-	-
B-pinene	978	0.5	1.6	-	-	-
Myrcene	983	0.2	3.5	3.1	-	-
p-cymene	1018	0.5	2.4	0.8	-	-
γ-terpinene	1062	0.2	1.0	-	-	-
MTO		80.4	60.3	91.4	93.4	100.0
1,8-cineole	1031	73.8	59.7	91.4	-	-
Linalool	1088	0.3	0.3	-	2.1	-
Trans-pinocarveol	1138	0.4	-	-	2.9	-
Borneol	1165	0.2	-	-	1.1	-
Terpinen-4-ol	1174	0.5	-	-	1.9	10
A-terpineol	1185	4.8	0.3	-	85.4	88.7
Alcohol (M 152)	1242	0.4	-	-	-	1.3
STH		0.5	-	-	5.4	-
B-bisabolene	1509	0.2	-	-	1.8	-
Δ- cadinene	1536	0.3	-	-	3.6	-
Aromatic compounds		tr	-	-	1.1	-
Phenyl ethyl acetate	1589	tr	-	-	1.1	-
Total % of identified compounds		99.2	99.9	100	99.9	100

*Retention indices on HP-1

in inhibiting mycelia growth of *P. angolensis* than that of *C. rigidus*. Plates with essential oil at a concentration lower than 1000 mg/l did not prevent fungal growth under the test conditions. Percentage of growth inhibition of the two essential oils were significantly ($P < 0.05$) influenced by incubation time and essential oil concentration. Myce-lia growth was considerably reduced with increasing con-centration of essential oils while their growth increased with incubation time. The MICs of the two essential oils varied with incubation time. It ranged from 5000 mg/l after 10 days of incubation, to 6500 mg/l after 40 days for *C. rigidus*, and from 4000 mg/l after 10 days of incubation to 6000 mg/l after 40 days for *C. citrinus*. This could be due to the fact that during a relatively long incubation period some volatile components in these oils may evaporate from the media, leading to decrease in their concen-tration.

The evaluation of the antifungal activity of these frac-tions enabled us to have an idea on the nature of the active components of our oils. The percentage inhibitions of each essential oil and its fractions at different concen-trations are represented in Tables 4 and 5.

Essential oil of *C. rigidus* (Table 4) presents a total inhi-bition at 6500 mg/l while fractions E (94.7% of 1.8-

cineole) and J (100% of 1.8-cineole) present it at 6000 mg/l. Fraction D which had a lower content of 1,8-cineole than the preceding ones (81.8%) but a α- terpineol (3.4%) content higher than the others, allowed total inhi-bition at 4000 mg/l. This enabled the assumption of a combined action from these two components or of a synergy between them.

In addition, an analytical comparison of the percentage inhibition of fraction J containing 100% of 1.8-cineole and synthetic 1.8-cineole on *P. angolensis* at different concentrations was conducted. It showed that the first induces total inhibition at 6000 mg/l while the second does so at 5500 mg/l.

Essential oil of *C. citrinus* (Table 5) and fraction E (91.4% of 1,8-cineole) present a total inhibition at 6000 mg/l while fraction L (88.7% of α-terpineol) presents it at 1500 mg/l. On the other hand, fraction D (59.7% 1.8-cineole and 0.3 % of α-terpineol) did not show a total inhibition for the concentrations lower or equal to 7000 mg/l. Considering that fraction 12 which is most effective, is primarily made up of α-terpineol (88.7%) and of terpinen-4-ol (10%), this study surmises that these com-pounds which are of alcoholic nature (recognized antimi-crobial) are partly responsible for the antifungal action.

Table 3. Percentages of growth inhibition of essential oils of *C. rigidus* and *C. citrinus* on *P. angolensis* at different concentrations.

Concentration (ppm)	*Callistemon rigidus*	*Callistemon citrinus*
1000	7.8 ± 1.3[a]	5.6 ± 2.5[a]
2000	25.5 ± 12.6[a]	10.0 ± 5.1[b]
3000	30.0 ± 1.3[a]	11.5 ± 2.2[b]
4000	34.4 ± 6.4[b]	43.2 ± 1.3[a]
5000	53.5 ± 3.8[b]	65.3 ± 6.8[a]
5500	64.6 ± 4.4[b]	84.5 ± 0.0[a]
6000	80.1 ± 2.2[b]	100 ± 0.0[a]
6500	100 ± 0.0[a]	100 ± 0.0[a]
7000	100 ± 0.0[a]	100 ± 0.0[a]

Means within rows with the same letter do not differ significantly in Duncan Multiple Range Test ($P < 0.05$).
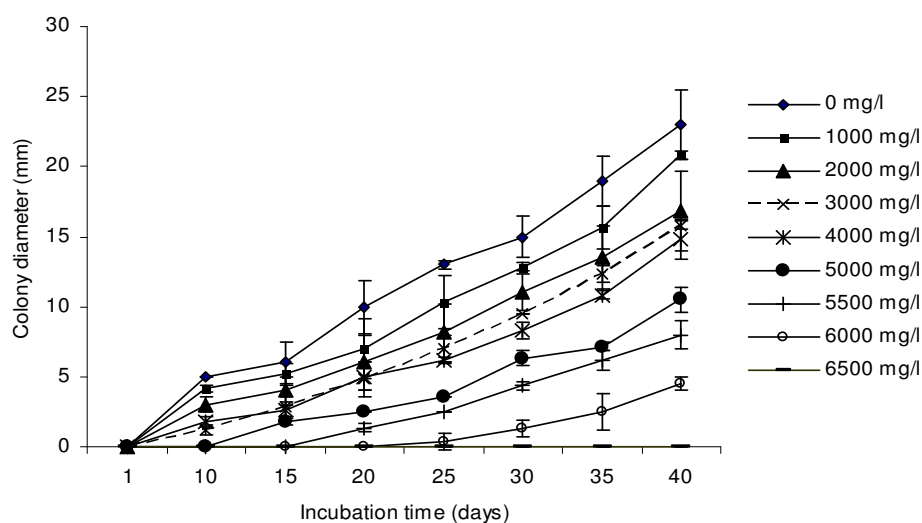

Figure 1. Effect of different concentrations of C. rigidus essential oil on P. angolensis growth.

This is more true since the fraction with high percentage of 1.8-cineole and without α-terpineol, nor terpinen-4-ol presented a concentration of total inhibition at 6000 mg/l, just like essential oil.

Pandey (1995) had already shown the fungicidal effect of the essential oil of a close species, *Callistemon lanceolatus*, on several fungi with an inhibiting minimal concentration of 2000 mg/l. This difference with the two essential oils tested in the present work can be explained by the difference in their composition which emanates from the different vegetable species.

In addition, Jazet et al. (2002) showed an inhibition of the growth of *P. angolensis* by essential oils of *Citrus* at varied degrees, with a more marked activity for oils resulting from *C. latifolia* and *C. limon* which respectively revealed a total inhibition at 2500 and 4000 mg/l. This difference in effectiveness is explained by a difference in chemical composition between our extracts and those of both *Citrus.*

In the research of the nature of inhibition, the mycelial discs used came from Petri dishes where no growth was observed and this for each extract of EO tested and some of their fractions. Indeed, the discs which came from the EO concentrations having shown a total inhibition until the 40th day were again sown in a non-built-in culture medium of EO. After 30 days of incubation, no growth was observed for all the mycelial discs having undergone beforehand total inhibition by the rough extracts. In the same way, the mycelial discs resulting from fraction 12 of *C. citrinus* and fraction 10 of *C. rigidus*
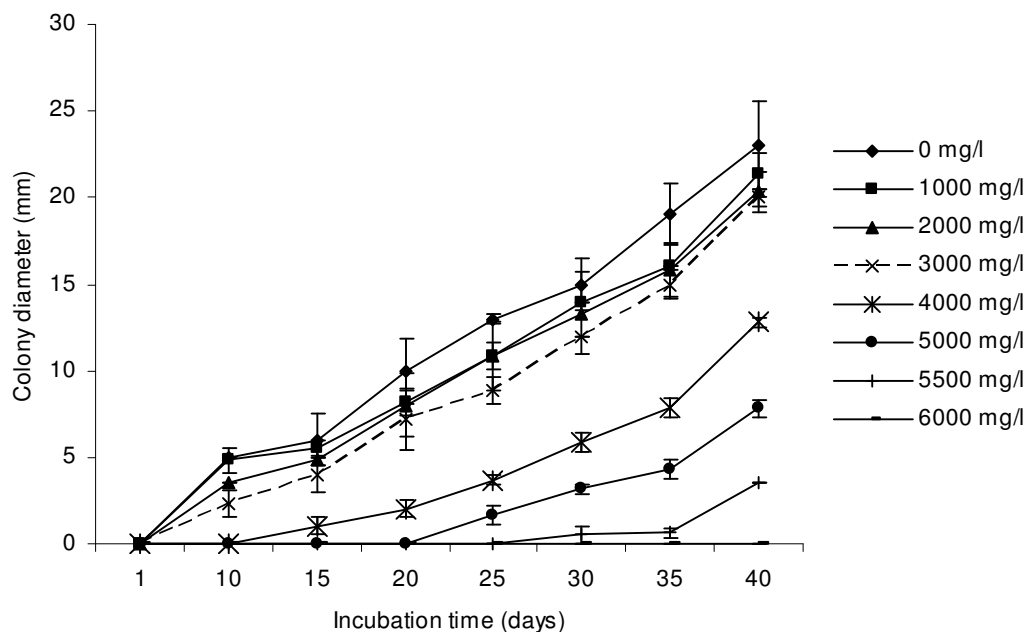
Figure 2. Effect of different concentrations of *C. citrinus* essential oil on *P. angolensis* growth.

Table 4. Percentages of growth inhibition of *C. rigidus,* its fractions and 1.8-cineole on *P. angolensis*.

Concentration (ppm)	Essential oil	Fraction D	Fraction E	Fraction J	1,8-cineole
1000	7.8 ± 1.27^d	16.7 ± 1.3^a	10.0 ± 1.3^c	12.3 ± 1.3^b	13.2 ± 1.8^b
2000	25.5 ±12.6^{ab}	27.0 ± 0.0^a	14.5 ± 1.3^c	17.1 ± 0.0^b	16.4 ± 2.2^b
3000	29.9 ± 1.3^b	39.6 ± 3.4^a	26.3 ± 2.5^b	27.7 ± 2.5^b	27.4 ± 3.7^b
4000	34.4 ± 6.4 ^{b}	100 ± 0.0^a	35.1 ± 3.4^b	31.4 ± 3.4^b	33.2 ± 1.2^b
5000	53.5 ± 3.8 ^{b}	100 ± 0.0^a	46.9 ± 3.8^c	44.7 ± 3.8 ^{c}	55.8 ± 0.0^b
5500	64.6 ± 4.4 ^{c}	100 ± 0.0^a	70.2 ± 0.8^b	73.4 ± 4.4^b	100 ± 0.0^a
6000	80.1 ± 2.2 ^{b}	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a
6500	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a	100 ± 0.0^a

Means within rows with the same letter do not differ significantly in Duncan Multiple Range Test (P < 0.05).

Table 5. Percentages of growth inhibition of *C. citrinus* and its fractions on *P. angolensis*.

Concentration (ppm)	Essential oil	Fraction D	Fraction E	Fraction L
1000	5.6 ±1.6^b	5.6 ± 1.6^b	3.4 ± 1.3^c	52.1 ± 3.4^a
1500	8.4 ±1.7^b	7.8 ± 0.0^b	5.4 ± 1.3^c	100 ± 0,0^a
2000	10.0 ± 5.0 ^{b}	12.0 ± 1.1^b	14.5 ± 3.4 ^{b}	100 ± 0,0 ^{a}
3000	11.5 ± 2.2 ^{c}	15.9 ± 5.8 ^{bc}	17.4 ± 2.5 ^{b}	100 ± 0,0 ^{a}
4000	43.3 ± 1.3 ^{b}	27.7 ± 1.3 ^{d}	32.2 ± 3.4 ^{c}	100 ± 0,0 ^{a}
5000	65.3 ± 6.7 ^{b}	39.6 ± 3.4 ^{d}	46.2 ± 1.3 ^{c}	100 ± 0,0 ^{a}
6000	100 ± 0.0 ^{a}	60.2 ± 7.6 ^{b}	100 ± 0.0 ^{a}	100 ± 0,0 ^{a}
7000	100 ± 0,0 ^{a}	77.8 ± 7.1 ^{b}	100 ± 0.0 ^{a}	100 ± 0,0 ^{a}

Means within rows with the same letter do not differ significantly in Duncan Multiple Range Test (P < 0.05).

rich in α-terpineol (88.7%) and 1.8-cineole (100%) respectively, did not present any growth. Essential oils of the leaves of *C. citrinus* and *C. rigidus* would be fungicidal on this fungus. This would result fromthe fact that these two extracts are relatively rich in oxygenated com-pounds which according to Griffin and Wyllie (1999) are the origin of the fungicidal effect of essential oils. In conclusion, these two extracts could constitute an alternative to fungi-cides of synthesis usually used for citrus fruit crops.

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