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Full Length Research Paper

Essential oils of *Citrus aurantifolia* from Cameroon and their antifungal activity against *Phaeoramularia angolensis*

P. M. Jazet Dongmo[1,2], L. N. Tatsadjieu[3]*, E. Tchinda Sonwa[1], J. Kuate[4], P. H. Amvam Zollo[1] and C. Menut[5]

[1]ENSAI, P. O. Box 455, University of Ngaoundere, Ngaoundere, Cameroon.
[2]Faculty of Science, P. O. Box 24157, University of Douala, Douala, Cameroon.
[3]IUT, P. O. Box 455, University of Ngaoundere, Ngaoundere, Cameroon.
[4]Institute of Agricultural Research for Development (IRAD), Yaounde, Cameroun.
[5]IBMM-UMR 5247, ENSCM 8, Rue de l'Ecole normale, 34296, Montpellier, cedex 5.

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Essential oils of three varieties of *Citrus aurantifolia* (Bearss, Mexican and "Sans épines") grown in Cameroon were extracted by hydrodistillation with yields of 0.29, 0.57 and 0.25% respectively. The chemical analysis was carried out by gas chromatography and gas chromatography coupled with mass spectrometry. The three samples were found to be very rich in monoterpenes, and limonene was the major compound with 53.92, 43.53 and 51.98% respectively. The determination of the antifungal activity was done by the incorporation technique. The three extracts exerted an inhibiting action on the radial growth of *Phaeoramularia angolensis* which is fungicidal. The MIC obtained is 1.4 mg/ml for *C. aurantifolia* var. Mexican and 1.5 mg/ml for Bearss and "Sans épines" varieties. These three essential oils could constitute an alternative to chemical fungicides usually used for *Citrus* fruit crops. In addition, the essential oil of the Bears variety was fractionated and the fractions were tested in order to determine the active compounds. The most active fractions are rich in neral and geranial, compounds which could thus be responsible for the activity of the crude extract.

Key words: Essential oil, *Citrus aurantifolia*, monoterpenes, sesquiterpenes, *Phaeoramularia angolensis*, antifungal activity.

INTRODUCTION

The *Citrus* fruits make up a group of plants of great importance. In 1989, they represented the first fruit-bearing product by their volume of exploitation with 65 million tons ahead of the grape. Citrus is actually the world' second fruit crop after banana by the volume produced (more than 108 million tons in 2006) (FAO, 2006). Citrus fruits are rich in mineral salts and vitamin C. They can be consumed fresh or after transformation into juice, jam and liquor (Loeillet, 1991). Essential oils obtained from Citrus are of great commercial interest due to their chemical composition responsible for their odorous properties, pharmaceutical, and organoleptic interest. Thanks to these properties, they constitute the raw materials for perfumery, cosmetic, food, chemical and pharmaceutical industries.

In tropical Africa particularly in Cameroon, efforts to develop this crop are hindered by many obstacles amongst which are parasitic diseases. Indeed, for a few decades' pathogenic fungi, *Phaeoramularia angolensis* has inflicted heavy losses on citrus fruit harvests, over 80% loss of total harvest in certain areas of the country (Kuate, 1997).

Analysis of the use of some chemical compounds against *P. angolensis* showed inhibitory effects of copper based fungicides on the fungus (Seif and Hillocks, 1993). This chemical control remains however difficult to manage, not only because of numerous possible resistances but also because of the harmful effects on man and the environment (Blasco et al., 2005; Jawich, 2006). Due to these adverse effects there is urgent need to look for an alternate antifungal agent to replace these chemical fungicides.

*Corresponding author. E- mail: tatsadjieu@yahoo.fr.

The antimicrobial activities of essential oils has been scientifically confirmed by many researchers, thus could serve as possible alternates for antibiotics in infectious pathologies (Carson and Riley, 1995; Joulain and König, 1998; Lambert and Skandamis, 2001) and fungicides in phythopathology (Jazet Dongmo et al., 2002). The aim of the present work was to test the effects of essential oils extracted from the fresh leaves of three varieties of *Citrus aurantifolia* on the growth of *P. angolensis* and to identify their active components.

MATERIALS AND METHODS

Plant material

Fresh leaves from *C. aurantifolia* var Bearss, Mexican and "Sans épines" were collected from the experimental plot of the Institute of Agricultural Research for Development (IRAD), Nkolbisson, Yaounde in March 2006.

Fungal material

Phaeoramularia angolensis was the isolate CMR4 obtained from *Citrus sinensis* var Valencia late and maintained in the IRAD Regional Biological Control and Applied Microbiology Laboratory at Nkolbisson Yaoundé. It was grown on Potatoes dextrose agar (Difco, Detroit, MI) at 22°C for 15 days and stored at 4°C until used.

Extraction and fractionation

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 used. The fractionation of the essential oil was performed using the column chromatography with Silica Gel G60 (63-200µm). Fractions 0-13 were obtained using hexane/ether system as mobile phase.

Chemical analysis

Gas chromatography (GC) analysis

Essential oil (10 µl) was dissolved in pentane (100 µl) and 2 µl of the solution was injected into a GC-17A with Flame Ionization Detector (FID) and C-R6A-chromatopac integrator (Shimadzu Co, Japan). The column used was SUPELCOWAX fused silica (film thickness: 0.2 µm, Supelco USA, 60 m x 0.25 mm). Column temperature was programmed to range from 40 to 250 °C with a rate of 6°C/min. Injector and detector temperatures were 220 and 280 °C, respectively. The carrier gas was hydrogen. Quantification was carried out by % peak area calculations (GC/FID using a non-polar column).

Gas Chromatography – Mass Spectrometry

For GC/MS analysis, a GC–17A with a QP5050 mass spectrometer (Shimadzu Co, Japan) was used. Helium was used as carrier gas at a flow rate of 0.6 ml/min; the mass spectrometer was operated at 70 eV. Column temperature was programmed from 70 to 200°C at 10°C/min; injector temperature was 250°C. Mass spectra correlations were carried out with Wiley, NBS, NIST and private aroma library spectra.

Identifications were determined by comparing the retention time of each compound with that of known compounds (Jennings and Shibamoto, 1980; Joulain and König, 1998; Adams, 2001).

In vitro antifungal test

The antifungal assay was performed by the agar disc diffusion method (de Billerbeck et al., 2001). The PDA (Potatoes dextrose agar) medium with different concentrations of essential oils (1, 2, 3, 4 and 5 mg/ml) was prepared by adding appropriate quantities of essential oil to melted SMKY agar, followed by manual rotation of Erlenmeyer to disperse the oil in the medium. About 20 ml of the medium were poured into glass Petri–dishes of 55 mm at a rate of 10 ml per dish and allowed to rest for solidification. Each Petri–dish was inoculated at the centre with a mycelial disc (6 mm Ø) taken at the periphery of a *Phaeoramularia angolensis* colony grown on PDA agar for 15 days. Control plates (without essential oil) were inoculated following the same procedure. The dishes were incubated in an inverted position at 22°C in the dark. After 10 days, mycelial growth was observed with the diameter measured along two perpendicular lines passing through the centre of the dish, with a 5 days regular interval for up to 40 days. Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration of essential oil in which no growth occurred. Citral was also assayed for comparison activity.

The antifungal index (AI) was calculated as follows (Sheng-Yang et al., 2005):

$$AI = 1 - \frac{Go}{Gc}\, x\, 100,$$

G_o = diameter of growth zone in the test plate
Gc= the diameter of growth zone in the control plate.

Each experiment was performed three times, and the data were averaged.

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

Extraction yields

The essential oil yields obtained were 0.29% for *C. aurantifolia* var. Bearss , 0.57% for *C. aurantifolia* var. Mexican and 0.25% w/w for *C. aurantifolia* var. "Sans épines". These yields varied for the same species of *Citrus* according to the variety.

Chemical composition of essential oils

The chemical compositions of the three essential oils are presented in Table 1 along with those of the fractions obtained from *C. aurantifolia* var. Bearss.

We noticed that the three varieties of *C. aurantifolia* contained high percentage of limonene (53.92% ,43.53% and 51.98% respectively for the Bearss, Mexican and

Table 1. Chemical composition of essential oils of the leaves of *C. aurantifolia* var. Mexican, "Sans épines", Bearss (and its fractions).

Compounds	IK	Mexican	"Sans épines"	Bearss				
				Essential oil	Fraction 2	Fraction 5	Fraction 8	Fraction 12
Monoterpenes hydrocarbons		**54.33**	**60.34**	**61.48**	**88.66**	**14.74**	**55.35**	**1.87**
α-Pinene	934	0.34	0.34	0.33	0.59	0.51	0.40	-
Camphene	965	2.25	2.98	-	-	-	-	-
Sabinene	969	1.18	0.47	2.06	1.16	-	2.96	-
β-Pinene	978	0.32	0.43	0.97	0.97	-	0.61	0.72
Myrcene	982	1.47	1.58	1.58	1.58	1.12	-	0.68
δ-3- carene	1011	0.30	-	-	-	-	-	-
Limonene	1029	43.53	51.98	53.92	85.79	12.35	50.59	1.05
Z-β-ocimene	1038	2.73	2.56	2.06	-	1.27	-	-
γ-Terpinene	1055	0,26	-	-	-	-	-	-
Terpinolene	1087	1.50	-	-	-	-	-	-
Isocamphene	1146	0.45	-	0.56	-	-	-	-
Oxygenated monoterpenes		**39.34**	**35.03**	**35.81**	**7.61**	**80.21**	**40.04**	**97.14**
Linalool	1088	-	1.14	1.20	-	1.41	1.06	-
E-pinocarveol	1136	1.72	1.12	1.32	-	3.58	-	-
Borneol	1164	0.64	-	0.76	-	-	-	-
Terpinen-4-ol	1175	0.63	-	-	-	-	-	-
Myrtenal	1180	0.33	-	-	-	-	-	-
α-terpineol	1184	0.35	0.48	0.42	-	2.04	-	6.55
Citronellol	1216	1.27	1.37	0.67	2.02	-	-	-
Nerol	1218	2.65	3.96	1.38	1.22		-	18,53
Neral	1225	10.00	7.72	9.88	2.33	-	15.16	33.19
Geraniol	1242	4.02	4.65	1.33	-	1.38	1.69	33.70
Geranial	1253	12.57	10.92	12.26	-	0,82	20,47	4,15
Neryl acetate	1344	1.92	1.31	4.56	1,12	45,12	1,01	1,02
Geranyl acetate	1362	3.24	2.36	2.03	0,92	25,86	0,65	-
Sesquiterpenes hydrocarbons		**5.29**	**3.80**	**2.63**	**1.49**	**0.72**	**0.99**	**-**
β-elemene	1397	1.03	0.40	2.02	0.81	-	0.59	-
β-caryophyllene	1434	2.62	2.20	0.61	0.68	0.72	0.40	-
α- humulene	1469	0.45	0.36	-	-	-	-	-
germacrene D	1499	0.54	0.42	-	-	-	-	-
β- bisabolene	1509	0.40	0.42	-	-	-	-	-
δ- cadinene	1540	0.25	-	-	-	-	-	-
Oxygenated sesquiterpenes		**0.77**	**0.81**	**-**	**-**	**-**	**-**	**-**
Caryophyllene oxide	1595	0.43	0.50	-	-	-	-	-
α- eudesmol	1632	0.34	0.31	-	-	-	-	-

"Sans épines"); followed by geranial and the neral (12.26% and 9.88% for Bearss,12.57% and 10.00% for Mexican, 10.92% and 7.72% for "Sans épines"). The high content of limonene in these oils 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 tumours in rats. They also have a direct tumorstatic effect, meaning they inhibit the growth of tumours, 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).

The fractionation of *C. aurantifolia* var. Bearss enabled us to have four different fractions. Fraction 2 was rich in monoterpenes hydrocarbons with limonene (85.79%) as major compound. Fraction 5 was, rich in oxygenated monoterpenes, it was mostly consisted of neryl acetate (45.12%) and geranyl acetate (25.86%). Fraction 8 was also rich in monoterpene hydrocarbons (50.59% of limo-

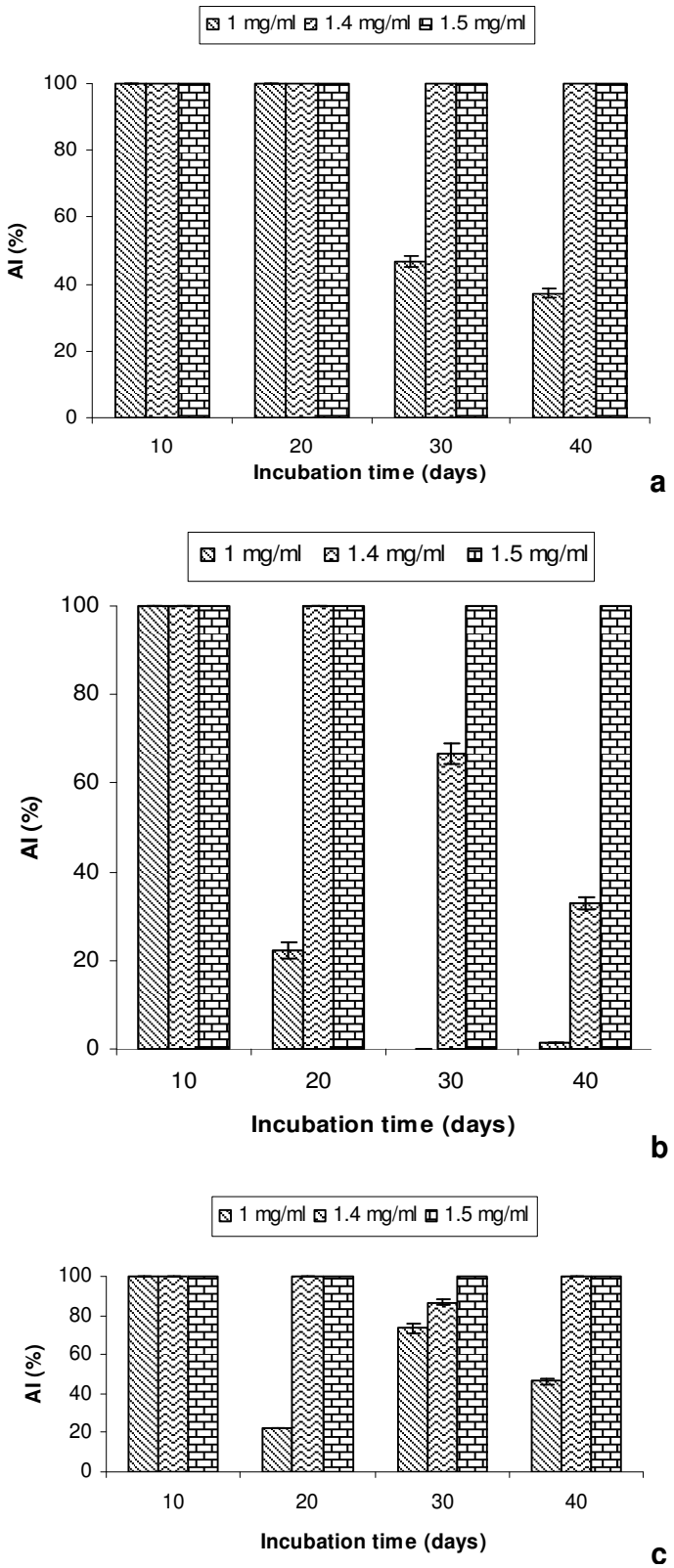

Figure 1. Antifungal activity of *C. aurantifolia* against *P. angolensis*
a: var. Mexican
b: var. Bearss
c: var. "sans épines"

nene) but it contained significant proportions of neral (15.16%) and geranial (20.47%). Fraction 12 was dominated by oxygenated monoterpenes with nerol (18.53%), neral (33.19%) and geraniol (33.70%).

Antifungal activity

Figure 1 shows the antifungal activities of the fresh leaves of *C. aurantifolia* var. Mexican, *C. aurantifolia* var. Bearss and *C. aurantifolia* var. "Sans épines" respectively. Mycelial growth was considerably reduced with increasing concentration of essential oil while their growth increased with incubation time. The MIC of the three essential oils were 1.4, 1.5 and 1.5 mg/ml after 40 days of incubation respectively for *C. aurantifolia* var. Mexican, *C. aurantifolia* var. Bearss and *C. aurantifolia* var "Sans épines". To the best of our knowledge, there has not been a relevant study investigated the effectiveness of *C. aurantifolia* and its fractions against *P. angolensis*. The effect of citral and the main fractions of *C. aurantifolia* var. Bearss were also tested against *P. angolensis*. Citral which constituted our reference sample compared to the fractions was seen to be very active against *P. angolensis* with a MIC of 400 mg/l. Fraction 8, was twice more effective than the crude essential oil of *C. aurantifolia* var. Bearss, followed by fraction 12, which activity was almost identical to that of the crude essential. Fraction 2 was far less active. The antimicrobial activity of citral has already been proven (Onawunmi et al., 1984; Mishra et al., 1988; Knobloch et al., 1989). Fraction 2 which was the least effective (4.5 mg/ml) contained a small proportion of citral (2.43 %). It however contained limonene (89.48%) as main component, thus limonene could not be responsible for the activity of the essential oil. Fraction 12 which contained more citral (37.34 %) than the fraction 8 (35.63 %) was less active. This could be explained by the difference in geranial contain. Fraction 12 has 4.15 % geranial while the fraction 8 has 20.47 %. We can say that geranial presents in the essential oil of *C. aurantifolia* would be most active. We do not want to exclude the idea that the activity of essential oil would be reinforced by other minority compounds which would act in a synergistic way. Moreover, the fact that the fraction rich in limonene (majority compound of C*itrus*) was revealed not to be very active confirmed the fact that the activity of essential oils should not be reduced to the activity of its majority compounds, or those likely to be active. In this way, the value of an essential oil depends on its "totum", i.e. the totality of its components and not only to its majority compounds (Lahlou, 2004).

At the different MIC of the three essential oils, the mycelial discs were transferred into PDA without essential oil and no growth was observed until 40 days of incubation indicating a lethal effect at these concentrations. This indicates that our extracts were fungicidal, it did not only stop the growth but killed the germ. This could be explained by the very rich presence in the oil of aldehyde

which are powerful antimicrobial agents (Franchomme et al., 1990). According to the same authors, the aldehyde group combined with it double bonds, as it is the case with the neral and the geranial, is strongly electronegative; electronegative compounds being able to induce reactions of electron transfer and do react with vital nitrogen compounds in the microbial cell such as nucleic proteins and acids.

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