Archives of Current Research International

21(1): 12-23, 2021; Article no.ACRI.65721
ISSN: 2454-7077

Chemical Composition of Essential Oil of *Ageratum conyzoides* with Antifungal Activity on the *Lasiodiplodia theobromae* Strain in the Region of Kisangani and DR Congo

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Authors' contributions

This work was carried out in collaboration among all authors. Author JTKK wrote the first draft of the manuscript, designed the study and wrote the protocol. Authors OO, PTM, PV and GH managed the analyses of the study and directed the bibliographical research. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ACRI/2021/v21i130222
Editor(s):
(1) Dr. M. A. Elbagermi, Misurata University, Libya.
Reviewers:
(1) Guido Laercio Bragança Castagnino, Federal University of Bahia, Brazil.
(2) Farah Saeed, Dow University of Health Sciences, Pakistan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/65721

Original Research Article

Received 10 November 2020
Accepted 16 January 2021
Published 17 January 2021

ABSTRACT

Aims: To determine the chemical composition of essential oil of *Ageratum conyzoides* with antifungal activity on the strain of *Lasiodiplodia theobromae* in the Kisangani region.
Location and Duration of Studies: Faculty of Sciences of University of Kisangani (Democratic

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Republic of Congo) and Faculty of Biosciences Engineering of University of Ghent (Belgium), between May to November 2019.
Methodology: The essential oil from the leaves of *A. conyzoides* was extracted by hydrodistillation. Potato dextrose agar (PDA) was used as a culture medium. *In vitro* evaluation of its antifungal activity was performed on PDA medium and expressed as percentage of inhibition (PI).
Results: The extracted essential oil (with a yield of 0.63%) showed a PI of 91.63% on the strain of *L. theobromae* after two days of incubation. It consists of at least 23 compounds, of which seven are in the majority (abundances greater than 1.5% and represent 92.05%), namely Precocene I (38.33%), Beta-caryophyllene (26.51%), Beta-sesquiphellandrene (8.63%), Beta-cubebene (7.91%), Alpha-muurolene (4.95%), 1.5.9.9-Tetramethyl-1.4.7-cycloundecatriene (3.04%), Cis-beta-farnesene (2.99%). The hydrocarbon sesquiterpenes are the most frequent compounds (58.95%).
Discussion: Compared to the *A. conyzoides* saponins the PI obtained from the essential oil is higher. The essential oil chemotype obtained from the leaves of *A. conyzoides* is of the Precocene I type, similar to the oils from the stems and flowers of the same plant in West Africa.
Conclusion: The essential oil of *A. conyzoides* has a very high inhibitory power on *L. theobromae*. It mainly contains the Precocene and the hydrocarbon sesquiterpenes. Assessment of the antifungal activity of each separate molecule should be considered.

Keywords: Ageratum conyzoides; Lasiodiplodia theobromae; Essential oil; Antifungal activity; Chemotype.

1. INTRODUCTION

The population of world in general and of Africa in particular, rely on agriculture as a source of income [1,2]. Among the crops found in the tropics is *Theobroma cacao*, which tends to be predominant for its beans for making chocolate and extracting vegetable fats (butters) [3]. As of today, Africa alone holds nearly 70% of world production [4].

Around 1980, it was observed in Cameroon, the unusual rotting of cocoa orchards, affecting 100% of cocoa trees in some plantations [5]. In 1985, *Lasiodiplodia theobromae* was identified as a phytopathogenic fungus responsible for this brown rot [6,7]. Other studies had revealed the presence of this germ on cocoa in South America, Ecuador, India, Western Samoa, the Philippines [8-10] and recently in the Kisangani region in the Democratic Republic of the Congo, DRC [11-14]. This endophyte can cause tree death [15].

The use of synthetic fungicides to fight against phytopathogenic microorganisms generates (residual) pollution and the development of resistant strains. This is how the food industry and regulatory bodies have imposed restrictions on the use of certain synthetic food additives [16]. This currently arouses an attraction towards biofungicides, based on plants.

Since ancient times, plants, in this case aromatic plants, have been used as medicines [17]. Evidence of the antimicrobial activity of essential oil plants was first carried out by Delacroix in 1881 [18]. Essential oils, in addition to their use in cosmetics, are known for their healing, antiseptic, anti-inflammatory, antipyretic, antispasmodic, insecticide, bactericide, fungicide, anti-sickling, anti-tumor properties, etc. [19,20].

Preliminary ethnobotanical studies and surveys have been revealed that essential oils are also effective to preserve food products, in particular peanuts, meats, poultry, cold meats, vegetables, salads, yoghurts, fish and fruits [21-23]

Nowadays, essential oils are in high demand and are experiencing constant growth on the market because of their application in human consumption. Essential oils have the advantage of being natural, less expensive and effective, with less risk of developing a resistant strain of pathogenic microorganisms [24, 25].

Among the aromatic plants, is *Ageratum conyzoides*, an herb recognized for its multiple therapeutic effects (medico-magical, mental, ocular instillations, cutaneous, pneumonia etc) [26,27].

Several studies have been carried out to highlight its antimicrobial activities [28,29]. And recently in our series of studies on plants with

antifungal activity on the strain of *L. theobromae*, the non-volatile extracts of *A. conyzoides* were tested [12,14].

Several studies have proven the antifungal activity of many essential oils such as that of oils extracted from *Achillea*, *Achillea fragrantissima* [30], *A. terefolia* [31] and *A. milefolium* [32], against the pathogenic yeast *Candida albicans* and others [33]. However, to our knowledge, there are no data on the antifungal activity of essential oils on the strain of *L. theobromae*.

It is to overcome this deficiency that this work was undertaken with the aim to determine chemical composition the essential oil of *Ageratum conyzoides* with antifungal activity on the strain of *Lasiodiplodia theobromae*.

2. MATERIALS AND METHODS

2.1 Study Area

The Kisangani region was the study environment for this work. The city of Kisangani is located at 0º31' North latitude and 25º11' East longitude, 428 meters above sea level and is the capital of the Province of Tshopo in the DRC [34,35].

2.2 Plant Material

The leaves of *A. conyzoides*, growing in the Masako Forest Reserve (0°36'N; 25°13'E), were collected from May to June, dried for three weeks at room temperature (25 to 30°C), crushed and sifted. The powder was used for the identification of sterols and terpenes before the extraction of essential oil [36].

2.3 Essential Oil Extraction

The essential oil of *A. conyzoides* has been extracted by hydro-distillation [37]. In fact, 550g of powder was mixed with seven liters of water and distilled to obtain 2L of distillate. The essential oil was wheedle, dried (by five decigrams of magnesium shavings) and stored at 4°C.

2.4 Fungal Strain

The strain of *L. theobromae* was isolated from cocoa pods naturally affected by brown rot. The protocol for obtaining the strain was applied to Potato dextrose agar (PDA) medium [11,12].

2.5 Evaluation of Antifungal Activity

This evaluation was carried out in six repetitions according to the universal protocol for essential oils [23,38]. The mycelial growth of the *L. theobromae* strain was expressed by calculating percentage of inhibition (PI). In fact, 2.40 mL of the mixture consisting of essential oil and the 5% solution of tween 80 (proportion 1:1) were added 120mL of culture medium cooled to 45°C after sterilization. Thus 12 mL of this homogenized mixture were poured into each 90 mm diameter Petri dish. A 5 mm diameter mycelial explant was placed on the culture medium in each Petrie dish. Mycelial diameter was measured every 24 hours until the dish was filled. The control was produced under the same conditions, but without extract.

The calculation of PI was performed by the formula

$$\mathrm{PI} = \frac{(\text{Diameter of negative control} - \text{Diameter Extract})}{\text{Diameter of negatif control}} \times 100$$

2.6 Chemical Composition Determination

The essential oil molecules of *A. conyzoides* were identified by gas chromatography coupled with an electronic impact mass spectrometer (CG-MS-EI), brand Hewlett-Packard Plus + als, equipped with a selective mass detector n°5973 with electronic impact and an apolar capillary column model Phenomenex ZEBRON ZB-5MS (30 m X 0.25 mm ID), 0.25 µm thick of stationary phase.

Helium, carrier gas was used with a flow rate of 1 mL/min for one hour. The split mode was 1/20 for an injection volume of 1µL. The column was heated first at 50°C for 5min then raised from 5°C / min to 300°C (5min). The injection and detection temperatures were all 280°C. The molecules were ionized by an electronic impact with energy of 70eV. Detection has been optimized for m/Z molecules of 40-600.

To perform CG-MS-EI, a drop of essential oil was mixed with 10mL of hexane and the hexane was used as a blank for this analysis. The essential oil molecules were identified by comparing their mass spectra to those of the National Institute of

Standards and Technology, NIST database [38,39].

3. RESULTS AND DISCUSSION

3.1 Identification of Terpenes

Terpenes and Sterols are very abundant in the leaves of *A. conyzoides*. These secondary metabolites were also identified by N'guessan, [40], thus confirming our previous results [14]. However, these results are contrary to those of Bouquet and Debray [41]. The habitat and the geographical location would be the basis of this diversity of results.

3.2 Extraction Yield of Essential Oil

The leaves of *A. conyzoides* gave a yield of 0.63% W/W of essential oil after hydro-distillation. This yield is greater than 0.58% for the leaves of the same plant obtained by Wandji and Sood [42,43] and than 0.20% of *Ageratum houstonianum* found by Tedonkeng [19]. Differences in extraction yield can be caused due to the plant species, the stage of plant development, the period and geographical area of harvest, as proven in certain studies [44-49]. Moreover, the drying time and the method of extraction are among many factors that can also have a direct impact on the yields of essential oil [50-54].

Compared to stems (0.19%), flowers (0.22%) and roots (0.18%) [38,42,43], the leaves of *A. conyzoides* have a high essential oil content. Indeed, the leaves are part of the organs, sites of various vital reactions and metabolisms of the phytochemical groups characteristic of the plant.

In addition, *A. conyzoides* has an essential oil content slightly higher than that of *Croton hirtus* (0.60%) [38] and higher than that of *Seclerocarya birrea* (0.24%) [55]. However, *A. conyzoides* has a low yield compared to those from the leaves of *Ocimum basilicum* (0.65%) [56], *Tetraclinis articulata* (0.75%), *Hyptis suaveolens* (0.88%), *Psidium guajava* (0.78%) and *Eucalyptus camaldulensis* (1.38%) [48,49,55].

3.3 Organoleptic and Physicochemical Properties

Table 1 gives some organoleptic and physicochemical aspects of the essential oil of *A. conyzoides.*

The essential oil of *A. conyzoides* is characterized by a lime green color, strong and persistent odor as well as a bitter flavor. That of *Cardiospermum grandiflorum* also has a strong and persistent odor, but it is dark yellow in color [38] while that of *Cymbopogon gigateus* varies from pale yellow to yellow brown. Furthermore, in terms of density, the essential oil of *A. conyzoides* has a higher density than that of *Cymbopogon gigateus* (0.945) [57]. However, the miscibility of *A. conyzoides* oil with 95% ethanol makes it possible to pack it for multiple uses.

3.4 Percentage of Growth Inhibition of *L. theobromae*

The essential oil of *A. conyzoides* has a PI of 91.63% after two days of incubation on the strain of *L. theobromae*. Fig. 1 illustrates the antifungal effect of this essential oil.

The essential oil of the leaves of *A. conyzoides* has a slightly higher PI ($P= 4.187e^{-05}$) than that of its saponins (84.44%) [14]. It showed antifungal activity on the strain of *L. theobromae*, like the essential oils of *Ocimum gratissimum*, *Ocimum canum*, and *Syzygium aromaticum* which have a very pronounced activity on the strains of toxigenic molds isolated from peanuts [23,58].

Indeed, essential oils are known for their antiaflatoxinogenic properties (by their reactions with the mycotoxin), inhibition of the production of mycotoxin by the fungal species [23], their ability to damage the cellular enzymatic system and to provide profound changes in the cellular energy balance at the level of the mitochondria, thus disrupting the functioning of the nuclear membrane and endoplasmic reticulum. Some oils can also inhibit the synthesis of DNA, RNA, proteins and polysaccharides [59-61]. The chemical composition and the nature of the major constituents define the antimicrobial characteristics of essential oils [47,62].

3.5 Chemical Profile

Table 2 gives the chemical profile of the essential oil of the leaves of *A. conyzoides*, the mass to charge ratio (m/Z) as well as the Chemical Abstracts Service numbers (N°CAS) of each molecule.

It appears from this table that the essential oil of the leaves *A. conyzoides* contains 23 molecules of m/Z 137 to 297. Toure has identified 50

respectively in the flowers and stems of the same plant (for m/Z from 33 to 450) [38]. Indeed, the number of molecules identified depends on the organ of the plant, the method of analysis (equipment and protocol), etc.

3.6 Percentage of Molecules Identified

Fig. 2 gives the percentage of different essential oil molecules identified from the leaves of *A. conyzoides*.

It emerges from this figure that the Precocene I (17598-02-6) is the most abundant molecule with 38.22%, followed by Beta-caryophyllene (87-44-5), 26.51% while Bicyclo [3,1,0] hexane,1-methyl-6-(1-methylethylidene) and 1,5,5-Trimethyl-6-methylene-cyclohexene are the least abundant (respective contents of 0.06 and 0.10%). According to the order of magnitude of the concentrations [63], the oil of the leaves of *A. conyzoides* consists of seven major molecules (abundances greater than 1.5%) and sixteen minorities (abundances between 0.05 and 1.5%).

The majority molecules represent 92.36% and their retention times ranging from 23.862 to 27.304 minutes. The antifungal activity of the essential oil of *A. conyzoides* on the strain of *L. theobromae* is due to Precocene I and Beta-caryophyllene. Indeed, the activity of molecules depends on the oxygen composition, the lipophilic nature of their hydrocarbon skeleton and the hydrophilic nature of their functional groups as well as the chemical structure [64,65].

Precocene I is the only chromene present in the essential oils of the flowers (58.78%) and stems (76.46%) of *A. conyzoids* from the Center-West of Cote d'Ivoire [38], but also in the leaves according to our results (for that of the Kisangani region/DRC) although at a relatively low percentage (38.22%). As in other studies, the essential oils of *A. conyzoides* in Vietnam and the Fiji Islands, contain Ageratochromene (Precocene II) and/or derivatives. Those of Pakistan or India contain Precocene I and II and those of West Africa are characterized by a very high content of Precocene I: 87% in the Republic of Congo, 86% in Burkina Faso, 80.29% in Ghana, 82.2% in Nigeria, 85.6% in Benin, 80% in Ivory Coast and 81% in Cameroon [66].

A. houstonianum oil contains 73.38% of Precocene I followed by Beta-cayophyllen (19.41%) and finally Precocene II 1.20% [19]. Thus, the chemotype of the oil of *A. houstonianum* is of the Precocene I and II type, while that of the leaves of *A. conyzoides* (in the Kisangani region of central Africa) is of the Precocene I type, like that of the stems and flowers of *A. conyzoides* from West Africa [38].

In addition, Beta-caryophyllene is abundant in leaves (26.51%) according to our results, but less abundant in flowers (15.20%) and stems (8.60%) according to Toure [38]. Aalbersger found 19.50% Beta-caryophyllene in the oils from the flowers of *A. conyzoides* from Nigeria [61]. The same molecule has an abundance of 8.13% in the essential oil of the leaves of *A. conyzoides* in Burkina Faso, 6.00% in that of Ivory Coast and 20.00% in the Hymalayan region [67]. Germacrene D is almost absent in the stems, present in the flowers [38] and also in the leaves (0.18%). The essential oils of the flowers of *A. conyzoids* originating in Nigeria contain 3.90% of Germacrene D [68].

Beta-cubebene is less abundant in the stems (0.22%) and flowers (1.06%), but abundant in the leaves (7.91%) according to our results. Beta-farnesene is less abundant in the flowers (1.58%) and stems (0.54%), but abundant in the leaves (2.99%). Alpha-muurolene trace in the flowers (0.08%), but 4.95% in the leaves. Gamma cadinene has an abundance of 0.14 and 0.07% respectively in the flowers and stems, but Beta cadinene 1.13% in the leaves. Beta-Sesquiphellandrene 8.32% in leaves in the present work but 1.82 and 0.88% respectively in flowers and stems [38].

Compared to the oil from the leaves of *A. conyzoides*, that of *S. birrea* contains more Alpha-muurolene, (25.03%), less Beta-caryophyllene (3.22%), more Alpha-copaene (1.24%) while that of *P. guajava* contains less Beta-caryophyllene (8.13%) [55].

Table 1. Some organoleptic and physicochemical aspects of essential oil of *A. conyzoides*

Organoleptic qualities		Physico-chemical qualities	
Color	Lime-green	pH	5.50
Odor	Characteristic, strong and persistent	Density	0.978
Flavor	Bitter	Miscibility with ethanol 95%	Positive

Table 2. Lists of molecules identified in the essential oil of *A. conyzoides*

N°	Retention time/min	Compounds	m/Z	N°CAS
1	10.152	Bicyclo [3.1.0] hexane, 1-methyl-6- (1-methylethylidene)	136	24524-57-0
2	11.927	1.5.5-Trimethyl-6-methylene-cyclohexene	136	514-95-4
3	21.018	Bornyl acetate	196	125-12-2
4	22.749	Alpha-cubebene	204	17699-14-8
5	23.537	Alpha-copaene	204	3856-25-5
6	23.754	Beta-bourbonene	204	5208-59-3
7	23.862	Beta-cubebene	204	13744-15-5
8	24.765	Beta-caryophyllene	204	87-44-5
9	24.963	Germacrene D	204	23986-74-5
10	25.013	Alpha-bergamotene	204	17699-05-7
11	25.522	Cis-beta-farnesene	204	28973-97-9
12	25.637	1.5.9.9-Tetramethyl-1.4.7-cycloundecatriene	204	1000062-61-9
13	25.828	Precocene I	190	17598-02-6
14	26.559	Cadina-4(14),5-diene	204	54324-03-7
15	26.674	Alpha-muurolene	204	31983-22-9
16	26.782	Alpha-farnesene	204	502-61-4
17	26.89	Beta-bisabolene	204	495-61-4
18	27.157	Beta-cadinene	204	523-47-7
19	27.304	Beta-sesquiphellandrene	204	20307-83-9
20	28.748	Caryophyllene oxide	220	1139-30-6
21	30.784	7-tert-Butyl-3.3-dimethyl-1-indanone	216	56298-78-3
22	36.694	Palmitic acid	256	57-10-3
23	39.544	Phytol	297	150-86-7

Caption: m/Z: Mass to charge ratio CAS N °: Applied Chemistry Standards Number

3.7 Classes of Identified Molecules

The classification of the various constituents of essential oil of *A. conyzoides* as well as their contents, crude formulas and frequencies are given in Table 3.

This table indicates that the hydrocarbon sesquiterpenes are in the majority (58.95%) with 15 types of constituents followed by a type of Precocene I chromene (38.33%). On the other hand, cycloalkyl acetate and monoterpenes are the least abundant, i.e. 0.19% (1 type of constituent) and 0.16% (2 types of constituents) respectively.

This distribution of molecules is the reverse of those of the oils of the flowers and stems of *A. conyzoides* [38], the major classes that consist of Precocene I chromene (58.78 and 76.46% respectively) followed by the hydrocarbon sesquiterpenes (30.66 and 14.06%). This difference is due to the climate, the vegetative cycle and the sampling region of the plant studied [54,69].

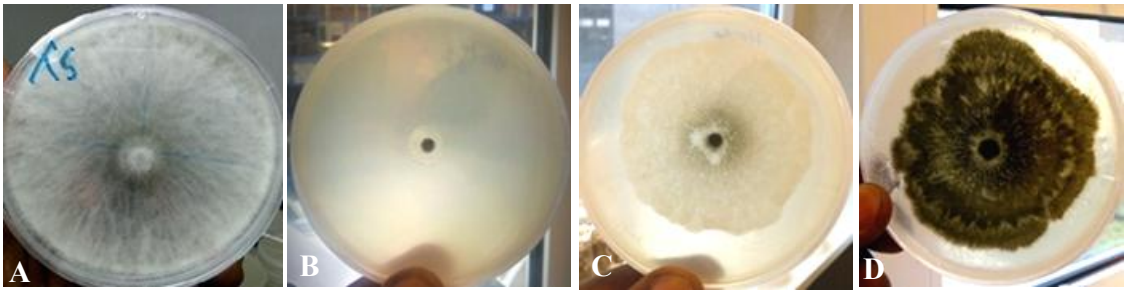

Fig. 1. *In vitro* evaluation of the antifungal activity
A: Control strain of *L. theobromae* after 2 days. B, C and D: inhibition of strain of *L. theobromae* by the essential oil of *A. conyzoides* leaves, observed respectively after 2, 6 and 9 days of incubation

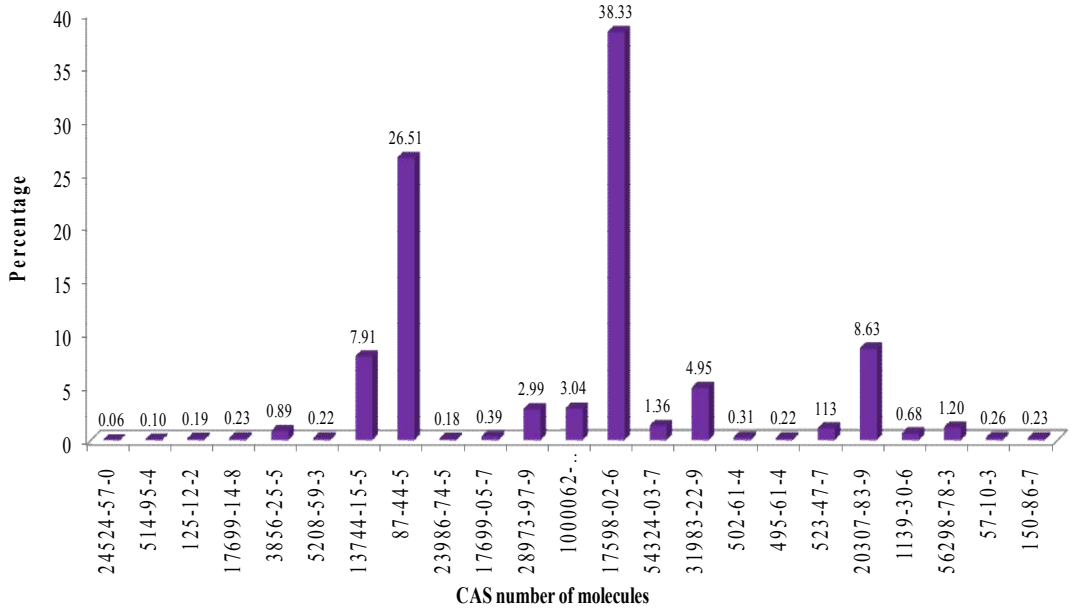

Fig. 2. Percentage of identified essential oil molecules from the leaves of *A. conyzoides*

Table 3. Classification of the different essential oil molecules of *A. conyzoides*

N°	Class	Percentage	Formula	Frequency
1	Carboxylic acid	0.26	$C_{16}H_{32}O_2$	1
2	Alkene-ol	0.23	$C_{20}H_{40}O$	1
3	Benzocyclo-one	1.20	$C_{15}H_{20}O$	1
4	Chromene	38.33	$C_{12}H_{14}O_2$	1
5	Cycloalkyl acetate	0.19	$C_{12}H_{20}O_2$	1
6	Monoterpenes	0.16	$C_{10}H_{16}$	2
7	Hydrocarbon sesquiterpenes	58.95	$C_{15}H_{24}$	15
8	Oxygenated sesquiterpenes	0.68	$C_{15}H_{24}O$	1

The essential oils of *S. birrea* and *P. guajava* also contain sesquiterpenes as the major constituent [55]. The oil from the leaves of *A. conyzoides* contains fifteen different types of the hydrocarbon sesquiterpenes, while that of *Tetraclinis articulata* from the Frenda region of Algeria contains eleven [48].

4. CONCLUSION

The objective of this study was to determine chemical composition the essential oil of *Ageratum conyzoides* with antifungal activity on the strain of *Lasiodiplodia theobromae* in the Kisangani region. It appears that sterols and terpenes are very abundant in the leaves of *A. conyzoides*. The essential oil, which has an extraction yield of 0.63%, has a percentage inhibition of 91.63% on the strain of *L. theobromae* after two days of incubation. This oil consists of at least 23 different compounds, the main ones include Precocene I (38.33%), Beta-caryophyllene (26.51%), Beta-sesquiphellandrene (8.63%), Beta-cubebene (7.91%), Alpha-muurolene (4.95%), 1.5.9.9-Tetramethyl-1.4.7-cycloundecatriene (3.04%), Cis-beta-farnesene (2.99%) , Cadina-4 (14), 5-diene (1.36%). Hydrocarbon sesquiterpenes represent 58.95% of all identified molecules, consisting of fifteen different types of compounds. The evaluation of the antifungal activity of these molecules separately on the strain of *L. theobromae* should be undertaken.

ACKNOWLEDGEMENT

- VLAAMSE INTERUNIVERSITY COOPERATION PROGRAM RAAD (VLIR-UOS): for financial support
- DIEDERIK LEENKNECHT of the University of Ghent: for his technical assistance
- NKFUTELA EWALA Michel for his moral and scientific contributions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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