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Chemical composition and Biological activities of the Essential oil extracted from the Fresh leaves of *Chromolaena odorata* (L. Robinson) growing in Benin

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Abstract

The chemical composition of the essential oil of Chromolaena odorata (L. Robinson) (Asteraceae) of leaves collected in Benin was analyzed by GC and GC/MS. Twenty three compounds were identified and quantified in the essential oil, which represented 99.4 % of the total constituents. The main constituents found in the oil were α-pinene (20.7%), pregeijerene (14.6%), geijerene (12.0%), β-pinene (10.3%), germacrene-D (9.7%). The antibacterial activitiy of this oil was found to be relevant while the antifungal and antiradical activities were low.

Keywords: antimicrobial, antiradical, anti-inflammatory activities, essential oil, α-pinene, pregeijerene, *Chromolaena odorata*.

Introduction

Chromolaena odorata (L.) R.M. King (Syn: *Eupatorium odoratum* L.) is an invading aromatic species of African vegetation. It is known in Benin in the traditional pharmacopeia for its many medicinal virtues. Previous studies indicated the presence in its organs of steroids[1], triterpenes[2], flavonoïdes[3-5], alkaloids[6] and essential oils[7-12]. We noted in the oil collected at Houin (Benin) β-caryophyllene (21%) and germacrene-D (15.3%)[13]. Bicyclogermacrene (12.55%), geigerene (11.85%), (Z)-β-farnesene (9.98%) and α-pinene (9.36%)[14]. Pregeijerene (19.9%) steady of α–pinene (17.9%); β-caryophyllene (21.0%) accompanied of germacrene-D (15.3%) were the main constituents of *C. odorata* collected in Abomey-Calavi and Houin (Benin) respectively[15]. The essential oil of the leaves of *C. odorata* was shown more toxic on *Rhipicephalus lunalatus,* ectoparasite of the dwarf goat of Guinea[14]. The physico-chemical properties of investigated essential oils allowed their best characterization[15]. The oil from *C. odorata* also had been exploited as insecticide[16, 17], ovicide and larvicide[17]. The leaves extract of *C. odorata* got antifungal property[18]. Nowadays, the interests for essential oil extracted from aromatic plants are multiple and diversified. Based on their therapeutic properties and the chemical substances isolated from their volatile parts, it may allow further application in particular biological activities. This study aims to investigate the biological properties such as antimicrobial, antiradical and anti-inflammatory, but also make a review on the chemical composition of its essential oil.

Material and Methods

Plants material and Isolation of the Essential oils: The plant material was collected at Godomey (Benin) in January 2010. A voucher specimen was deposited in the herbarium of the University of Abomey-Calavi. Batches of 200 g of fresh leaves were submitted to hydrodistillation for 2h using a Clevenger-type apparatus; after decantation, the oils were dried over anhydrous Na_2SO_4 and stored in sealed vials below 10°C until using.

Chemical analysis of Essential oil: Quantitative and qualitative analyses of the essential oils were carried out by gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS). GC/FID analyses were performed using a Varian CP-3380 GC equipped with a DB1 (100% dimethylpolysiloxane) fitted with a fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 µm) and supelcowax 10 (polyethylene glycol) fused capillary column (30 m x 0.25 mm, film thickness 0.25 µm); temperature program 50°-200°C at 5°C/min, injector temperature 220°C, detector temperature 250°C, carrier gas N_2 at a flow rate of 0.5 mL.min^{-1}. Diluted samples (10/100, v/v, in methylene chloride) of 2.0 µL were injected manually in a split mode (1/100). The percentage compositions were obtained from electronic integration measurements without taking into account relative response factors. The linear retention indices of the components were determined relatively to the retention times of a series of *n*-alkanes (C_9-C_{20}).

GC/MS analyses were performed using a Hewlett Packard apparatus equipped with a HP1 fused silica column (30 m x 0.25 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/min; injector temperature was 220°C. Helium was used as carrier gas at a flow rate of 0.6 mL.min^{-1}, the mass spectrometer was operated at 70 eV. 2.0 µL

of diluted samples (10/100, v/v, in methylene chloride) were injected manually in the split mode (1/100). The identification of individual compounds was based on the comparison of their relative retention times with those of authentic samples on the DB1 column and by matching the linear retention indices and mass spectra of peaks with those obtained from authentic samples and/or the NBS75K.L and NIST98.L libraries and published data[19, 20].

Biological Evaluation: Antibacterial activity: Essential oil emulsion: 2 mL of Mueller Hinton broth added with 0.02 g/L of phenol red were added to 40 µL of essential oil and 2 drops of Tween 80 and has been introduced in an hemolyse test tube and homogenized.

Preparation of Bacteria Suspensions: This preparation was carried out from the three stocks of tested bacteria. A pure colony of each stock was suspended in 5 mL of Mueller Hinton broth. After incubation at 37°C for 2 hours, we obtained 10^6 CFU/mL corresponding to the scale 2 of McFarland standard.

Determination of Minimal Inhibitory Concentration (MIC): The method used was reported by Yehouenou et al [21]. 100 µL of medium Mueller Hinton broth containing of phenol red to 0.02 g/L were distributed in all the 96 wells of a microplate. 100 µL of essential oil emulsion (initial solution) were added to the well of the first column except that of the second line and successive dilutions of reason 2 were carried out well by well till the 12th one and the remaining aliquot (100 µL) were rejected. 100 µL of Mueller Hinton which not containing phenol red were introduced on the first well of the first columns and successive dilutions of reason 2 were carried out as before. All the wells of the second column received 100 µL of bacteria suspension except the first line which represents the negative control and the second line, the positive control. The microplate one was finally covered with parafilm paper and was incubated at 37°C during approximately 18 hours.

Antimicrobial assay: It concerned to test the sensitivity of the stock E. coli ATCC 25922 in the presence of certain specific antibiotic discs (diameter = 6mm) such as ceftriazone 30 µg, gentamycine 10 µg, nalidixic acid 30 µg and chloramphenicol 30 µg on the one hand and that of the stock S. aureus ATCC 25923 on the other hand using the antibiotic discs which are specific for it such as lyncomycine and erytromycine 15 µg and tetracycline 30 µg. The activity of essential oil was evaluated in comparison of those of antibiotics discs by measuring the diameters of inhibition of the stocks. The essential oil was spread on sterile disc of 6 mm of diameter at same concentrations with the antibiotic disc.

Antifungal activity: Preparation of the Culture medium: 11.5 g agar of yeast extract (yeast extract AGAR) and 10 g of anhydrous glucose are mixed with 500 mL of distilled water for the preparation of culture medium. After sterilization and addition of oxytetracycline (0.1%) 5 mL, this medium was cast in limp of Petri dish 9 cm in diameter at a rate of 17 mL.

Detection of the Moulds: A quantity of vegetable weighed from gardening culture, fresh tomato fruits and banana leaves was diluted in sterile peptone water in order to detecte fungi responsible of their deterioration. 30 min after homogenizing each sample, 0.1 mL of the inocula was spread out on the sterilized mould medium (yeast extract glucose agar: YEGA) and uniformly. The present limp was incubated at 25° ± 1°C five days aware from day light. The stocks of fungi (Penicillium digitatum and Aspergillus ochraceus) were detected and identified according to Samson et al., 1995 [22].

Transplantation and Mycelial growth: The moulds detected after examination and identification then, were transplanted (subcultured) using a disc of 6 mm in diameter which carried spores from the anamorph mould on the surface of petri dish containing the former medium YEGA with tested essential oil at different concentrations (1.33 $mL.L^{-1}$, 2.66 $mL.L^{-1}$, 5.32 $mL.L^{-1}$ or no (positive control). The moulds subcultured were incubated at 25° ± 1°C. The mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc, from the first day till the tenth one at, least 10 days [23]. The antifungal activity was evaluated by the following equation[24]:

$$I = (1 - \frac{d}{dc}) \times 100$$

with I : antifungal index; d : diameter of growth of petri dish treated out of essential oil; dc : diameter of growth of the control (witness) (petri dish without essential oil).

Test of determination of the fongiostatic or fungicidal activity: With the experimental concentrations where neither growth nor germination was observed, we tested the fungiostatic or fungicidal activity. This test consisted in taking the mycelial disc not germinated at the end of the incubation of the petri dish and reintroducing it in a new culture medium (former one) without natural extract. If the mycelial growth was always inhibited, the fungicidal activity of the natural extracts and in the contrary case, it's spoken about fongiostatic activity[25].

Free radical-scavenging activity: DPPH test: Antiradical activity was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the Mellors and Tappel method[26] adapted to essential oil screening [27].

1,1-diphenyl-picrylhydrazyl [1898-66-4] was purchased from Sigma-Aldrich and the solutions were prepared with analytical grade solvents purchased from standard commercial sources.

DPPH, was dissolved in ethanol to give a 100 µM solution. To 2.0 mL of the ethanolic solution of DPPH were added 100 µL of a methanolic solution of the antioxidant reference BHT (butylated hydroxytoluene) at different concentrations. The essential oil was tested with the same manner. The control, without antioxidant, was represented by the DPPH ethanolic solution containing 100 µL of methanol. The decrease in absorption was measured at 517 nm after 30 min, at 30°C. All

the spectrophotometric measures were performed in triplicate with a SAFAS UV mc2 spectrophotometer, equipped with a multicells/multikinetics measure system and with a thermostated cells-case. The free radical-scavenging activity of each solution was calculated according to the following equation:

$$SC\% = \frac{A(blank) - A(sample)}{A(blank)} \times 100$$

Antiradical activity, defined as the concentration of test material required to cause a 50% decrease of the initial DPPH absorbance, was determined graphically and expressed as SC_{50} (mg. L^{-1}).

Anti-inflammatory activity: Lipoxygenase test: Soybean lipoxygenase (EC.1.13.11.12) was purchased from Fluka whereas nordihydroguaiaretic acid (NDGA) [500.38.9] and linoleic acid sodium sulfate (822-17-3) were obtained from Sigma Chemical Co; potassium phosphate buffer 0.1 M, pH= 9 was prepared with analytical grade reagent purchased from standard commercial sources. Deionized water was used for the preparation of all solutions. Lipoxygenase was known to catalyse the oxidation of unsaturated fatty acids containing 1,4-diene structures. The conversion of linoleic acid to 13-hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at 234 nm. Nordihydroguaiaretic acid (NDGA), a known inhibitor of soybean lipoxygenase, was used as a reference drug. The experimental conditions were adapted from those previously used and fully described [28]**.**

Statistical analysis: Data were subjected to analysis of variance (ANOVA). They were expressed as the mean ± standard error of triplicate measurements; standard deviations did not exceed 5 %.

Results and Discussion

Chemical composition of *Chromolaena odorata* essential oil: The essential oil was obtained in 0.1 % yield from the leaves of *C. odorata*; this yield was lightly weak to that observed with the same species collected in Benin[14] and high to that observed with the species collected in Cameroon[15]**.** The chemical composition of the essential oils is given in table-1.

Globally, the essential oil was dominated by hydrogenated monoterpens and sesquiterpens. It was deduced from this table that the oil of *Chromolaena odorata* was characterized by 20.7 % of α-pinene, accompanied of pregeijerene (14.6 %), geijerene (12.0 %), β-pinene (10.3 %) and D-germacrene (9.7 %).

Antibacterial activity: Two microbial stocks were used in this study. The Minimal Inhibitory Concentration (MIC) values were determined for all. The essential oil of the leaves of *Chromoleana odorata* almost displayed an antimicrobial activity interesting against *Staphylococcus aureus* ATCC 25923 with MIC equal to 1.28 ± 0.06 mg/mL and low activity towards *Escherchia coli* ATCC 25922 with MIC equal to 5.11 ± 0.25 mg/mL compared to the standards use in preservation of cheese according to Smith *et al*., 2001 [29] (table-2). In spite of a lack of antibiotic capacity (theoretically calculated) the MIC determined for this essential oil showed an interesting antimicrobial activity.

The hydrocarbon terpens were at high amount (98.7 %) and stood for a weak potential on the wall cell of *E. coli* while this activity was effective on the one of *S. aureus* ATCC 25923. The main chemical substances isolated from the essential oil made of α-pinene, β-pinene, geijerene, pregeijerene in proportions of 20.7 %, 10.3 %, 12.0 %, and 14.6% respectively and germacrene D in proportion of 9.7% and *trans*-β-caryophyllene (6.5 %) were the major lipophilic components that displayed diversely this antibacterial activities against the two tested strains. Burt (2004) had reported that as far as non-phenolic components of essential oils are concerned the type of alkyl group has been found to influence activity[30]. Enzymes such ATPase located in the cytoplasm membrane were influenced by cyclohydrocarbons the accumulation of lipophilic hydrocarbon molecules could distort the lipid protein interaction, but alternatively, direct interaction of lipophilic compounds with hydrophobic parts of the protein is possible. Our tested essential oil composed of this lipophilic components act alone but also each other in the strains cell parts with a synergy that influenced diversely the integrity of the wall cell. This justifies *S. aureus* ATCC 25923 cell was more sensitive to the essential oil than *E. coli* ATCC 25922 one.

Antibiotic capacity of the Extracts: - The averages of the diameters of the halos of incubation measured in mm are consigned in the table-3.

These results showed that *E. coli* ATCC 25922 was very sensitive to chloramphenicol, gentamycine and ceftriazone, but resistant to nalidixic acid. On the other hand the essential oil of *C. odorata* presented an intermediate inhibiting action on the stock compared to gentamicine, chloramphenicol, ceftriazone and with the nalidixic acid, whereas *S. aureus* ATCC 25923 was very sensitive to lyncomycine, erythromycine and tetracycline, while the oil showed an inhibiting activity on *S. aureus* ATCC 25923 compared to lyncomycine, erythromycine and with Tetracycline.

Antifungal activity: Figures -1 and 2 translated the mycelial reduction ratio according to the number of days of setting into contact with the essential oil of *Chromolaena odorata* met with the stocks of *Aspergillus ochraceus* and *Penicillium digitatum.*

With the concentration of 1.33 mL/L and of 2.33 mL/L, the mycelial ratio reduction on Aspergillus ochraceus to the third day of essential oil were respectively 40 and 70 % whereas this rate was 90 % with the concentration of 5.32 mL/L in the same time. We observed there after a tendency to the decrease of the MRR (mycelal reduction ratio) of the stock to different concentration from the essential oil and from the fourth ninth day up zero value (MRR = 0 %) ninth day with all the concentrations tested.

Table-1
Chemical composition of essential oils of *Chromolaena odorata* collected in Benin.

RI*	Component	Percent Composition	Identification methods
936	**α-pinene**	**20.7**	GC, MS, RI
967	sabinene	1.5	GC, MS, RI
977	**β-pinene**	**10.3**	GC, MS, RI
980	myrcene	2.3	GC, MS, RI
1023	limonene	1.8	GC, MS, RI
1037	γ-terpinene	3.3	GC, MS, RI
1133	myrtenol	0.6	GC, MS, RI
1144	**geijerene**	**12.0**	GC, MS, RI
1244	isogeijerene C isomere	0.9	GC, MS, RI
1252	isogeijerene C	0.6	GC, MS, RI
1262	isogeijerene	1.1	GC, MS, RI
1273	pregeijerene isomere	1.3	GC, MS, RI
1300	**pregeijerene**	**14.6**	GC, MS, RI
1384	α-copaene	2.1	GC, MS, RI
1392	β-elemene	0.9	GC, MS, RI
1468	*trans*-β-caryophyllene	6.5	GC, MS, RI
1483	α-humulene	2.0	GC, MS, RI
1492	β-cubebene	1.3	GC, MS, RI
1497	**germacrene-D**	**9.7**	GC, MS, RI
1502	bicyclogermacrene	0.6	GC, MS, RI
1504	γ-cadinène	0.6	GC, MS, RI
1507	aromadendrene	0.9	GC, MS, RI
1527	δ-cadinene	3.7	GC, MS, RI
	Total identified	99.3	
	Monoterpene hydrocarbons	39.9	
	Oxygenated monoterpenes	0.6	
	Sesquiterpene hydrocarbons	58.8	

RI[a], Retention index relative to *n*-alkanes (C_9-C_{20}) on a DB1 capillary column (100% dimethylpolysiloxane); Identification methods: GC, identification based on retention times of authentic compounds, MS, identification based on computer matching of the mass spectra of peaks with NBS75K.L, NIST98.L libraries and published data [19,20]. RI, tentative identification based on comparison of retention index of the compounds with published data[19,20].

Table-2
Antimicrobial activity (Minimal Inhibitory Concentration: MIC value, mg/mL) of essential oil of leaves of Chromoleana odorata

Microbial stock	Minimal Inhibitory Concentration (MIC) (mg/mL)
Escherichia coli ATCC 25922	5.11 ± 0.25
Staphylococcus aureus ATCC 25923	1.28 ± 0.06

Table-3
Evaluation of the sensitivity of the microbial stocks tested with respect to antibiotics of reference and of the essential oil of *Chromoleana odorata*

	***E. coli* (Diameter mm)**				***S. aureus* (Diameter mm)**		
	Chloramphenicol	**Gentamycine**	**Nalidixic Acid**	**Ceftriazone**	**Lyncomycine**	**Erythromycine**	**Tetracycline**
	18.0 ± 0.7	18.0 ± 0.7	00.0 ± 0.0	21.0 ± 0.8	24.0 ± 0.9	24.0 ± 0.9	30.0 ± 1.2
E.O	14.02 ± 0.8	12.5 ± 0.5	14.0 ± 0.7	14.0 ± 0.8	28.0 ± 1.1	28.0 ± 1.1	30.0 ± 1.2

EO: Essential oil.

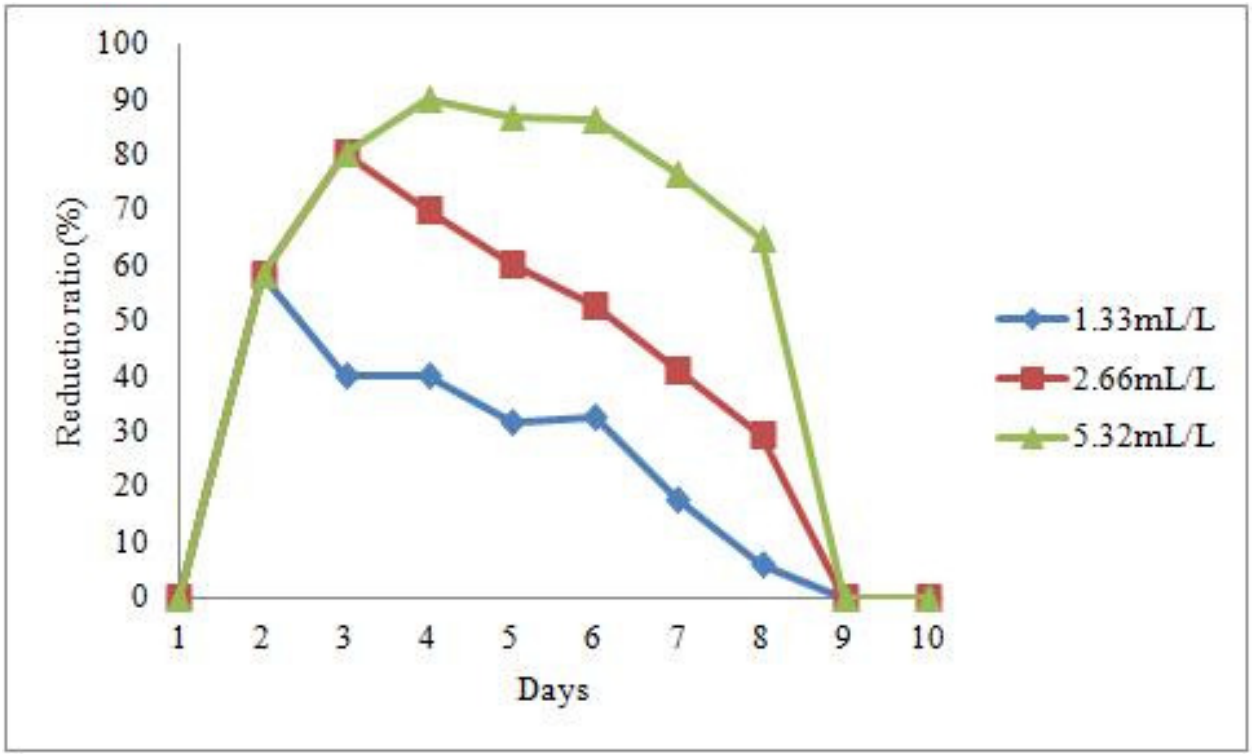

Figure-1
Action of the oil essential of *Chromolaena odorata* with various Concentrations on the mycelial growth of *Aspergillus ochraceus*

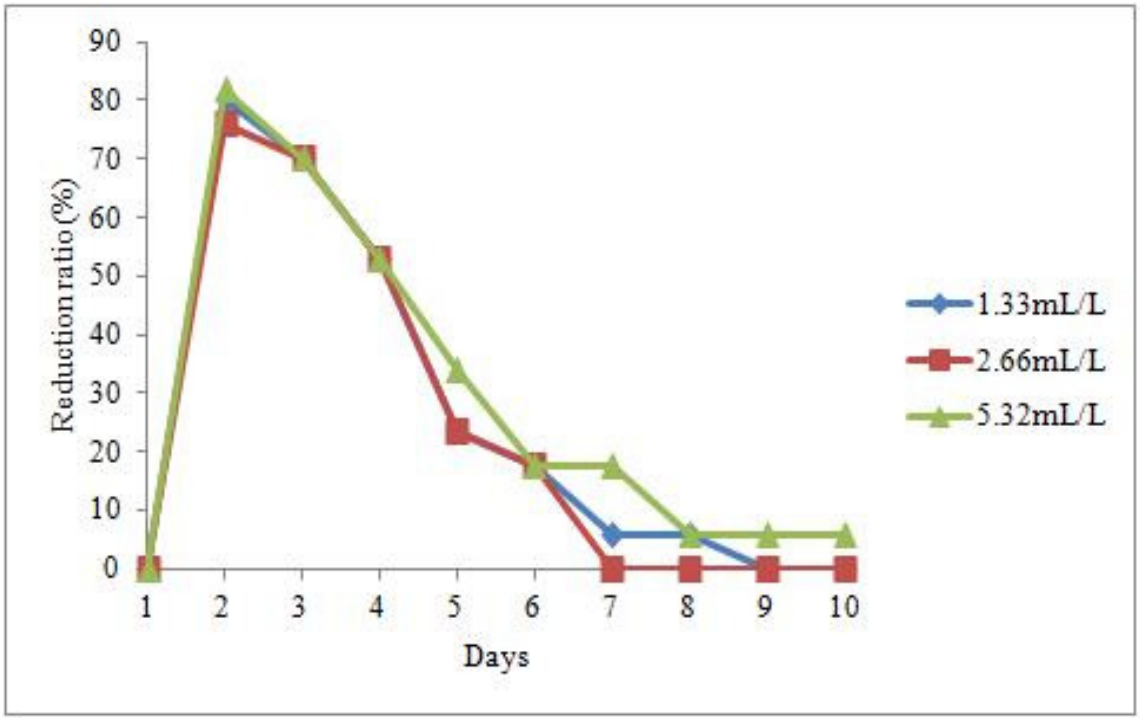

Figure-2
Action of the oil essential of *Chromolaena odorata* with various Concentrations on the mycelial growth of *Penicillium digitatum*

As for the stock of *Penicillium digitatum*, the same tendencies were observed for essential oil with the characteristic that the inhibiting activity was less marked with the various concentrations (MRR = 80 % to 5.32 mL/L, MRR= 20 % with 2.66 mL/L and MRR = 20 % with 1.33 mL/L) to the third day, with the same tendency to the cancellation (MRR = 0 %) of the inhibiting activity of essential oil to all the concentrations tested to the ninth day.

The essential oil of *Chromolaena odorata* got a temporary fongistatic activity during the first 4 days with all the concentrations tested on the two stocks of deuteromycetes (*Aspergillus ochraceus* and *Penicillium digitatum*), which became exhausted as of the fifth days until its cancellation to the ninth day. The MRR passed from 40 % to 0 % to all the concentrations tested from the fifth to the ninth day. The oil thus did not have an inhibiting activity on the fungic stocks tested.

This absence of fongistatic activity of the essential oil of *Chromolaena odorata* was inherent to its chemical composition rich in hydrogenated monoterpens and sesquiterpens. Parallely the antifungal activity of the essential oil effective the three first days on *P. digitatum* at 5.32 mL/L and the five days after contact with *A. ochraceus* but the progressive decreasing of this activity (MRR from 90 % to 0 % with 9 days) indicated that the hydrogenated terpens didn't allow than attachment with the main lipid ergosterol localized in the membrane bilayer of the moulds after three days, at least after five days and their insufficient action in destroying this lipid provocate the new arise of mycelia growth and this latter explains the weak antifungal activity of the oil observed on two moulds at any concentration [31].

Antiradical activity: Significant free radical scavenging activity was observed for oil sample; this was compared to the commercial antioxidant BHT (butylated hydroxytoluene), which was widely used as a reference. SC_{50} (*C. odorata*) = (4.9 ± 0.30) g/L; SC_{50} (BHT) = (7.50 ± 0.45) mg/L. The antiradical activity observed with the essential oil was weak.

Anti-inflammatory activity: The result obtained from the lipoxygenase tests performed on this essential oil of *Chromolaena odorata* did not present any anti-inflammatory activity at 10 ppm, the limiting concentration of its solubility. Those combination with a significant antimicrobial activity could explain the interesting activities described for this botanical species in traditional medicinal.

Conclusion

The chemical composition of essential oil of *Chromolaena odorata* from Benin was investigated. It was mainly composed of α-pinene and pregeijerene, similar to the previous one described by the literature. The volatile extract showed an interesting antimicrobial activity while the fongistatic one was less relevant. The essential oil got a weak antiradical activity and did not possess any anti-inflammatory activity. Considering its high content in monoterpene and sesquiterpene hydrocarbons, the leaves of *C. odorata* were exploited for its many medicinal virtues.

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