

## Chemical composition, free radical scavenging and antifungal activity of *Zanthoxylum leprieurii* essential oils against *Epidermophyton floccosum* and *Trichophyton rubrum*

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**ABSTRACT-Background:** These investigations were carried out to analyze the chemical composition, to evaluate the radical scavenging, and the anti-fungal activities of essential oils from leaves and fruits of *Zanthoxylum leprieurii*.

**Methods:** Oils were obtained by hydro distillation using Clevenger-type apparatus. The compounds were identified by gas chromatography and gas chromatography coupled with Mass Spectrometry. Antifungal activity of the essential oils was tested *in vitro* against *Epidermophyton floccosum* and *Trichophyton rubrum* while free radical scavenging activity evaluation was done using 2,2-diphenyl picrylhydrazyl method.

**Results:** Results showed that, the major components of the leaves oil were (E)- $\beta$ -ocimene (91.5 %) while the most abundant component from fruits oil were (E)- $\beta$ -Ocimene (90.3 %). The total growth inhibition of the pathogens by fruits essential oil occurred at 4.0 mg/ml and 3.0 mg/ml respectively, for *Epidermophyton floccosum* and *Trichophyton rubrum*. The leaves oil exhibited total growth inhibition at 4.0 mg/ml against both pathogens. These oils also showed antioxidant activities with SC<sub>50</sub> values at 2.0 mg/ml and 5.0 mg/ml respectively, for fruits and leaves. There were positive and significant correlations between mycelia growth inhibition of both pathogens and the antioxidant activities of the tested oils noted.

**Conclusion:** These results showed that, *Z. leprieurii* essential oils could be used as a resource of antioxidant and antidermatophytic compounds with may find applications in pharmaceutical industries.

**Key words:** Essential oil, Antidermatophytic activity, Antioxidant activity, *Zanthoxylum leprieurii*

### I. INTRODUCTION

Cutaneous mycosis is a human dermatophytosis which is the most common infection in skin and nail worldwide especially in Sub-Africa countries including Cameroon. This infection is often caused by filamentous fungi belonging to the genera *Epidermophyton*, *Trichophyton* and *Microsporium* [1]. Among them, *E. floccosum* and *T. rubrum* are widespread in most countries of the world, accounting for 5 % of all dermatophytes isolated [2]. Some preliminary works in Cameroon found that these two fungi were the most prevalent cutaneous mycosis. These fungi are anthropophilic dermatophytes that are transmitted between individuals by contact, particularly in community swimming pool areas, common showers, and gym facilities. The clinical presentation predisposing factors for mycosis include immunosuppression, diabetes mellitus, advanced age nail trauma, and poor peripheral circulation [3]. Free radicals are produced in normal and or pathological cell metabolism. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in triggering many diseases including dermatosis. Exogenous chemical and endogenous metabolic processes in the human body might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage [4]. The clinical care of cutaneous mycosis is based on combination of a topical and systemic chemical antifungals treatment. However, synthetic antifungals suffer from various weaknesses in terms of toxicity, lack of fungicidal efficacy, cost and emergence of resistant strains caused by the frequent use of some of them [5]. In spite of the recent introduction of new antifungal drugs, they are still limited in number. Hence, there is a great demand for novel antifungal agents, justifying the intense search for new drugs that are more effective and less toxic than those already in use [6,7]. An alternative should be used for plant derivate products such as the essential oils (EO) to control dermatophytes infections.

Essential oils are the odorous, volatile products of plant secondary metabolism normally formed in special cells or groups of cells or as glandular hair, found on any organ of the plant including roots, stem, leaves, flowers and fruits. EO have demonstrated various biological activities including anticancer, antiviral, antibacterial, antifungal and antioxidant activities [8,9,10]; as well as their ability to facilitate the passage of active molecules through the human tissues [11]. *Zanthoxylum leprieurii* is a large species of the family of *Rutaceae* which is geographically distributed across Africa. In traditional Cameroon medicine, this plant is used for the treatment of intestinal worms, abdominal pain, hyperhermia, diarrhea and certain mycosis [12]. Previous works have demonstrated the antimicrobial activities of *Zanthoxylum leprieurii* essential oil from Cameroon [13]. However, in the best of our knowledge there is no report on the antifungal properties of this oil against *E. floccosum* and *T. rubrum*. The aim of this study was to analyze the chemical composition of *Zanthoxylum leprieurii* essential oils and to evaluate their antioxidant and antifungal activities against *E. floccosum* and *T. rubrum*, the two most common causal agents of cutaneous mycosis.

## II. MATERIALS AND METHODS

### 1. Plant material

Leaves and fruits of *Z. leprieuri* were collected at Bamena (West Region of Cameroon) on July 2014. The herbarium/plants were identified at the National Herbarium where voucher specimen was deposited under the reference 2713/SRFK/CAM.

### 2. Essential oils extraction

About 200-400 g of air-dried leaves and fruits (after grounded) were coarsely crushed. Each sample was distilled for about 4 h using Clevenger-type apparatus. Essential oils collected were dried over anhydrous sodium sulphate, stored in an amber-colored flask and kept at 4°C until use.

### 3. Essential oils analysis

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

### 4. Gas chromatography

Each 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 DB-5, film thickness 0.25 µm); temperature program 50°C-200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas N<sub>2</sub> 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.

### 5. Gas chromatography coupled with mass spectrometry

GC-MS analyses were carried out using a furnace Focus GC (Thermo) apparatus equipped with a TG-5MS5MS column (30 m x 0.25 mm, film thickness, 0.25 µm) and interfaced with a quadruple detector (DSQ II). Column temperature was programmed from 60 to 200° C at 10° C/min; injector temperature was 220° C. The injections were carried out in mode SPLIT (Ratio: 1/100). Helium was used as carrier gas at a flow rate of 1.2 ml/min; the mass spectrometer was operated at 70 eV. The components were identified based on the comparison of their retention indices and their mass spectra with those given in the literature [14].

### 6. Fungal pathogen

*Epidermophyton floccosum* and *Trichophyton rubrum* used in this study were isolated from male patient at the General Hospital of Douala (Cameroon). Skin and nail scales of fungal lesion from patient were cultured on Sabouraud Dextrose Agar supplemented with chloramphenicol. After 21 days of incubation at 28 °C, the mycelia emerging from tissues were transferred into fresh Sabouraud medium. The operation was repeated many times to obtain pure culture of each isolate. Identification of both fungi isolate was based on macroscopic and microscopic criteria according to the method described by [15,16]. The cultures were stored at 4°C and sub-culture every three month.

### 7. Antifungal assay

The inhibition of mycelia growth was performed using agar incorporation method [17]. The test was carried out in 90 mm Petri dishes containing SDA medium supplemented with chloramphenicol. Four millimeters diameter disc was taken from the margin of 9 days old pure culture of each fungus, seeded at the center of Petri dish and incubated at 28±2°C. Oils were previously mixed with DMSO (Dimethylsulfoxide) to obtain a final concentration of 10 % (v/v) and added into the medium to produce final concentrations of 0.5, 1, 2, 3, 4 and 5 mg/ml. Plate containing SDA with DMSO was negative control while griseofulvine served as positive control. Each treatment consisted of four Petri plates and the experiment repeated twice. After 14 days of incubation, the antifungal activity of each essential oil was evaluated by calculating the percentage of inhibition (% I) of mycelia growth using the following formula: % I = ((Do – De)/Do) x 100 where Do is the diameter of the mycelia growth in the negative control and De the diameter of the mycelia growth in each oil supplemented plates. Fungistatic or fungicide effect of oils was determined by transferring the discs from the plate where no growth occurred in to the new SDA medium.

### 8. Antioxidant assay

This was carried out by scavenging of the stable 2,2-diphenylpicrylhydrazyl (DPPH) radical according to the method describe by Brand-William [18]. Ten microlitres of various concentrations (1 to 5 mg/ml) of each essential oil in methanol was added to 1990 µl of a 10 mg/ml methanol solution of DPPH. After 60 min incubation period at room temperature, the absorbance was read at 517 nm. Percentage inhibition of the DPPH free radical (I %) was measured using the following equation: (%) I = (A<sub>DPPH</sub> – A<sub>sample</sub>/A<sub>DPPH</sub>) x 100, where A<sub>DPPH</sub> is optical density of the positive control, A<sub>sample</sub> is optical density of the essay (essential oil). Butyl Hydroxy Toluene (BHT) was used as a standard chemical reference antioxidant at the various concentrations ranging from 0.04 to 0.4 mg/ml.

### 9. Statistical analysis

Data were analyzed using SPSS version 16.0. Results were expressed in term of means. Multiple comparisons of mean values were set up using one-way parametric ANOVA. The DUNCAN test was used to appreciate the differences between the means at p<0.05. The relationship between the different parameters was done using Pearson correlation.

## III. RESULTS AND DISCUSION

### 1. Extraction and chemical composition of the essential oils

Essential oils were obtained by hydrodistillation with yield of 0.025 % and 1.45 % respectively, from leaves and fruits (Table 1). Twenty one and twenty six compounds were identified representing 96.0% and 100 % volatile oil from leaves and fruits respectively. The major component of the leaves oil was (E)- $\beta$ - Ocimene (91.5 %) while (E)- $\beta$ -Ocimene (90.3 %) is the major components of the fruit oil. However, some minor components were present only in the leaf oil ( $\alpha$ -Pinene, Limonene, (2E)-Octen-1-ol, Linalol, Nobornyl acetate, Octyl acetate, Neryl acetate and Germacrene B) and fruit oil ( $\alpha$ -Terpinene,  $\beta$ -Phellandrene,  $\gamma$ -Terpinene, Cis-Linalool axide,  $\alpha$ -Pinene oxide, Cis-para-menth-2-enol, Allo-ocimene, Myrtenol,  $\alpha$ -Terpineol, Myrtenal, Pinocarvone,  $\gamma$ -Curcumene and Trans  $\beta$ -Guiaene). The essential oils of *Z. lepriurii* from different countries including Cameroon have been already studied. The analysis reveals a great degree of variability and plasticity in *Zanthoxylum* essential oil yield and composition[19, 20]. From leaves oil of Costa Rica, [20]obtained 1.35% yield, and a total of 12 components. The major components were citronellal (23.3%), limonene (16.7%), trans-pinocamphone (11.6%), and  $\beta$ -pinene (10.8%). Fogang[34] obtained (E)- $\beta$ -ocimene (29.4%), myrcene (28.6%), limonene (13.6%) and  $\alpha$ -pinene (8.1%) as the major compounds in leaves oil from Cameroon.[35]studied *Z. lepriurii*volatile oils from leaves and fruits of Cameroon. Their results showed that the oil distilled from the leaves contained only hydrocarbons. Among them,  $\alpha$ -pinene (40.4%), myrcene (27.1%) and trans- $\beta$ -ocimene (22.4%) were the most important. The composition of the fruit oil is more diverse, with a fairly large proportion of oxygenated products (46%) among which citronellol, geraniol and their acetates are the most abundant. Globally, these components were quantitatively and qualitatively different from those obtained in our study. In general, it is well demonstrated that, variation in yield and chemical composition could be explain by the extrinsic and intrinsic parameters such as the age of the plant, organ, soil, climate and ecological factors [21,22].

Table 1: Chemical composition of leaves and fruits essential oils of *Zanthoxylum lepriurii*

N°	Compounds	*KI	Relative Percentage (%)	
			Leaves	Fruits
1	$\alpha$ -Thujene	925	0.3	0.2
2	$\alpha$ -Pinene	943	0.1	-
3	Camphene	954	0.1	0.1
4	$\beta$ -Pinene	977	0.1	0.1
5	Octanol	983	0.1	0.3
6	Myrcene	990	-	2.3
7	$\alpha$ -Phellandrene	1000	0.8	0.1
8	$\alpha$ - Terpinene	1004	-	0.1
9	p-Cymene	1022	0.1	0.8
10	$\beta$ -Phellandrene	1029	-	0.1
11	Limonene	1032	0.2	-
12	(E) - $\beta$ -Ocimene	1039	<b>91.5</b>	<b>90.3</b>
13	(2E)-Octen 1-ol	1059	0.4	-
14	$\gamma$ -Terpinene	1055	-	0.5
15	Terpinolene	1087	0.2	0.1
16	Cis-Linalool oxide (furanoid)	1084	-	0.1
17	Linalol	1090	0.1	-
18	$\alpha$ -pinene oxide	1093	-	0.5
19	Pinocarveol	1109	0.4	2.8
20	cis-p-Menth-2-en-1-ol	1118	-	0.1
21	$\alpha$ -Campholenal	1124	-	0.3
22	Norbornyl acetate	1127	0.2	-
23	Allo-ocimene	1128	-	0.2
24	t-Pinocarveol	1132	0.6	0.2
25	Acetate	1186	0.1	0.1
26	Myrtenol	1190	-	0.1
27	$\alpha$ -Terpineol	1192	-	0.1
28	Myrtenal	1194	-	0.2
29	Octylacetate	1196	0.1	-
30	Pinocarvone	1343	-	0.1
31	Neryl acetate	1345	0.2	-
32	$\gamma$ -Curcumene	1472	-	0.1
33	Germacrene B	1475	0.2	-
34	Trans- $\beta$ -guaiene	1512	-	0.1
35	(2E, 6E)- Farnesyl acetate	1840	0.1	-
36	Farnesyl acetone	1850	0.1	-
Total			96.0	100
Yield (%)			0.025	1.45

\*KI Kovats index was determined by Gas Chromatography on a DB<sub>5</sub> column

## 2. Effects of essential oils on mycelia growth of *E. floccosum* and *T. rubrum*

Essential oils from leaves and fruits of *Z. lepriurii* showed activity in inhibiting the growth of *E. floccosum* and *T. rubrum* (Table 2). In fact, results showed that, there are significant differences ( $p < 0.05$ ) in the mycelia growth of each EOs supplemented samples compared with the negative control. Total inhibition of mycelia growth of *T. rubrum* occurred at 3.0 mg/ml and 4.0 mg/ml by EOs from fruits and leaves respectively while *E. floccosum* was completely inhibited at 4.0 mg/ml by the oils. Maximal inhibition by Gliseofulvin was observed at 5 mg/ml i.e 75.0% for *T. rubrum* and 65.35% for *E. floccosum*.

Many studies have also been reported on the antifungal activities of *Zanthoxylum* essential oil[23,24,25,26]. The antifungal properties of our samples could be due to the complexity of their chemical composition and structure of their different

constituents. It could also be due to the presence of the main compound such as ocimene or to a possible synergistic effect of the minor or major compounds in the essential oil [27,28]. The antifungal activities of compounds present in our tested essential oils may be explained to their lipophilic character and ability to disrupt the cellular membrane permeability and lead to the death of the cell [11].

Table 2 Mycelia growth inhibition of *T. rubrum* and *E. floccosum* by leaves and fruits essential oil of *Z. leprieurii*

Oils	<i>T. rubrum</i>			<i>E. floccosum</i>		
	Leaves	Fruits	Griseofulvine	Leaves	Fruits	Griseofulvine
Conc (mg/ml)	Inhibition percentage (%)					
0.0	0.0 <sup>f</sup>	0.0 <sup>f</sup>	0.0 <sup>d</sup>	0.0 <sup>f</sup>	0.0 <sup>f</sup>	0.0 <sup>c</sup>
0.5	15.0 <sup>e</sup>	16.0 <sup>d</sup>	56.0 <sup>c</sup>	24.0 <sup>d</sup>	21.66 <sup>d</sup>	50.0 <sup>b</sup>
1.0	30.33 <sup>d</sup>	43.33 <sup>c</sup>	58.0 <sup>c</sup>	37.0 <sup>c</sup>	41.0 <sup>c</sup>	52.0 <sup>b</sup>
2.0	69.0 <sup>c</sup>	64.66 <sup>b</sup>	64.0 <sup>b</sup>	37.33 <sup>c</sup>	41.0 <sup>c</sup>	55.5 <sup>b</sup>
3.0	88.0 <sup>b</sup>	100.0 <sup>a</sup>	67.33 <sup>b</sup>	58.66 <sup>b</sup>	56.33 <sup>b</sup>	58.5 <sup>ab</sup>
4.0	100.0 <sup>a</sup>	100.0 <sup>a</sup>	70.0 <sup>a</sup>	100.0 <sup>a</sup>	100.0 <sup>a</sup>	61.5 <sup>a</sup>
5.0	100.0 <sup>a</sup>	100.0 <sup>a</sup>	75.0 <sup>a</sup>	100.0 <sup>a</sup>	100.0 <sup>a</sup>	65.33 <sup>a</sup>

In the same column, values followed with the same letter are not significantly different ( $p \leq 0.05$ ) according to Duncan multiple range test.

### 3. Antioxidant activities of essential oils

Oils from leaves and fruits of *Z. leprieurii* showed effective free radical scavenging in DPPH assay (Figure 1). The antioxidant activities increase significantly with the concentration of each essential oil. The oils from fruits exhibited a remarkable antioxidant effects at low concentration with  $SC_{50}$  at 2 mg/ml, while  $SC_{50}$  from leaves oils was 5 mg/ml. Nevertheless, at all concentration tested, the chemical standard antioxidant BHT had a great effect compare to our oils sample and exhibited the most less  $SC_{50}$  (0.25 mg/ml). It has been demonstrated that plant extract and volatile essential oil can exhibit significant antioxidant activities [29,30,31]. This property could be assigned to many compounds such as aromatic and oxygenated monoterpenes [32,33,34] which are presents in our essential oils. Similarly, [11] showed that, antioxidant and antimicrobial activities of essential oil could be related to the presence of a variety of volatile molecules such as terperne and terpenoid, phenol derived aromatic components and aromatic components. Free-radicals are generated continuously in the body due to metabolism and diseases [35]. In order to protect themselves against free radicals, organisms are endowed with endogenous (catalase, superoxide dismutase, glutathione peroxidase/reductase) and exogenous (C and E vitamins, carotene, uric acid) defense systems. These defense systems are not sufficient in critical situations such as oxidative stress, contamination, microbial infections, where the production of free radicals significantly increases [36]. In our study, results showed that there are positive and significant correlations between the mycelia growth inhibition of *E. floccosum* and antioxidant activity of fruits ( $r^2 = 0.524$ ;  $p = 0.025$ ) and leaves Eos ( $r^2 = 0.734$ ;  $p = 0.01$ ). Positive correlation were also observed between *T. rubrum* growth inhibition and antioxidant properties ( $r^2 = 0.959$ ;  $p = 0.000$ ;  $r^2 = 0.974$ ;  $p = 0.000$ ) respectively, from fruits and leaves Eos. [37] studied the oil from *Zanthoxylum* sp. and found that, it had antifungal and strong antioxidant activity. There is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants.

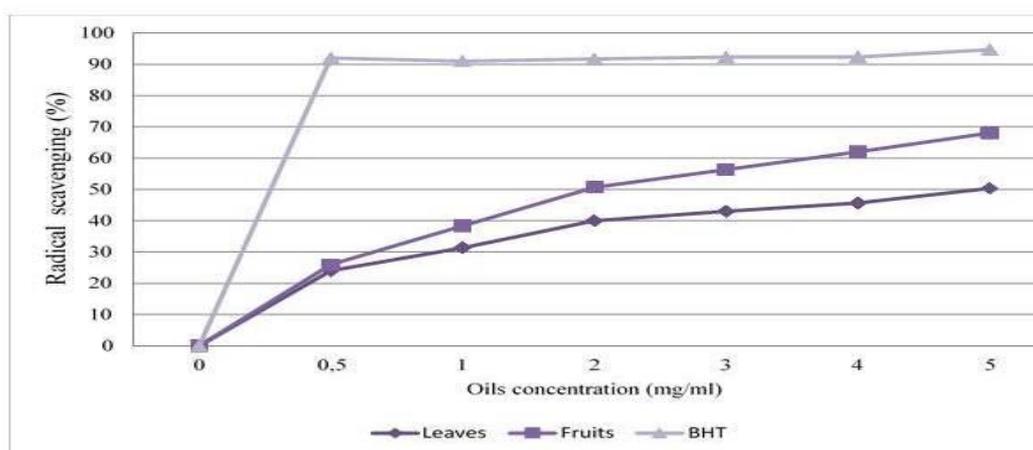


Figure 1: Antioxidant activities of leaves and fruits essential oils of *Z. leprieurii*

## IV. CONCLUSION

As our results is concerned, leaves and fruits of *Z. leprieurii* essential oils significantly inhibit the mycelia growth of *E. floccosum* and *T. rubrum*. They also have antioxidant properties. These could be assigned to the synergistic effects of minor and major components in the oils. This finding demonstrated that, *Z. leprieurii* essential oils could be served as alternative to

chemical fungicide to fight against dermatophytosis. Moreover, these oils are potential natural antioxidant. However, *in vivo* tests are needed.

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