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ANTIRADICAL, ANTIOXIDANT ACTIVITIES AND ANTI-INFLAMMATORY POTENTIAL OF THE ESSENTIAL OILS OF THE VARIETIES OF *CITRUS LIMON* AND *CITRUS AURANTIFOLIA* GROWING IN CAMEROON

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ABSTRACT

Essential oils of the leaves of Eureka, Lisbon and Meyer varieties of Citrus limon, as well as the Mexican, "Sans épines" and Bearss varieties of Citrus aurantifolia of Cameroon were extracted by hydrodistillation, with respective yields 0.64%, 0.90% and 0.46% 0.57%, 0.25% and 0.29%.

Chemical composition analysis was carried out by gas chromatography and gas chromatography coupled with mass spectrometry. As a whole, the six samples are very rich in monoterpenes, and limonene; thus proved to be the main compounds.

Antiradical and antioxidant properties of our samples were evaluated by the Diphenyl Picryl Hydrazyl (DPPH) method and the β-carotene decolouration method respectively. Citrus aurantifolia var. "Sans épines" proved to be the better antiradical oil with an SC_{50} value of 3.4g/l against 7.02 mg/l for the BHT used as reference. As antioxidant, the same extract was more effective with an IC_{50} of 0.26 mg/l against 0.10 mg/l for the BHT.

In addition, anti-inflammatory activity of the extracts of Citrus limon var. Meyer and Citrus aurantifolia var. "Sans épines" were measured by an enzymatic method based on the inhibiting action of the substance to be tested on the oxidation of linoleic acid by 5-lipoxygenase of soya beans. This measurement gave IC_{50} value of 46.5 ppm and 49.35 ppm respectively for Meyer and "San épines" against 0.7 ppm for NDGA.

Keywords: *Citrus limon* - *Citrus aurantifolia* – Essential oil - Chemical composition - Antiradical –Antioxidant -Anti-Inflammatory

1. INTRODUCTION

Free radicals are responsible for the destruction of biologically important organic molecules as well as the degradation (oxidation reactions) of lipid food, thus provoking certain diseases such as cardiovascular disease, diabetes, cancer, and also cause food losses. Similarly, certain components of food like, linoleic acid, can support the growth of carcinogenic cells under the action of lipoxygenase (Willet, 1994). Agro-food, pharmaceutical and cosmetic industries use synthetic antioxidant and antilipoxygenasic substances to protect their products from oxidation thus prolonging their shelf life and to inhibit the action of lipoxygenase. However, conscious of the harmful effects of these synthetic substances to man as well as to the environment (Hudson, 1990), studies are accentuated in the research for natural substances with multiple properties following the example of essential oils of the aromatic and/or medicinal plants. Already published work showed that essential oils constitute a good source of natural antioxidants (Cuvelier *et al.*, 1992; Botsoglou *et al.*, 2003; Gulluce *et al.*, 2003). Moreover, these essential oils are a potential reserve for natural care and represent a hope for medicine by their anti-inflammatory activity (Alexander, 2001; Baylac and Racine, 2003). It is thus in the same light that we were interested in the essential oils of *C. limon* and *C. aurantifolia*. *C. limon* is a first order anti-scorbutic. Against dysentery, one takes a seat bath with lemon water and boiled root pulp is employed against blennorrhoea while the maceration of the yellow bark of lemon or peeling is recommended against colics (Raponda-Walker and Silans, 1961). Its essential oil has inhibiting effects against microorganisms and anti-carcinogenic effects (Morris *et al.*, 1979). Significant work had been carried out on essential oils of *C. limon*, notably those of Mwaiko (1992). Mwaiko and Savaeli (1994) showed the effect of essential oils of *C. limon* against mosquitoes and those of Wattenberg *et al.* (1985), who discovered that the oil of *C. limon* inhibited the formation of stomach and lung tumours in rats. Matsuura *et al.* (2006) tested the inhibitory activity of tyrosinase of essential oils of thirty citrus. The Eureka and Lisbon varieties were most effective against the oxidation of L-dihydroxy phenylalanine. This activity was attributed to citral (neral and geranial) and myrcene because these compounds showed a strong inhibitory activity.

As for *C. aurantifolia*, it alleviates anxiety and nervousness. The plant also relieves stress related disorders such as insomnia or nervous originated digestive disorders. It also possesses anti-inflammatory potential (digestive system). The essence of "lime" has antispasmodic virtues that are being experienced during spasm of the digestive system (distension, diarrhoea). Finally, it has an anticoagulant property, which renders it very valuable for people with cardiovascular risks. It is also used against fever, headaches and cold (Chellaiah *et al.*, 2006). Some work had been published on the volatile extracts of *C. aurantifolia.* For example, the works of (Jantan *et al.*, 1995) and of Gancel *et al.* (2002) on samples harvested respectively in Malaysia and France showed that the main compounds of essential oils of the leaves of *C. aurantifolia* are geranial, limonene, and neral.

The present study aims at determining the antiradical, antioxidant and anti-inflammatory properties of the essential oils of the leaves of the varieties of *C. limon* and *C. aurantifolia* of Cameroon.

2. MATERIALS AND METHODS

2.1. Plant Material

Fresh leaves from the six varieties were collected from the experimental garden of the Institute of Agricultural Research for Development (IRAD), Nkolbisson, Yaounde in March 2006.

2.2. Extraction of Essential Oils

The plant samples were hydro-distilled for 5 hours using a Clevenger-type apparatus. The afforded oils were dried over anhydrous sodium sulphate and stored at 4°C until use for further experiments. The extraction yields were calculated in percent (w/w) relatively to the starting plant material.

2.3. Analysis of the Essential Oils

The essential oils were analysed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS).

2.4. Gas Chromatography

The oil was analysed on a Varian CP-3380 GC with flame ionisation detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25µm); temperature program 50°C-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.

2.5. Gas Chromatography/Mass 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 0.25µm) and interfaced with a quadrupole detector (GC- quadrupole MS system, model 5970). Column temperature was programmed from 70°C-200°C at 10°C/min; injector temperature was 200°C. Helium was used as carrier gas at a flow rate of 0.6 ml/min, the mass spectrometer was operated at 70eV.

2.6. 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 (Jennigs and Shibamoto, 1980; Joulain and Konig, 1998; Adams, 2007) with the data bank NBS75K and with the stored laboratory mass spectral library.

2.7. Determination of the Antiradical Activity

The antiradical activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), which was dissolved in ethanol to give around 100 µM solution. The accurate DPPH concentration (C_{DPPH}) was determined by spectrophotometric method following the equation A_{517}= 9832 x C_{DPPH}, where 9832 is the molecular extinction coefficient of DPPH determined independently in ethanol. To 2.0 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, was represented by the DPPH ethanolic solution containing 100 µl of methanol. The decrease in absorption was measured at 517 nm after 2 hours, at room temperature. The decrease in absorption induced by the test compound was calculated by substracting that of the control. The concentration required for 50% reduction (50% scavenging concentration, SC_{50}) was determined graphically. All the spectrophotometric measurements were performed using a SAFAS UV-mc^2 Spectrophotometer, equipped with a multi-cells / multikinetic measure system and with a thermostated cell-case (Brand-Williams *et al.*, 1995; Cotelle *et al.*, 1996; Mellors and Tappel, 1996; Nyegue, 2006).

2.8. Determination of Antioxidant Activity (Cotelle *et al.*, 1996; Agnaniet *et al.*, 2004)

The antioxidant activity was evaluated using a β -carotene/ linoleate model system.30 A solution of β -carotene from Fluka (7235-40-7) was prepared by dissolving 2.0 mg of β-carotene in 10 ml chloroform. 1.0 ml this solution was pipetted into a round-bottomed flask which contained 20 µl purified linoleic acid from Avocado (60-33-3) and 200 mg Tween 40 emulsifier from Aldrich (9005-66-7). After chloroform was removed under vacuum using a rotary evaporator at 40 °C, 50 ml aerated distilled water was added to the flask with vigorous shaking. The antioxidant activity was evaluated by measuring, at 470 nm, the kinetics of discoloration of β -carotene in the absence (control) and presence of the antioxidant solution (10 µl methanolic solutions containing different concentrations of essential oil or BHT for comparative purposes) at 50 °C.

A blank was prepared under the above conditions but without β-carotene. All the kinetics obtained were done according to the following equation:

$$A = A_0 e^{-kt} + C$$

Where A_0 is the absorbance at time zero, C the absorbance at infinite time and k the degradation rate constant of β -carotene, from which inhibition percentages (Ip) were calculated through the following relation:

$$Ip = 100.\ (k_0 - k)/k_0$$

Where k_0 and k are the degradation rate constants of β -carotene in the absence and in presence of inhibitor. The plot of the inhibition percentage as a function of the inhibitor concentration allowed the evaluation of the IC_{50} of the sample.

2.9. Determination of the Anti-Inflammatory Activity

Lipoxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid was followed spectrophotometrically by the

appearance of a conjugate diene at 234 nm. Nordihydroguiaretic acid (NDGA), a known inhibitor of soybean lipoxygenase was used as a reference drug (Alitonou, 2006).

The reaction was initiated by the addition of aliquots (50 µl) of a Soybean lipoxygenase solution (prepared daily in potassium phosphate buffer 0.1 M pH 9 in a sufficient concentration to give an easily measurable initial rate of reaction) to 2.0 ml of sodium linoleate 100 µM in phosphate buffer; the enzymatic reactions were performed in the absence or the presence of the inhibitor and their kinetics were compared. The inhibitors were dissolved in ethanol such that an aliquot of each (10 µl) yielded a final concentration of maximum 100 ppm in each assay (the solubility of the essential oil was verified in this range of concentrations by the determination of its specific extinction coefficient).

The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control (using 10 µl of ethanol instead of 10 µl of the inhibitor - ethanol solution). Each inhibitor concentration was tested in triplicate and the results averaged; the concentration that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration.

The anti-inflammatory activity of the essential oil from *C. limon* var. Eureka and *C. limon* var. Lisbon was evaluated comparatively to that of NDGA.

3. RESULTS AND DISCUSSION

3.1. Extraction Yield

The hydrodistillation of the leaves of *C. limon* gave essential oils of yellow color with respective yields of 0.64%, 0.90% and 0.46% for the Lisbon, Eureka and Meyer varieties. The leaves of the Eureka variety are thus two times richer than those of the Meyer variety, and 1.4 times richer than those of the Lisbon variety.

As regards *C. aurantifolia*, the leaves of the Mexican, “Sans épines” and *Bearss* varieties gave essential oils of yellow color with respective outputs of 0.57%, 0.25% and 0.29%. The leaves of the Mexican variety are thus 2 times richer in essential oil than those of “Sans épines” and *Bearss*. As a whole, *C. limon var*. Eureka is the richest variety in essential oil.

3.2. Chemical compositions

The results of the chemical analysis are consigned in table 1

Table-1. Results of the chemical analysis of essential oils of the samples

	Compounds	IK	*C. limon* var. Lisbonne	*C. limon* var. Eureka	*C. limon* var. Meyer	*C. aurantifolia* var. Mexicaine	*C. aurantifolia* var. "Sans épines"	*C. aurantifolia* var. Bearss
1	α-pinene	9.37	1.5	1.5	0.6	0.3	0.3	0.3
2	camphene	9.68	1.3	1.6	-	2.3	3.0	-
3	sabinene	972	3.9	2.9	1.3	1.2	0.5	2.1
4	β-pinene	980	16.0	17.6	0.8	0.3	0.4	1.0
5	myrcene	985	1.6	1.5	1.8	1.5	1.6	1.6
6	Δ^{3-} carene	1011	-	1.0	-	0.3	-	-
7	α-terpinene	1013	0.6	-	-	-	-	-
8	p-cymene	1019	-	-	0.2	-	-	-
9	limonene	1031	40.6	37.1	81.4	43.5	52.0	53.9
10	(Z)-β-ocimene	1041	2.3	2.3	1.6	2.7	2.6	2.1
11	γ-terpinene	1056	0.4	0.3	-	0.3	-	-
12	terpinolene	1086	-	0.3	1.6	1.5	-	-
13	linalool	1088	0.4	1.1	-	-	1.1	1.2
14	α-pinene oxide	1100	1.2	-	-	-	-	-
15	citronnelal	1138	0.8	0.5	35	1.7	1.1	1.3
16	isocamphene	1148	0.3	0.4	-	0.5	-	0.6
17	borneol	1165	0.5	0.5	-	0.6	-	0.8
18	terpinen-4-ol	1175	0.6	0.5	-	0.6	-	-
19	myrtenal	1180	-	-	-	0.3	-	-
20	α-terpineol	1186	1.1	0.5	-	0.4	0.5	0.4
21	citronellol	1219	1.6	0.3	0.6	1.3	1.4	0.7
22	nerol	1221	3.1	2.0	0.4	2.7	4.0	1.4
23	neral	1228	7.5	9.8	0.4	10.0	7.7	10.0
24	geraniol	1244	2.7	1.6	0.3	4.0	4.7	1.3
25	geranial	1255	9.2	12.2	0.5	12.6	10.9	12.3
26	citronellyl acetate	1336	-	-	12	-	-	-
27	neryl acetate	1347	1.0	2.0	1.0	1.9	1.3	4.6
28	geranyl acetate	1364	0.8	1.6	0.3	3.2	2.4	2.0
29	β-elemene	1397	-	-	0.9	1.0	0.4	2.0
30	β-caryophyllene	1434	0.8	0.7	1.1	2.6	2.2	0.6
31	α- humulene	1468	-	-	0.2	0.5	0.4	-
32	germacrene D	1499				0.5	0.4	-
33	β-bisabolene	1513	0.3	-	0.3	0.4	0.4	-
34	δ- cadinene	1540	-	-	-	0.3	-	-
35	caryophyllen oxide	1595	-	-	-	0.4	0.5	-
36	α- eudesmol	1632	-	-	-	0.3	0.3	-

It can be deduced from the table that the 3 varieties of *C. limon* studied are very rich in limonene (40.6 %, 37.1 % and 81.4 % respectively for the Lisbon, Eureka and *Meyer* varieties). In addition, β-pinene (16.0 % and 17.6 %), neral (7.5 % and 9.8 %) and geranial (9.2 % and 12.2%) are relatively abundant in the extracts of the Lisbon and Eureka varieties.

For *C. aurantifolia*, the three varieties studied contain a high percentage of limonene (43.5%, 52.0% and 53.9% respectively for the Mexican, "Sans épines" and *Bearss* varieties); followed by

geranial and *neral* (12.6 % and 10.0 % for Mexican; 10.9 % and 7.7 % for "Sans épines"; 12.3 % and 10.0 % for *Bearss*), which is not in accord with the results of who obtained as majority compound geranial (19.4 %) by analyzing the essential oil of the leaves of *C. aurantifolia* collected in Malaysia, which is concordant with the results of .

3.3. Antiradical Properties of Essential Oils

The antiradical properties of the studied samples were determined and compared with an antiradical of reference which is BHT (Butylated hydroxytoluene).

As regards to the essential oils studied, the results obtained are materialized by figures 1 and 2.

Figure-1. Antiradical activities of the essential oils of Citrus limon

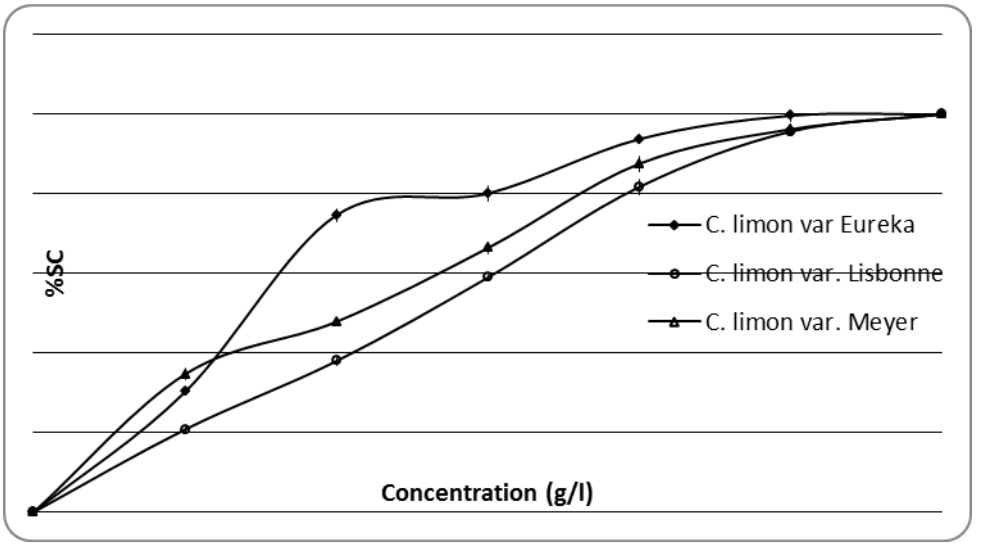
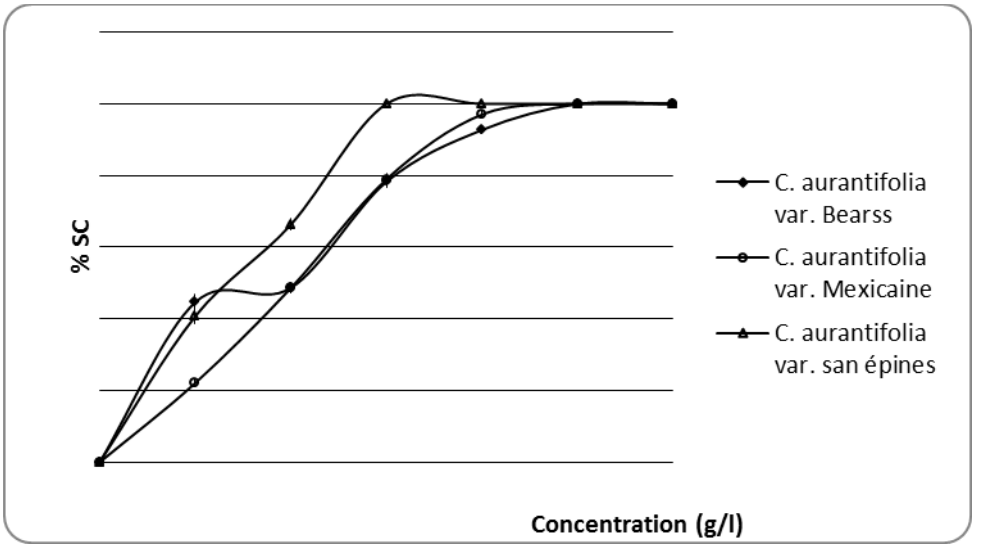


Figure-2. Antiradical activities of the essential oils of Citrus aurantifolia



From these figures it can be seen that the percentages of trapping of free radicals grow with concentrations of the various essential oils in the reaction medium until a stationary state.

These figures enabled us to obtain the SC_{50} of the studied varieties. These values are gathered in table 2 as well as the effective concentrations (CE_{50}) and the antiradical capacities.

Table-2. Values of SC_{50}, CE_{50} and antiradical capacity of the studied samples

Samples	SC_{50} (g/l)	Efficient Concentration CE_{50} (g /mol of DPPH)	Antiradical power
BHT	$7.0\ 10^{-3}$	89	$1.13\ 10^{-2}$
C. limon var. Lisbonne	7.8	$9.9\ 10^{4}$	$1.01\ 10^{-5}$
C. limon var. Eureka	3.6	$4.6\ 10^{4}$	$2.19\ 10^{-5}$
C. limon var. Meyer	5.5	$6.9\ 10^{4}$	$1.45\ 10^{-5}$
C. aurantifolia var. Bearss	5.1	$6.48\ 10^{4}$	$1.54\ 10^{-5}$
C. aurantifolia var. *Mexicaine*	5.1	$6.48\ 10^{4}$	$1.54\ 10^{-5}$
C. aurantifolia var. sans épines	3.4	$4.24\ 10^{4}$	$2.36\ 10^{-5}$

The results of *C. limon* show that the extract of the Eureka variety presents a more significant antiradical capacity (SC_{50} = 3.6g/l, CE_{50} = 4.6 x 10^4 g/mol and AC = 2.19 x10 $^{-5}$) than the Meyer variety (SC_{50} = 5.5g/l, CE_{50} = 6.90 x 10^4 g/mol and AC = 1.45 x 10^{-5}) and Lisbon (SC_{50} = 7.8g/l, CE_{50} = 9.9 x 10^4 g/mol and AC = 1.01 x 10^{-5}). However, these antiradical capacities are much lower than that of the *BHT* which presents an SC_{50} of 7.02 mg/l.

As regards *C. aurantifolia*, it was noticed that the "Sans épines" variety has the strongest activity (SC_{50} = 3.35 g/l, CE_{50} = 4.24 x 10^4 g/*mol* and AC = 2.36 x 10^{-5}); while the *Bearss* and Mexican varieties have similar activities (SC_{50} = 5.13 g/l, CE_{50} = 6.48 x 10^4 g/mol and AC = 1.54 x 10^{-5}).

3.4. Antioxidant properties of essential oils

The same compound (BHT) was used as reference. The results obtained made it possible to plot the curves of figures 3 and 4

Figure-3. Antioxydant Activities of *C. limon* var. Eureka and *C. limon* var. Lisbon

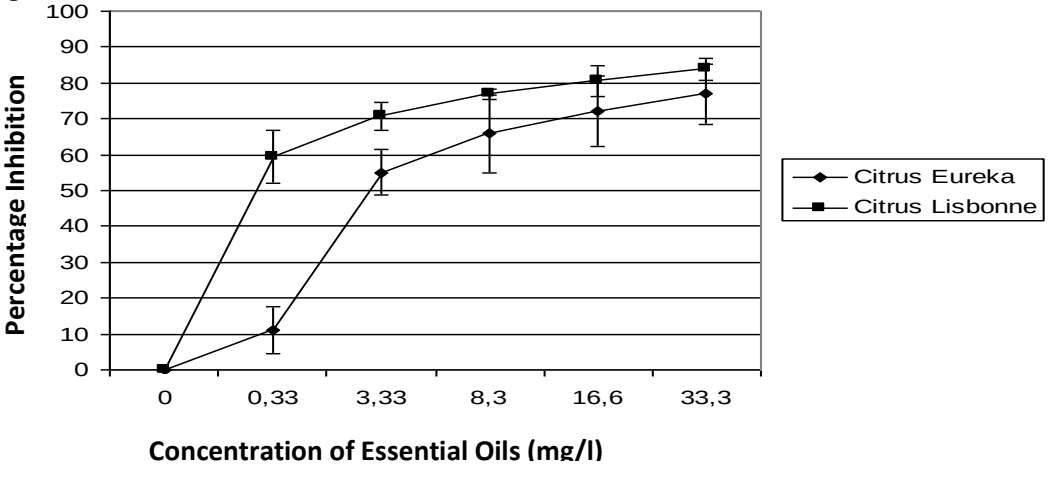
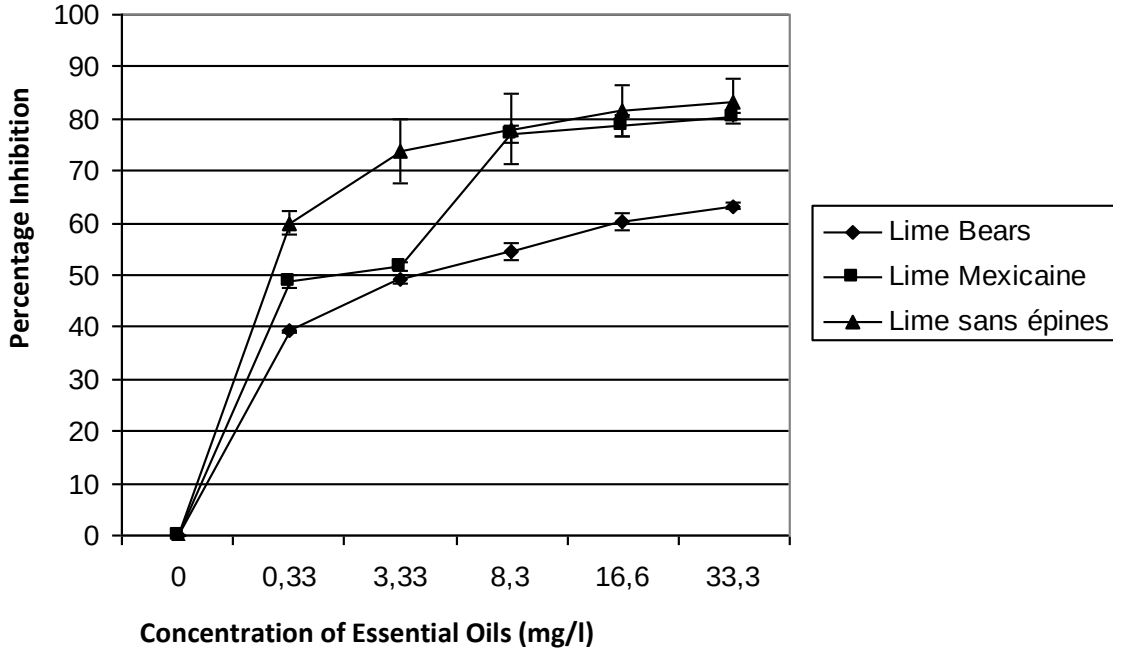


Figure-4. Antioxidant Activities of *C. auratifolia* var. Bearss, *C. aurantifolia* var. Mexican and *C. aurantifolia* var. "Sans épines"



These figures show that the percentages of inhibition grow with the concentrations of the different essential oils in the reaction medium until a stationary state.

These figures enabled us to obtain the IC_{50} in mg/l of varieties studied. These values are shown in table 3 as well as the value obtained with BHT, antioxidant molecule of reference used.

Table-3. Values of IC_{50} of the studied samples

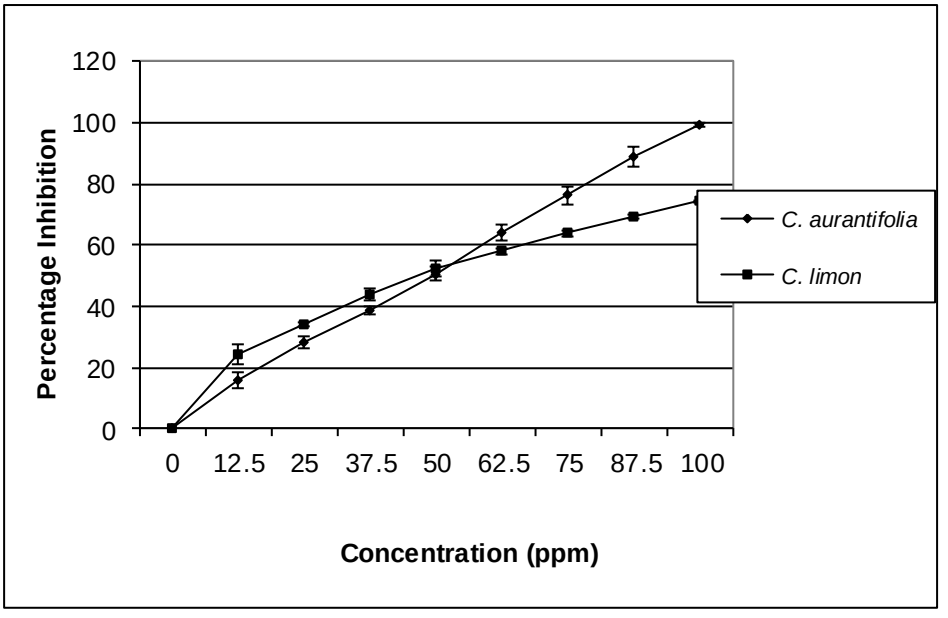
Samples	IC_{50} in mg/l
BHT	0.10 ± 0.0007^{a}
C. aurantifolia var. Bearss	4.32 ± 1.02^{d}
C. aurantifolia var. Mexican	1.62 ± 0.37^{b}
C. aurantifolia var. "sans épines"	0.26 ± 0.028^{a}
C. limon var. Eureka	3.12 ± 0.34^{c}
C. limon var. Lisbon	0.28 ± 0.06^{a}

These results show that essential oils of *C. aurantifolia* var "sans épines" and *C. limon* var Lisbon are very effective, as effective as the BHT thus, according to the Duncan test of classification, there is no significant difference between their IC_{50} values.

3.5. Anti-inflammatory Properties of Essential Oils

The anti-inflammatory activity of the extract of *C. limon* var Meyer and *C. aurantifolia* var. "sans épines" was studied and the results compared with a reference, NDGA (Nordihydroguaieretic). The results are illustrated on the figures below.

Figure-5. Anti-inflammatory activity of *C. limon* var. Meyer and *C. aurantifolia* var. Sans épines



IC_{50} *C. limon* var. Meyer =46.5 ppm

IC_{50} *C. aurantifolia* var. "Sans épines" = 49.35 ppm

IC_{50} NDGA= 0.7 ppm

C. limon var. Meyer (IC_{50}= 46.5 ppm) is less active than NDGA (IC_{50} = 0,7 ppm), which is almost similar to the result of *C. aurantifolia* var "Sans épines" (IC_{50} = 49.35ppm), that is, less active than NDGA. Nevertheless, these results show a strong activity of these extracts compared to other extracts that have already been studied (Ndoye, 2001; Nyegue, 2006), from where a possible exploitation in the field.

In conclusion, these essential oils are of unquestionable interest and prove to be good anti-inflammatory drugs. They can thus be used both for therapeutic and cosmetic purposes.

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