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Antidermatophyte Activity of Essential oils of Fresh *Zanthoxylum zanthoxyloides* Lam Leaves and Fruits Against *Trichophyton rubrum* and Antifungal Ointment Formulation Test

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Abstract:- Dermatophytes are fungi responsible for superficial mycoses called dermatophytoses. The aim of this work was to evaluate the anti-dermatophyte activity in vitro of essential oils from the leaves and fresh fruits of *Zanthoxylum zanthoxyloides*. For this purpose, the essential oils from the leaves and fresh fruits of *Z. zanthoxyloides* harvested in Bamena were extracted by hydro-distillation using a Clevenger. The extraction yields were 1.12% for the fruits and 0.04% for the leaves. The chemical composition of these extracts was determined by gas chromatography (GC) and by gas chromatography coupled with mass spectrometry (GC / MS). The results of this analysis made it possible to identify 59 compounds in the essential oil of fruits, with citronellol as the major compound (18.63%) while in the leaf extract 58 compounds were identified, with as major compound, citronellol acetate (23.4%). The essential oils of *Z. zanthoxyloides* showed MICs of 3600, 4200 and 4420 ppm respectively for the essence of the leaves, fruits and the mixture of fruits and leaves, the dermal application of essential oils did not cause any modification behavior including no erythema or edema on the skin, no convulsions, no tremors, or diarrhea. Food and water consumption remained normal in the treated groups during the experiment compared to the first day of the study. A formulation trial of an antifungal ointment and conformity tests led to the production of a product whose pharmaceutical form was called: ZANTHOZOLE ointment.

Keywords: *Dermatophytes; Essential Oils; Acute Dermal Toxicity; Ointment Formulation.*

I. INTRODUCTION

Plant resources take a preponderant place in human life. In Africa, rural populations have been able to conserve and transmit a great deal of knowledge about local plant biodiversity and continue to use the resources offered by plants on a daily basis (Bognounou and Guinko, 2006). The World Health Organization (WHO) estimates that nearly 80% of the African rural population uses medicinal plants for first aid. In Cameroon, the richly represented plant resource is full of several aromatic plants which have demonstrated an interest. In this rich flora, we should mention the plants of the genus *Zanthoxylum*, with some interesting species that are *Zanthoxylum leprieurii*, *Zanthoxylum macrophylla* and *Zanthoxylum zanthoxyloides*, which are used in traditional medicine to combat several dermatophytic diseases. (Tchiégang and Mbougueng, 2005). Dermatoses are affections of the skin, and by extension those of the nails or the hair (Samuel *et al*., 2000). Nowadays, they present an increasingly high prevalence in developing countries and this increase is due to the ever-increasing rate of degenerative diseases and the poorly adapted or incomplete treatment responsible for several cases of resistance (Sepahvand *et al*., 2009). In some regions of Cameroon, the prevalence of dermatophytosis in children is around 31% (Maslin et al, 2005), thus constituting a public health problem. In view of the increasingly high incidence of dermatophytosis, a team of researchers, particularly those from the Biochemistry laboratory at the University of Douala, decided to focus their work on the best use of Cameroonian flora. It is with this in mind that several studies have been carried out on some interesting species of *Zanthoxylum* and the results obtained are promising (Tchoumbougnang, (1997, 2005), Tatsadjieu *et al*., 2003, Kuiate, 2005, Nyegue, 2006, Jazet *et al*., 2008). In 2018, Tchabong *et al*. demonstrated that essential oils of the

Zanthoxylum genus exhibited interesting antifungal activity on three dermatophytes (*Microsporum gypseum, Epidermophyton floccosum* and *Trichophyton rubrum*).

II. MATERIALS AND METHODS

1. Materials

1.1. Plant Material

The plant material consisted of essential oils extracted from *Zanthoxylum zanthoxyloides*, the characteristics of which are presented in Table I.

Table I: Summary of Plant Information

Species	Botanical Family	Studied organs	Harvest date	Harvest site	Herbarium specimen number
Z. zanthoxyloides	Rutaceae	Leaves and fresh fruit	18/02/17	Bamena	2713/SRFK

1.2 Material for extraction

The essential oils extracted using a Clevenger type device (Photo 1) were used as extraction material.

Photo 1: Clevenger equipment

1.3 Fungal Material

The microorganism used is a dermatophyte belonging to the genus *Trichophyton* (Photo 2). It comes from the fungal bank of the Biochemistry Laboratory of the Faculty of Sciences of the University of Douala and had the code BD 023.

Photo 2: *Trichophyton rubrum* on Sabouraud Dextro Agar medium with chloramphenicol

1.4. Animal material

A total of 5 guinea pigs weighing on average 350-450 g were used for each test. They were all female, nulliparous and not pregnant. The animals were reared in the animal house of the biochemistry unit of the University of Douala under the temperature conditions of the experimental room (22 ° C ± 3). These animals were placed individually in cages lined with wood chips (Photo 3) and received a diet consisting of fodder and food supplements rich in protein, vitamins and calcium.

Photo 3: guinea pig (*Cavia porcellus*)

2. Methods

2.1. Analysis of the chemical composition of essential oils

The essential oils obtained were analyzed by CPG and by CPG / MS. This method is based on the separation of vaporized compounds by heating.

2.2. Preparation of Culture Medium and Antifungal Tests

The culture of the fungal species and the antifungal tests were carried out on Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol as an antibacterial. For the preparation of the medium, 65.5 g are dissolved in distilled water to obtain 1 L of medium. Complete dissolution is made by bringing the mixture to the boil (activation of the culture medium) in a water bath. The culture medium thus prepared is distributed into the 15 ml glass flasks, then sterilized in an autoclave for 15 min at 121 ° C.

2.3. Preparation of media supplemented with essential oils

Under the laminar flow hood, the stock solutions of the essential oils were prepared beforehand by dissolving the essences in dimethylsulfoxide (DMSO) in the proportions

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(1/9) (v /v). This DMSO surfactant allows a better diffusion of essential oils in the SDA culture medium. Subsequently, the Essential Oil (ET) / DMSO solution obtained, at varying concentrations, is supplemented with SDA media so as to obtain concentrations (Table II). The whole is homogenized by stirring and poured into Petri dishes 90 mm in diameter at a rate of 10 ml per dish. All these operations were carried out in a microbiological hood near the Bunsen burner.

Table II: Essential oils solutions concentration / DMSO

Concentration (ppm)	DMSO (µL)	HE (µL)	DMSO+HE (µL)	SDA (µL)	Total volume (µL)
Control (-)	0	0	0	10000	10000
TDMSO		0	0		10000
500	60.4	6.4	66	9934	10000
1000	117	13	130	9870	10000
2000	200	26	260	9740	10000
4000	500	53	530	9470	10000
8000	1000	106	1060	9000	10000

2.4. Inoculation

After solidification of the medium in the Petri dish, a 2 mm mycelium disc from a 4 to 5 day pre-culture of the germs studied is placed in the center of each of the dishes. These dishes are then sealed with parafilm and incubated at room temperature (25 ° C) in an inverted position. The growth of the mycelium is monitored by means of measurements taken, at the same time, every two days, of the growth diameter along two perpendicular lines passing through the center of the box. These measurements are carried out for three weeks using a graduated ruler or until the "negative control" dishes are completely invaded by the mycelium.

2.5. Evaluation of the antifungal properties of essential oils

The antifungal activities of essential oils from the fruits and leaves of *Zanthoxylum zantholoides* were evaluated using the agar incorporation method as described by Lahlou (2004). This technique consists of following the mycelial growth of a microbial culture disc, seeded in the center of the Petri dishes containing the culture medium supplemented with essential oils at different concentrations.

2.6. Criteria for selecting animals for study

Selection of species

Inclusion criteria: Any nulliparous, non-pregnant female guinea pig with a weight range of 350 to 450 g.

Exclusion criteria: Any guinea pig weighing less than 350g.

2.7. Dermal irritation tests

The evaluation of the degree of dermal irritation was carried out on guinea pigs as an animal model using the EOs of the fresh fruits and leaves of *Zanthoxylum zanthoxyloides*. This method is equivalent to the OECD TG 404 guideline (OECD, 2002). For this, 20 guinea pigs (females) were used and each animal served as its own control. They were divided into four groups of five animals.

Group 1: animals tested with the essences of leaves and fresh fruits at a volume of 1 ml;
Group 2: animals tested with the essences of leaves and fresh fruits at a volume of 2 ml;
Group 3: animals tested with the essences of leaves and fresh fruits at a volume of 3 ml;
Group 4: animals tested with the essences of leaves and fresh fruits at a volume of 4 ml;

On day D0, the hair was shaved at two sites of approximately 6 cm2 on the dorsal part of each animal (Photo 4a). The right site served as a negative control and the left site was the test site. Each guinea pig was locked in a cage for 24 hours.

On day D1, 1mL, 2mL, 3mL, 4mL of essential oils were applied to the test site of animals in groups 1, 2, 3 and 4 and the skin was covered with a compress band and a non-irritating adhesive plaster (Photo 4b). The control sites were treated with distilled water and covered as indicated above.

After 24 hours of exposure, the covers were removed and the test sites rinsed with distilled water, then they were dried. Animals were examined for the presence of erythema and edema using the Draize Skin Irritation Score System (0, no erythema and no edema; 1, just few edema and d erythema noticeable; 2, well-defined erythema or slight edema; 3, moderate to severe erythema or moderate edema; 4, erythema or severe edema) at increasing intervals of 1, 24, 48 and 72 h (Draize, 1959).

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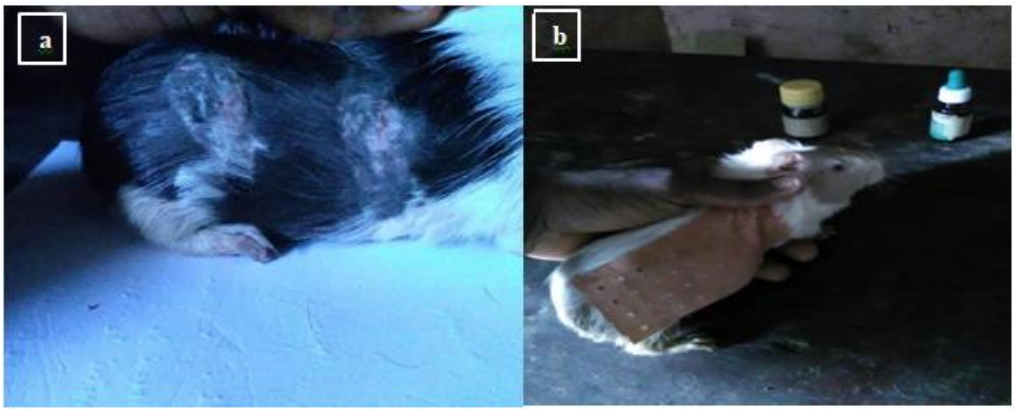

Photo 4: Skin irritation test.

2.9. Acute dermal toxicity study

Twenty female guinea pigs were randomly assigned to four groups of five animals. The test substance was applied uniformly over an area approximately equal to 10% of the total body area and the test essential oils were applied so as to form as thin and uniform a film as possible. The treated part was further suitably covered so as to hold the gas dressing and the test substance in place. The OECD method (2002) was used to assess acute dermal toxicity. A single dose per group of animals was administered as follows: Group 1: animals receiving a dose of 1 mL of essential oils (ET) of leaves and fresh fruits; Group 2: animals receiving a dose of 2 mL of essential oils (ET) of leaves and fresh fruits; Group 3: animals receiving a dose of 3 mL of essential oils (ET) of leaves and fresh fruits; Group 4: animals receiving a dose of 4 mL of essential oils (ET) of leaves and fresh fruits. The control sites were treated with olive oil at different volumes including 1mL, 2 mL, 3 mL and 4 mL. Animals were observed frequently on the first day and then careful clinical examinations were performed at least once per working day. Further observations were made daily, taking measures in such a way that the relatively small number of animals during the study could not be a handicap.

2.10. Composition and formulation of the Ointment

The ointment consists of:

essence of essential oil of fruits of *zanthoxylum zanthoxyloides* ……..0.0375%;

essential oil essence of *zanthoxylum zanthoxoloides* leaf …… 0.009375%;

pure glycerin, Lanolin, Sodium benzoate, White petroleum jelly and orange oil.

The ointments were triturated with a pestle, in a porcelain mortar, the amount of essential oil extract from the fruits and leaves of *Zanthoxylum* corresponding to 5 g and the amount sufficient for 95 g of the excipient. The excipient was added in small quantities until a homogeneous mixture was obtained. A spatula was used to loosen the ointment from the pestle and put the ointment in the glass jars (P. Picerno, Sanogo *et al*., 2010).

2.11.Cream compliance test

The variables or parameters examined with the naked eye included the macroscopic characteristics of the preparation, the homogeneity, the pH and finally the packaging.

2.11.1.Macroscopic characters

Macroscopic characterization of the ointments consisted of observing the color, consistency and odor of each ointment.

2.11.2. Homogeneity

The homogeneity of the ointments was checked by spreading them in a thin layer on a flat surface using a spatula. The regular distribution or not of the extracts in the excipients was noted.

2.11.3. Measurement of the pH of ointments

The pH was determined by measuring that of a tenth dilution of each ointment in hot distilled water.

III. RESULTS

1. Extraction efficiency of essential oils

The extraction yields as well as the color of the essential oils are presented in Table III.

It appears that the extraction yields of essential oils from the fresh fruits and leaves of *Zanthoxylum zanthoxyloides* are 1.12% and 0.04%, respectively.

Table III: Extraction yield and color of essential oils

Species	Organs	Extraction yield (%)	Color	physical aspect	Harvest date	Harvest site	Date of extraction
Zanthoxylum zanthoxyloides	Fruits	1.12	Pale yellow	Liquid	26/07/17	Bamena	05/12/17
	leaves	0.04	Light yellow	Liquid	26/07/17	Bamena	05/12/17

2. Chemical composition of essential oils

The analysis of the chemical composition made it possible to identify 59 compounds in the fruits and 58 compounds in the leaves with respectively as the majority essence in the fruit citronellol with a peak at 21.45 (18.63%), in the main essence leaves citronellol acetate with a peak at 26.61 at a percentage of 23.45%. The essences of *Zanthoxylum zanthoxyloides* consist almost exclusively of monoterpenes in a proportion of 53.45% and 63.47% respectively for the fruits and the leaves, while the proportions of sesquiterpenes hardly exceed 3.31% and 4.05% respectively. For the aforementioned plant parts. The hydrogenated monoterpenes represent a proportion of 28.87% and 53.45% respectively for the fruits and the leaves, while that of the oxygenated monoterpenes is exactly around 36.68% and 63.47%. Linear compounds for their part, although representing a rather mixed proportion of 0.23% in the leaves are totally absent in that of the fruits.

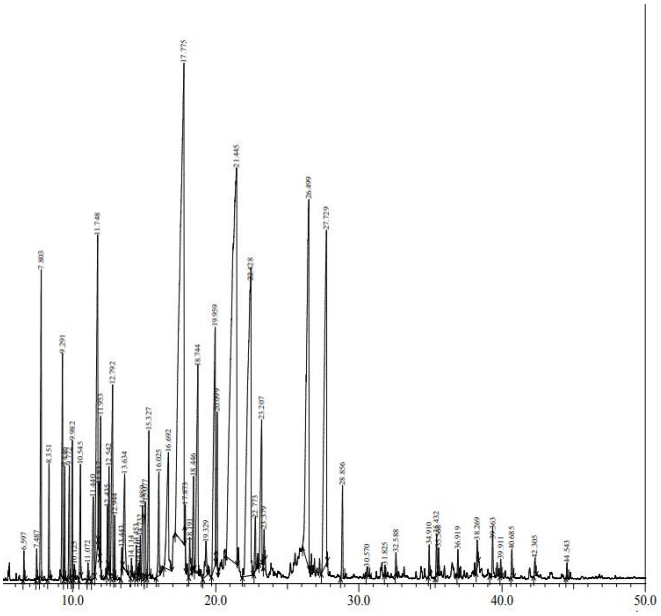

Figure 1: Chromatogram of essential oils of fruits of *Zanthoxylum zanthoxyloides*

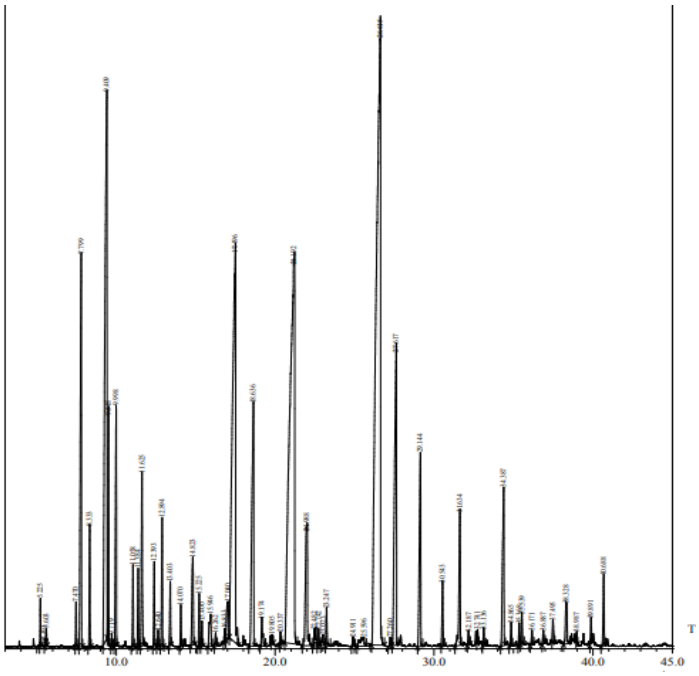

Figure 2: Chromatogram of essential oils of leaves of *Zanthoxylum zanthoxyloides*

3. In vitro activity of essential oils of fruits mixed with leaves, fruits and fresh leaves of Z. zanthoxyloides on Trichophyton rubrum

The evaluation of the antifungal activity gave the results below (photos 3).

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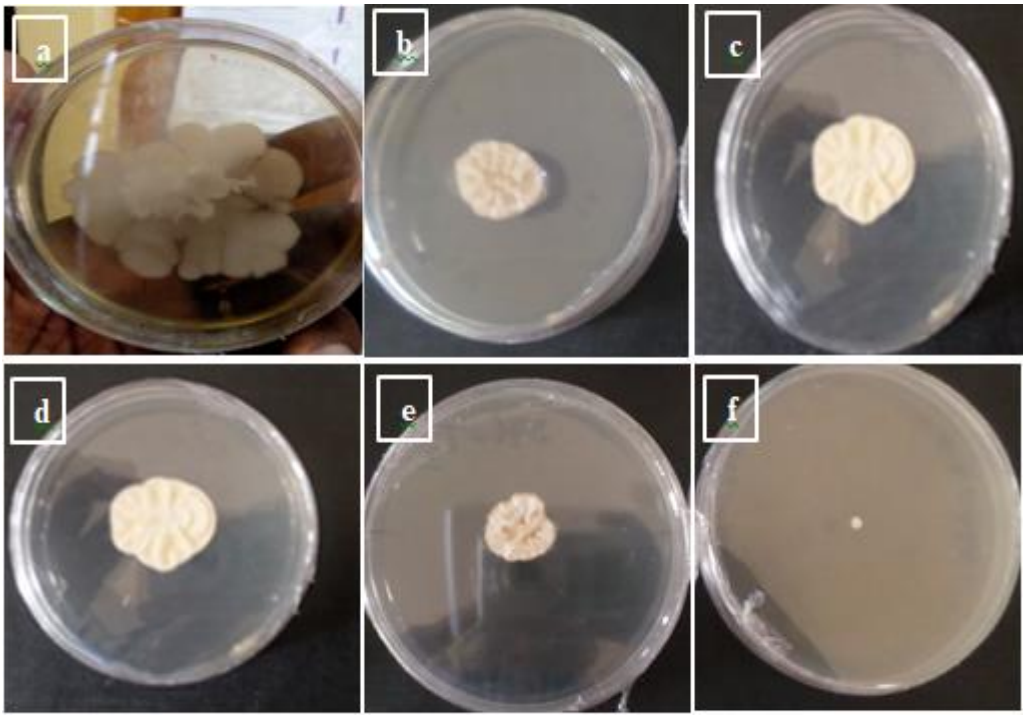

Photo 3: Minimum inhibitory concentration (MIC) of the essential oil of *Z. zanthoxyloides* on *Trichophyton rubrum*. a: controls b: 1000ppm leaves c: 1500ppm leaves d: 2000 ppm Fruits e: 3400 ppm fruits f: 4420 ppm MIC (leaves + fruits).

These results showed that at concentrations of 3600 ppm for the leaf essence, 4200 ppm for the fruit and 4420 ppm for the fruit and leaf mixture, no mycelial growth was observed for 7 days.

4. Assessment of dermal toxicity

Evaluation of the dermal toxicity of essential oils (Table IV) from the leaves and fresh fruits of *Zanthoxylum zanthoxyloides* and the combination of these according to the Draize protocol (1989) did not lead to any change in behavior, no appearance of erythema or edema on the skin of guinea pigs at these different doses compared to the control group. The results of the dermal irritation test according to the protocol of Draize (1989) recorded a score of zero, characteristic of the absence of erythema and edema.

Table IV: Results of dermal toxicity tests

Animals	Quantity HE	Dermal irritation score (DIS) as a function of exposure time (ET)				PII (Primary Irritation Index) = sum DIS/nb intervals (4)
		24h	48h	72h	96h	
G1	1 mL	0±0	0±0	0±0	0±0	0±0
G2	2 mL	0±0	0±0	0±0	0±0	0±0
G3	3 mL	0±0	0±0	0±0	0±0	0±0
G4	4 mL	0±0	0±0	0±0	0±0	0±0

5. Galenic formulation

5.1. Color, smell and consistency of the ointment

The ointment is light yellow in color (Photo 4) with a predominantly lemongrass odor. The ointment has a semi solid consistency. It appears moderately hard to the touch, but after taking it, it softens immediately on contact with the skin (T °> 30 ° C).

Photo 4: Color of the ointment

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5.2. Stability of the Ointment

Stored at laboratory temperature (room temperature, 28 ° C), the ointment is stable. But at a temperature above 30 ° C, it begins to melt. As for its evolution over time, the following observations were noted. Three batches of two jars were left to stand at laboratory temperature. They are then examined after 1 day, 1 week, 2 weeks, 1 month, 2 months and 3 months of conservation: ¬ permanently open batch: in the second week, a slight change in the surface of the ointment was observed. This corresponds to a slight oxidation of fatty acids. One of the pots in this lot must have been contaminated with mold; ¬ batch permanently closed until the end of the experiment: the ointment was kept intact; ¬ lot opened and scanned at each control: no change was observed.

5.3. Homogeneity

The preparation method made it possible to obtain very good homogeneity, able to guarantee an even distribution of the essential oil essence.

5.4. pH of the ointment

Measurement of the pH of the essential oil of the plant gave a value of between 4 and 5. The petroleum jelly used for the preparation has a pH of between 6 and 7.

5.6. Conditioning

Jars with a capacity of 30 mg were used. The labels used bear the following information for each jar:

- Name of the pharmaceutical form: ZANTHOZOLE ointment;
- Qualitative and quantitative composition:

Fruits essential oil essence of *Zanthoxylum zanthoxyloides* …… ..0.0375%

Leaves essential oil essence of Zanthoxylum zanthoxyloides ……. 0.009375%

Excipients: pure glycerin, lanolin, sodium benzoate, white petroleum jelly and orange oil

- Indication: local treatment of dermatophytes and superficial mycoses.
- Date of manufacture: February 2018.
- Expiration date: September 2020.
- Batch number for this study: it is noted "Lot n ° 0001".

Photo 6: Presentation of the primary packaging of ZANTHOZOLE ointment

Photo 7: Ointment presentation and instructions

IV. IV.DISCUSSION

1. Extraction performance of essential oils

It appears that the extraction yields of essential oils from the fresh fruits and leaves of *Zanthoxylum zantholoides* are 1.12% and 0.04%, respectively. These studies do not corroborate those obtained by Jazet in 2010 (7.22%) and Tchoumbougnang in 2005 (10, 50%) for fruits of the same species harvested respectively in Bana and Bagangté. These sensitive differences could find a possible explanation in the harvest period (years and seasons), the harvest site, the soil conditions, the extraction conditions and the nature of the vegetative material, given the fact that said material was relatively dry. instead of being perfectly fresh.

2. Chemical composition of essential oils

This work is different from that of Tchabon *et al* in 2018, on the other hand from that of Jazet in 2010 and finally that of Tchoumbougnang (2005). This difference could find an explanation at the level of the number of identified compounds which were only 27 in the work raised compared to that which emerges from the present work, namely 58 and 59. These results are similar to those obtained by Jirovertz *et al.* (1997) and Tasadjieu (2003) who have shown that the main constituents found in the EO of fruits and leaves were mainly composed of hydrogenated and oxygenated monoterpene including sesquiterpenes.

3. Evaluation of the effect of essential oils on germs

This work corroborates with the studies made by Jazet *et al* .; 2008 and Tatsadjieu (2003) who showed that species of the Zanthoxylum family have antifungal activities and inflammatory properties on fungal infections. This work is in line with that of Tchabon et al in 2018 who demonstrated antifungal activity on another species of this family, in particular Zanthoxylum leprieurii.

4. Galenic formulation

This work goes in the same direction as that of Sanogo rokia *et al* published in 2006 in Bamako which revealed that the ointments obtained with shea butter were of hard consistency and fairly satisfactory homogeneity. In addition, due to the presence of lanolin as an excipient, the ointments have a soft consistency and a satisfactory homogeneity. The ointments prepared with petroleum jelly have a semi-hard consistency and good homogeneity. The pH of the ointments obtained are practically identical (6 to 6.5) in the present study.

V. CONCLUSION

The extraction yield of the essential oils was 1.116% and 0.043% respectively for the fruits and the leaves. Likewise, the analysis of the chemical composition made it possible to identify 59 compounds in the fruits and 58 compounds for the leaves, of which the major compound for the fruits is Citronellol while for the leaves, it is acetate. of citronellol. The evaluation of the antidermatophytic activity of the essences of the fruits and leaves of Zanthoxylum zanthoxyloides showed that these essential oils are active vis-à-vis the dermatophyte studied, namely *Trichophyton Rubrum* with an MIC of 4420ppm for the mixture of the fruit essences. and leaves. These extracts were tested for dermal irritation and acute dermal toxicity using three month old female guinea pigs which showed no adverse effects in guinea pigs. A formulation trial of an antifungal ointment and compliance tests (pH, stability, homogeneity, organoleptic) led to the production of a product whose pharmaceutical form was called: ZANTHOZOLE ointment.

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