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Full Length Research Paper

Composition, radical scavenging and antifungal activities of essential oils from 3 *Helichrysum* species growing in Cameroon against *Penicillium oxalicum* a yam rot fungi

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The chemical composition of essential oils from leaves of *Helichrysum cameroonense* Hutch. and Dalziel, *Helichrysum cymosum* Sensu C. D. Adam*, Helichrysum globosum* Sch. Bip. ex A. Rich. and flowers of *Helichrysum cameroonense* was examined by means of GC and GC-MS. In addition, the radical scavenging and antifungal activities were assayed according to the DPPH (Diphenyl Picryl Hydrazyl) and dilution methods respectively. The results showed a predominance of monoterpenes in the oils of the leaves (89.5%) and flowers (62.9%) of *H. cameroonense* and of sesquiterpenes (58.0%) in that of *H. cymosum*. Extract from *H. globosum* was characterized by comparable proportions of monoterpenes (49.6%) and sesqiterpenes (49.1%). The major components were found to be α-pinene (46.4%) and camphor (11.5%) in the oil of leaves of *H. cameroonense* while α-pinene (31.4%) and guaienol (18.9%) were those from the flowers of the same specie. ∆-3-Carene (16.1%) and β-caryophyllene (12.0%) were the predominant compounds of the essential oils of *H. cymosum* and α-pinene (36.6%), valerianone (10.9%) were those of *H. globosum.* The radical scavenging activities of essential oils from the leaves of *H. cameroonense* and *H. cymosum* were found to be 4.9 and 6.3 g/l respectively while butylated hydroxy toluene (BHT) used as reference has a SC_{50} =7.0 mg/l, On another hand, the essential oils from the leaves of these *Helichrysum* species showed significant antifungal activities against *Penicillium oxalicum*, with percent inhibition at 5 mg/mL ranging from 54.7 to 100%. These results highlight the potential of some essential oils as an alternative tool to fight this pathogen which is one of the most prevailing causing agent of soft rot of post harvest yam tuber.

Key words: *Helichrysum,* Asteraceae*,* essential oil, radical scavenging activity, antifungal activity, *Penicillium oxalicum,* yams, Discoreaceae.

INTRODUCTION

Yams are monocotyledonous flowering plants belonging to the family Discoreaceae and to the genus *Dioscorea* (Coursey, 1967). Being the staple food for more than 400 million people, yams are one of the most important crop plants in Africa. The 6 west and central African countries from Côte d'Ivoire to Cameroon produce over 95% of the world's yam (FAO, 2003). Though this crop is of immense importance in Cameroon, it has not received the attention it deserves with regards to the large quantities of yam becoming unsuitable for use because of deterioration

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caused by pests and diseases in storage. Several pathogenic fungi of the genus *Penicillium* have been found associated with yam, causing root rots, decreasing their commercial and nutritive value and producing a number of toxic metabolites including aflatoxin (Adeniji, 1970). Application of fungicide during packing can reduce the rate of decay (Thayer, 1984; Crowe, 1980). However, fungicides have various setbacks such as development of new resistance strains in the treated fungi, environmental toxic residues and eventual toxicity to consumers. Therefore, biodegradables alternatives should be developed for reducing postharvest losses. For this end, selected plants and their essential oils have been evaluated as natural sources of compounds to combat a variety of fungi, insects and other storage pests (Singh and Upadhyay, 1993; Tapondjou et al., 2003; Ngamo et al., 2005). In the mountain region of west Cameroon, *Helichrysum* species are traditionally used for the protection of post-harvest food. Some species are also known in folk medicine for the treatment of acute hepatitis, fever, or edema (Bougatsos et al., 2004).

This paper then reports the phytochemical composition of essential oils from the leaves of *Helichrysum cameroonense*, *Helichrysum cymosum, Helichrysum globosum* and flowers of *H. cameroonense*. In addition, their antifungal activities were evaluated against *Penicillium oxalicum* isolated from a rot yam tuber. Since reactive oxygen species are well recognized to be the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis, *etc* (Halliwell and Gutteridge, 1999), we also evaluated the free radical scavenging of the oils of *H. cameroonense* and *H. cymosum,* plants that are expected to prevent these free radical mediated diseases. This is the first report of the chemical composition, the free radical scavenging and antifungal activities of essential oils from *H. cameroonense*.

MATERIALS AND METHODS

Plant material

Helichrysum species were collected in Bamumbou (Cameroon), a village located on the Bamboutos mountain grassland, particularly on broken rocky ground (1900 - 2500 m altitude) in September 2006. The specimens were identified at the Yaoundé national herbarium by M Ghogue. Voucher specimens were deposited at that herbarium under the following identification numbers: *H. cameroonense (*12164 SRF/CAM); *H. cymosum (*7514 NHC); *H. globosum (*11122/HNC. The whole plant samples collected in the morning at stage of maturity were dried at room temperature (20 - 25°C) for 48 h (light conditions); flowers were separated from the twigs before drying in the same conditions.

Isolation of essential oils

Air-dried plant materials (400 g) were subjected to hydrodistillation using a clevenger-type apparatus for 5 h. The oil layers obtained were dried over anhydrous Na_2SO_4. The yields were averaged over 3 experiments and calculated on the basis of dried weight material.

GC analyses

GC analyses were performed on a varian CP-3380 gas chromatograph equipped with flame ionization detectors using a fused silica capillary column (30 m x 0.25 mm i.d, 0.25 µm film thickness), coated with DB-1. The oven temperature was programmed from 50 to 200°C at 5°C/min. Injector and detector temperature were 220 and 250ºC respectively. The carrier gas was N_2 (0.8 ml/min). The linear retention indices of the components were determined relative to the retention times of a homologous series of *n*-alkanes and the percentage compositions were obtained directly from a Shimadzu C-R4A recorder by electronic integration measurements.

GC-MS analyses

GC-MS analyses were carried out using a Hewlett-packard apparatus equipped with an HP-1 fused silica column (30 m x 0.20 mm, film thickness, 0.25 µm) and interfaced with a quadruple detector (Model 5970). Column temperature was programmed from 70 to 200°C at 10°C/mn; injector temperature was 220°C. Helium was used as carrier gas at a flow rate of 0.6 ml/min, the mass spectrometer was operated at 70 eV.

Identification of oils components

The components were identified based on the comparison of their retention indices and their mass spectra with those given in the literature (McLafferty and Stauffer, 1989; Adams, 2001).

Determination of the radical scavenging activity

The radical scavenging activity was determined by the DPPH (2,2-diphenyl-1-1-picrylhydrazyl) method (Miliauskas et al., 2004). DPPH, a stable free radical, was dissolved in ethanol to obtain a 100 µM solution. To 2 ml of the ethanolic solution of DPPH was added 100 µl of a methanolic solution of butylated hydroxytoluene (BHT) set as reference at different concentrations. The oils were rigorously evaluated in the same conditions. Negative control consisted of the mixture of 2 ml of DPPH solution and 100 µl of methanol. The decreased in absorption was measured at 517 nm after 30 mn, at room temperature. The relevant decrease in absorption induced by the test compound was calculated by subtracting that of the negative control. The concentration required for 50% reduction (SC_{50}) was determined graphically. All the spectrophotometric measurements were per-formed on a SAFAS UV-mc^2 spectrophotometer, equipped with a multi-cells/ multikinetics measurement system and with a thermostated cell-case. Each experiment was done in triplicate.

Fungal strain and media

P. oxalicum used in the antifungal assays was isolated and purified from infected yams tubers that have been maintained at the laboratory of biochemistry of the Douala university. The mycopathogen was maintained on potato dextrose agar (PDA). The cultures were stored at 4°C and sub-cultured once a month.

Antifungal assays

The antifungal activity of the essential oils was evaluated by the method of dilution on a solid medium (Lahlou, 2004). The test was performed in sterile Petri dishes (90 mm diameter) containing PDA as medium. Different concentrations of essential oils (5, 2.5, 1.25, 0.625 and 0.312 mg/ml) were prepared by adding appropriate quantity of essential oil to melted medium, followed by gently agita-

tion to disperse the oil in the medium (Table 4). About 20 ml of the medium were poured into individual Petri dish. After solidification, each dish was inoculated at the center with a fresh mycelium culture of test fungus (6 mm in diameter) taken at the periphery of an *P. oxalicum* colony grown on PDA for 72 h. The Petri dishes were then incubated at 25°C and the colony diameter was recorded each day for 7 days. The negative control consisted only of solvent (without essential oil) and the positive control of amphotericine B, prepared at the same w/v concentrations as oils. All experiments were carried out in triplicate and reported data represent average values ± SD.

RESULTS AND DISCUSSION

The essential oils were yellowish with distinct sharp odours. They were obtained with yields ranging from 0.02 to 0.12% weight /weight (Table 1).

The chemical compositions are given in Table 1 where components are listed according to their order of elution on the DB-1 column. From these results, the oils from *Helichrysum* species were found to be quantitatively and qualitatively variable. Those from *H. cameroonense* (leaf and flower) were found to contain higher amounts of monoterpenes (62.9 - 89.5%) with α-pinene (31.4 - 46.4%) as the main constituent. In previous studies, many authors have identified this compound as the major constituent of different essential oils from *Helichrysum* species (Roberto et al., 2002; Bougatsos et al., 2004; Lourens et al., 2004). Beside this main component, the other important compounds that can be mentioned were δ-guaienol (18.9%) for the flower oil and camphor (11.5%) for leaf oil. Concerning the essential oil from *H. cymosum*, only β-caryophyllene (12.0%) and Δ-3-carene (16.1%) were found to an amount above 10%. The appreciable amount of β-caryophyllene is in agreement with previous reports on *Helichrysum dasyanthum* (13.3%), *Helichrysum felinum* (27.6%) and *Helichrysum petiolare* (22.4%) from South Africa (Lourens et al., 2004) as well as *Helichrysum odoratissimum* oils from Cameroon (Kuiate, 1999). The oil isolated from *H. globosum* was characterized mainly by terpenes, with similar quantities of monoterpenes (49.6%) and sesquiterpenes (49.1%). α-pinene (38.6%) and valerianone (10.9%) were found to be the main components. Other constituents were identified in appreciable amounts in the oil from *H. globosum*, including α-selinene (8.6%), Δ-3-carene (7.2%) and β-selinene (6.0%).

A comparative study of the essential oils analysed in this study highlighted the predominance of monoterpenes in *H. cameroonense* extracts (62.9 - 89.5%). Furthermore, these are the only samples containing fewer amounts of aliphatic hydrocarbons (1.1 - 1.2%). In the essential oil obtained from *H. cymosum,* sesquiterpenes (58.0%) were found to be more abundant. From the same study, α-pinene (6.8 - 46.4%) was found to be characteristic of all the studied *Helichrysum* oils.

The essential oils from the leaves of *H. cameroonense* and *H. cymosum* were also evaluated for radical scavenging activity. The results are summarized in Table 2. From these results, it is found that the radical scavenging activities of *H. cameroonense* (SC_{50} = 4.9 g/l) and *H. cymosum* (6.3 g/l) are less than that of butylated hydroxy toluene (BHT) used as reference (SC_{50} = 7.0 mg/l).

The antifungal activity of the essential oils was evaluated by means of dilution on a solid medium. The percentages of growth inhibition (Tables 3A and 3B) were obtained according to the method described by Lahlou (2004). From these results, the fungitoxic activity significantly varied from one essential oil to another. The extract from *H. cameroonense* showed more toxicity against the tested fungus, with 100% inhibition starting from 2.5 to 5 mg/ml. The other 2 oils from *H. cymosum* and *H. globosum* exerted a growth inhibition above 50% at the same concentrations.

Essential oils from various sources exhibit broad-spectrum antimicrobial activity. Their biological activities have been related to their chemical composition. Indeed, compounds such as α-pinene, limonene, α-terpineol, carvone, 1,8-cineole, ascaridole have been shown to exert various biological activities. These compounds increase fungal cell permeability and membrane fluidity and inhibit medium acidification. Moreover, terpenes are thought to induce alterations in cell permeability by inserting between the fatty acyl chains that make up the membrane lipid bilayers, disrupting lipid packing and causing changes to membrane properties and functions (Sikkema et al., 1995; Christine et al., 2002). This theory is strongly supported by data from previous studies demonstrating changes in permeability and increases in membrane fluidity after treatment with terpenes (Uribe, 1985; Bard et al., 1988; Hammer et al., 2004).

Concerning the *Helichrysum* oil samples reported in this study, they are qualitatively and quantitatively different. This could explain the variation in activities. On another hand, α-pinene (31.4 – 46.4%) which is prominently present in the oils from *H. cameroonense* and *H. globosum*, may significantly contribute to their antifungal activity as mentioned above and underlined by Bougatsos et al., (2004). In addition, the inhibiting activeties of these essential oils may not only be attributable to their major components. Indeed, there is another option of whole extract-action through a synergistic effect of individual compounds on each other (Valnet, 1980).

From an analytical comparison of statistical data between the activities of essential oils reported here and that of amphotericine B, they show comparable patterns at concentrations from 1.25 to 5 mg/ml ($P > 0.05$). But at lower concentrations, amphotericine B is more active ($p < 0.05$) (Table 4, Figure 1).

Conclusion

The results of this study highlight the volatile extracts from *Helichrysum* species as interesting growth inhibitors of *P. oxalicum*, one of the causative agents of soft rot

Table 1. Yields and percentage composition of essential oils of *Helichrysum* species from Cameroon.

Components	Percentages of constituents *				
	RI onDB-1	***H. cameroonense (leaves)***	***H. cameroonense (flowers)***	***H. cymosum (leaves)***	***H. globosum (leaves)***
Linear aliphati compounds		1.2	1.1	-	<0.1
Nonanal	1010	-	<0.1	-	-
Octen-3ol	1017	1.2	1.1	-	<0.1
Monterpenes		89.5	62.9	41.4	49.6
Monoterpene hydrocarbons		54.7	35.2	37.5	48.3
Bornylene	907	-	-	2.7	-
α-Thujene	930	<0.1	<0.1	-	-
α-Pinene	936	46.4	31.4	6.8	38.6
Camphene	949	-	-	7.4	-
Verbenene	953	2.6	<0.1	-	<0.1
Sabinene	971	1.1	<0.1	-	<0.1
β-Pinene	978	2.0	1.6	0.6	-
Myrcene	984	<0.1	1.0	-	<0.1
α-Terpinene	1000	-	-	1.6	-
p-Cymene	1016	<0.1	<0.1	0.6	-
Δ-3-Carene	1027	1.5	<0.1	16.1	7.2
limonene	1029	-	-	1.7	-
(Z)-β-Ocimene	1037	-	-	<0.1	-
(E)-β-Ocimene	1054	1.1	-	<0.1	-
γ-Terpinene	1086	-	1.2	-	-
Terpinolene	1089	-	-	<0.1	2.5
Oxygen-containing monoterpenes		34.8	27.7	3.9	1.3
1,8-Cineole	1031	-	-	1.7	-
Linalool	1091	-	<0.1	<0.1	-
Octyl acatate	1122	-	-	1.6	-
Campholenal	1027	1.0	3.1	-	-
Pinocarveol	1137	3.2	<0.1	-	-
Camphor	1141	11.5	1.7	-	-
α-Phellandrol	1158	5.3	3.0	-	-
Borneol	1165	-	-	<0.1	-
Pinocarvone	1173	-	3.8	-	-
Terpinen-4-ol	1174	3.3	<0.1	<0.1	-
α-Terpineol	1185	1.3	1.7	0.6	1.3
Myrtenol	1193	1.8	2.0	-	-
p-Cymen-8-ol	1197	4.1	2.8	-	-
myrtenyl acetate	1316	3.3	8.5	-	-
Geranyl acetate	1363	-	1.1	-	-
Sesquiterpenes		8.9	35.7	58.0	49.1
Sesquiterpene hydrocarbons		1.3	7.8	48.2	28.4
δ-Elemene	1346	-	-	1.9	-
α-Copaene	1389	-	1.4-	0.5	-
β-Elemene	1398	-	-	0.8	-
β-Caryophyllene	1435	1.3	3.8	12.0	3.8
Aromadendrene	1456	-	-	3.6	-
Methyl hexyl bourgene	1470	-	-	7.2	-
α-Humulene	1473	-	<0.1	5.6	3.0

Table 1. Contd.

γ-Selinene	1487	-	-	1.2	4.1
Germacrene D	1494	-	-	0.6	-
β-Bisabolene	1500	-	1.4	-	-
β-Selinene	1501	-	<0.1	5.7	6.0
Germacrene B	1507	-	-	2.3	-
γ-Cadinene	1509	-	-	-	2.9
α-Selinene	1511	-	<0.1	6.8	8.6
δ-Guaiene	1517	-	1.2	-	-
δ-Cadinene	1523	-	<0.1	-	-
Oxygen-containing sesquiterpenes		7.6	27.9	9.8	20.7
Nerolidol	1529	-	-	-	5.0
Spathulenol	1589	-	1.0	0.9	-
Caryophyllene oxide	1597	2.2	4.4	1.1	-
Globulol	1600	-	-	1.5	-
Humulene oxide	1608	-	1.4	0.9	-
α-Cadinol	1644	-	-	-	3.0
β-Eudesmol	1634	-	-	2.7	-
δ-Guaienol	1665	5.4	18.9	-	1.8
α-Eudesmol	1666	-	-	1.3	-
Valerianone	1686	-	2.2	1.4	10.9
Total identified		99.6	99.7	99.4	98.7
Yield of oils		0.04	0.02	0.10	0.12

Table 2. Scavaging capacity of BHT and *Helichrysum* oils expressed as SC_{50}.

BHT and essential oils	SC_{50}
BHT	7.0 mg/l
H. cameroonense	4.9 g/l
H. cymosum	6.3 g/l

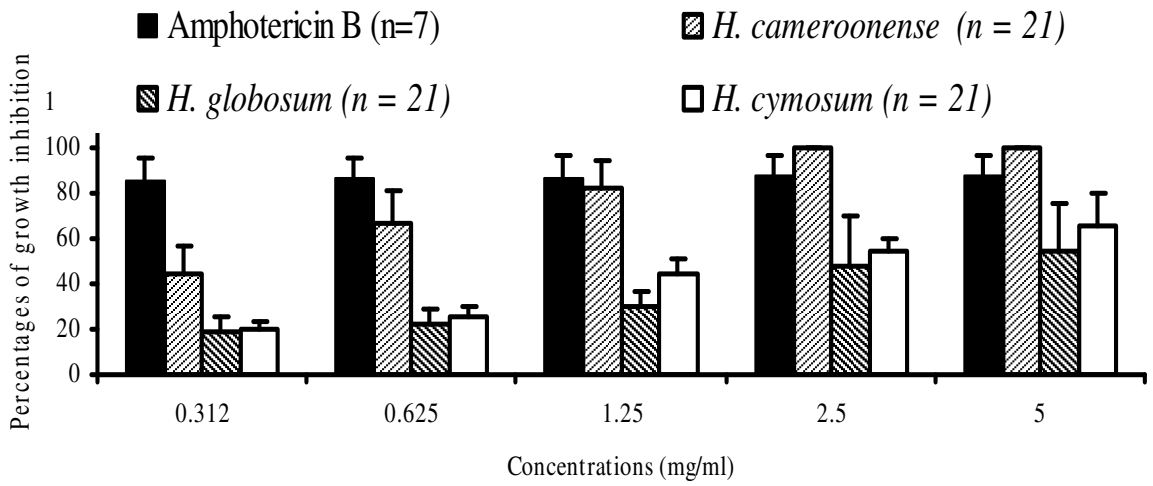

Figure 1. Effect on fungi growth inhibition of different concentrations of essential oils and amphotericin B.

Table 3a. Range and mean values of the percentages of growth inhibition of *P. oxalicum* at different concentrations of amphotericin B and essential oils.

Amphotericine B and essential oils	Concentrations								
	5 mg/ml			2.5 mg/ml			1.25 mg/ml		
	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD
A. Amphotericin B (n = 7)	76.52	100.00	87.9 ± 9.0	76.52	100.00	87.3 ± 9.2	76.52	100.00	86.8 ± 9.4
B. *H. cameroonense* (n = 21)	100.00	100.00	100.0 ± 0.0	100.00	100.00	100.0 ± 0.0	70.00	100.00	82.2 ± 11.8
C. *H. globosum* (n = 21)	35.71	100.00	54.7 ± 20.6	28.80	100.00	50.6 ± 22.8	15.38	43.85	29.6 ± 7.1
D. *H. cymosum* (n = 21)	56.18	100.00	66.0 ± 14.4	45.72	62.65	54.0 ± 5.6	34.69	55.25	44.5 ± 6.2
ANOVA Test $F_{(3 ; 66)}$		41.506 ***			70.359 ***			166.986 ***	

Table 3b. Range and mean values of the percentages of growth inhibition of *P. oxalicum* at different concentrations of amphotericin B and essential oils.

Amphotericine B and essential oils	Concentrations						Global comparison
	0.625 mg/ml			0.312 mg/ml			ANOVA Test
	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD	
A. Amphotericin B (n = 7)	76.52	100.00	86.5 ± 9.6	75.22	100.00	85.4 ± 10.3	$F_{(4 ; 30)}$ = 0.069 ns
B. *H. cameroonense* (n = 21)	56.95	100.00	66.8 ± 14.1	24.55	59.66	44.0 ± 12.5	$F_{(4 ; 100)}$ = 120.022 ***
C. *H. globosum* (n = 21)	14.29	33.85	22.3 ± 6.7	9.00	29.23	18.9 ± 6.2	$F_{(4 ; 100)}$ = 24.265 ***
D. *H. cymosum* (n = 21)	18.26	33.95	25.3 ± 4.3	15.24	26.50	20.2 ± 3.5	$F_{(4 ; 100)}$ = 126.551 ***
ANOVA Test $F_{(3 ; 66)}$		154.178 ***			135.376 ***		-

Table 4. Comparison of the activity of amphotericine B versus other essential oils at different concentrations (Dunnett test).

I. 5 mg/ml		II. 2.5 mg/ml		III. 1.25 mg/ml		IV. 0.625 mg/ml		V. 0.312 mg/ml	
A / B :	q' = 1.971 ns	A / B :	q' = 2.198 ns	A / B:	q' = 1.194 ns	A / B :	q' = 4.818 *	A / B :	q' = 11.139 *
A / C :	q' = 5.407 *	A / C :	q' = 6.883 *	A / C:	q' = 14.950 *	A / C :	q' = 15.689 *	A / C :	q' = 17.920 *
A / D :	q' = 3.566 *	A / D :	q' = 5.782 *	A / D:	q' = 11.062 *	A / D :	q' = 14.942 *	A / D :	q' = 17.556 *

ns = P > 0.05; * = P < 0.05 ; *** = P < 0.001

post harvest yam tubers. Considering the nutritional importance of yams for over 400 million people worldwide and the post harvest losses due to *P. oxalicum*, *Helichrysum* oils in particular and plant volatiles in a global view offer new alternative tool for the safeguard of yams during storage. Nevertheless, detailed studies are required in regard of toxicological aspects related to the activity of these oils and probably their shelf live.

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