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RESEARCH ARTICLE

CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OILS OF FOUR VARIETIES OF *LIPPIA MULTIFLORA* IN BENIN

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ABSTRACT
Present study involves the study of the chemical composition of the essential oils extracted from the leaves by gas chromatography and gas chromatography coupled with mass spectrometry of *Lippia multiflora* harvested in the regions of Kétou, Savalou, Bohicon and Mono and tested by the well diffusion method against pathogenic microorganisms. The essential oils studied are terpene compounds, aromatic compounds, aliphatic compounds and other natural substances. The inhibition zone diameters determined allowed us to evaluate their degree of germ sensitivity of the strains tested. Essential oils extracted from *Lippia multiflora* harvested in these areas have the most pronounced antimicrobial activity. In total, the essential oils tested have different and specifically a degree of sensitivity against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Proteus mirabilis* A24974, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* except that harvested in the Savalou who does not have no degree of sensitivity on *Pseudomonas aeruginosa*. This work paves the way for food preservation with extracted natural substances and also the formulation of natural antimicrobials for this fact.
Keywords: Antibacterials, Benin, chemical composition, *Lippia multiflora*, volatile extracts.

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INTRODUCTION

For thousands of years, plants have been occupied a prominent place in the life of man. All known civilizations have used either wild or cultivated plants to provide for their basic needs: food, shelter, clothing and also for their medical needs. The use of medicinal plants has been evolved gradually with the changing needs of humans. Over the centuries, a first distinction has been made between edible and toxic plants. Subsequently, accumulated empirical knowledge has been enabled humans to take plants as the essential source of drugs. The study of this knowledge by modern science gradually reveals some secrets of nature that allow man to continue his evolution. Until the beginning of the 20th century, almost all medicines were herbal.

Even today, modern medicine is highly dependent on plants whose therapeutic virtues have been confirmed. Whatever parts and shapes plants use, they are extremely rich in complex chemical structures. The metabolism of plants contains thousands of different constituents, which belong to very different chemical families, such as alkaloids, phenols, flavonoids, terpenoids, steroids.

These secondary metabolites, extraordinarily diversified, are widely exploited in various fields: in the culinary field as dyes and flavors, in the medicinal field as antibiotics, antioxidants, drugs, etc., and in the agricultural field as pesticides.

These are secondary metabolites of aromatic plants containing in high proportion very active molecules with antibacterial, insecticidal, fungicidal, acaricidal and cytotoxic properties[10]. Food-borne diseases are nowadays public health problems[23] notwithstanding all the modern innovations in the direction of the improvement and the performance of the techniques and the hygiene of the production of the food. It has been estimated that more than 30% of the populations in industrialized countries suffer from these diseases every year, and more particularly in the year 2000, two million deaths from diarrheal diseases worldwide[9].

Microbial activity was the primary mode of food deterioration and many microorganisms involved in these food-borne conditions most often contribute to the loss of quality and safety. In fact, essential oils (HE) are the most exploited thanks to their broad spectrum of known biological activities. Many volatile compounds of essential oils are nowadays common ingredients of pharmaceutical preparations such as pule gone, menthol and thymol[18]; α-pinene, a highly demanded ingredient in the international market, was used in the manufacture of vitamin E[12].

Numerous studies have made it possible, on the one hand, to systematically study essential oils extracted from aromatic plants commonly used in traditional pharmacopoeia in Benin [5] and[4].[3], and on the other hand to high light the effectiveness of certain essential oils in the context of integrated protection against certain insect pests of cereal stocks[16].

However, prior to current work, and no scientific study in Benin has focused on testing the efficacy of volatile extracts of aromatic plants against pathogenic microorganisms and alterations identified in foods. Faced with the resistance of microorganisms to synthetic antibiotics, current study undertook to propose a cheaper and more credible alternative solution with aromatic plants used in traditional medicine. Choice of current study, following an ethnobotanical survey, focused on *Lippia multiflora*[11]. The present study therefore aims to analyze the chemical compositions of the volatile extracts of the retained aromatic plants and to test their effectiveness against certain pathogenic and alteration microorganisms identified in staple foods in Benin.

It was a question, specifically to determine the antibacterial activities of the extracts from the diameters of zones of inhibition around the wells vis-à-vis microorganisms tested.

The search for these compounds was launched with the aim of discovering new effective therapies against diseases that have not been treated, and to reduce the use of synthetic products that are harmful to humans and their environment. Like antibiotics, antioxidants and pesticides.

Despite attempts to list plants and their extracts, the number of species studied remains relatively low given the growing need for natural compounds of aromatic and / or medicinal interest.

MATERIALS AND METHODS

Plant material

It consists of plant material (*Lippia multiflora)*, microbial strains (bacteria, yeast and mold), biological and chemical diagnostic equipment. Survey in current study was carried out in 2015 in the main towns of the four departments of Benin which are chosen because of their demography and their varied geographical areas. Present study carried out the survey in the areas devoted exclusively to the marketing of medicinal plants in the markets of these towns.

The concerned regions are: Plateau, Mono, Zou and Collines. Precisely, the survey was carried out in the towns of Kétou for the Plateau regions, Houéyogbé for Mono Bohicon and Djidja for Zou and Savalou for Collines. A specimen of the plant is deposited and identified by the national herbarium of the University of Abomey-Calavi (Benin)[7]. The plant materials used in present study were made of leaves or leaf stems of plant selected based on their frequency use against opportunistic infections and arterial hypertension. After harvesting the plants, their fresh samples were dried for ten to fourteen days in the dark in a constant-temperature cabinet (conditioned air).

Bacterial strains

The bacterial strains used consisted of 04 clinical strains (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and 03 reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and Proteus mirabilis A 24974).

Biological and chemical diagnostic equipment:

The equipment used in the laboratory consists of conventional glassware sterile single-use plates of 90mn diameter, a micropipette P100 Gilson brand and culture media. The culture media and reagents used in the microbiological diagnosis come from BIORAD, MERCK, OXOID, BASINGSTORE, BIOMERIEUX and DifCO laboratories. A 24-hour pure colony portion from the Mueller Hinton medium of each strain was emulsified in 5ml of physiological saline to obtain a turbidity of 0.5 on the Mc Farland scale. The chemical diagnostic equipment is a GC, a spectrometer, Clevenger for extraction of essential oils.

Methods

The survey was carried out using the method of [21]. This study was performed with 20 herbal medicine doctors chosen from the agenda of 'Programme National de la Promotion de la Médécine Traditionnelle'of Benin Ministry of Health. It was carried out in the selected areas in presence of people who master the mother tongues of these regions and the plants. The tools of the survey were composed of a questionnaire and interviews. During the survey, questions are asked are related to:

- Used plants to treat the hypertension high blood pressure
- Vernacular names of the used plants.
- Parts of the used plants
- How to make the medicines, and dosage?
- Administration and the remedies

Questions were asked to traditional herbal medicine doctors and the over-sixties people with traditional knowledge. A selection from the most used species and the helped pharmaco-chemistry point of view after consulting the different data banks, to retain only one species belonging to only one botanical family. This species was harvested from July to August 2015 in their natural habitat in Benin. The botanical determinations are carried out at 'Herbier National'of University of *Abomey Calavi* (Benin). This harvested species presents either a similar use in the other traditional pharmacopoeias or other popular uses.

Extraction of oils and calculation of yields

About 200 g of plant material from each plant were each subjected to hydrodistillation with a Clevenger type apparatus for 3 hours used in previous study[3,14,22]. The essential oil thus obtained in an essencier was collected in a small dark bottle containing anhydrous sodium sulphate (Na_2SO_4) and stored at 4°C until use. The yield R was expressed relative to the fresh plant material and its calculation was as follows:

$$R = \frac{M\ (HE)}{M\ (MV)} X100$$

Where, R: yield; M (HE): mass of essential oil obtained; M (MV): Mass of extracted plant material.

Chemical composition of essential oils Analysis by gas chromatography

The oils were analyzed using a VARIAN CP.3380 gas chromatograph equipped with two capillary columns (apolar and polar) and connected to a Varian integrator (Model C-R4A°); [22].

Analysis by gas chromatography and mass spectrometry

Technical characteristics of the equipment

All essential oils were analyzed by GPC / MS, which results in the separation of the constituents of the mixture on a column mounted in series with a mass spectrometer. Each component leaving the column passes directly into the ionization field of the spectrometer. The molecule subjected to the electronic impact is fragmented. The mass spectra obtained for each compound are compared with data from the literature as well as those from the laboratory data base. CPG/MS couplings were performed on a Hewlett Packard-Quadruple type device (Model 5970). The chromatograph was equipped with a fused silica capillary column 30 m long and 0.25 mm inside diameter with a dimethylpolysiloxane (DB-1) type grafted phase 0.25 μm thick.

Evaluation of the antibacterial activity of essential oils Isolation and purification of microbial strains:

The clinical strains were isolated from stool samples at the National Public Health Laboratory of Benin.

Sensitivity test by well diffusion

Each inoculum was seeded by swabbing onto petri dishes containing Mueller Hinton agar. Using the sterile pasteur pipette tip 6 mm diameter wells were dug. Then using a cone and a micropipette 50µl of each extract were deposited in the wells previously dug. A well containing sterile distilled water to serve as a negative control. The Petri dishes were left for 1 hour at room temperature for pre-diffusion of the substances, before being incubated at 37°C in an oven for 18 h. After the incubation period, the dishes were examined for measurements of the inhibition zones. The antibacterial activity of the extracts was determined from the diameters of inhibition zones around the wells as shown (Figure 1).

RESULTS AND DISCUSSION

Yields of the plants studied

The essential oil yields of plants of the family Verbenaceae, the yields of essential oil extracted from the leaves of *Lippia multiflora* (Kétou, Savalou, Mono and Bohicon) are relatively high and their yields are 0.58±0.02%, 1.07±0.03%, 1.70±0.04 and 2.18±0.04%, respectively.

Chemical composition of essential oils

Present work involves the study of the chemical compositions of essential oils according to its large botanical family of different regions[14].

Verbenaceae: This family includes *Lippia multiflora* (from Kétou, Savalou, Bohicon and Mono).

As shown in Table 3, the oils obtained do not have the same composition. There is a relatively large difference in composition in oils recovered from *Lippia multiflora* from Savalou, Djidja, Kétou and Mono. Indeed, the major components of *Lippia multiflora* essential oil of Savalou are: 1,8-cineol 50,6%, α-terpinol 13,8%, aromadendrene 6,2%, germacrene - D 2,2%, linalool 0.3%, Isoledene 0.2%, Pinocarvone 0.2%, (E) - 0.1% nerolidol, 0.1% 6-ol camphene, α-cubebene 0.1%, terpinene-4-ol 0.3%, sabinene 14.4%, α-pinene 5.7%, myrcene 2.9%, β-pinene 1.9%, γ-terpinene 0 , 3%, α-tujene 0.3%, δ-cadinene 0.2%). Those of Djidja are: linalool 91%, germacrene D 2.9%, α-caryophyllene 2.1%, allo-aromadendrene 1.9%, (E) -olidolidol 0.5%, δ-cadinene 0.4%, trans-β-ocimene 0.3%, α-copaene 0.2%, 1,8-cineol 0.2%, α-muurolene 0.1%, Romadendrene 0.1%, caryophyllene oxide 0.1%, 0.1% sabinene, 0.1% myrcene. Those of Mono are: caryophyllene oxide 10.25%, Methanethiol 08.12%, carvacrol 13%, β-Myrcene 1.98%, α-pinene 4.5%, Beta Pinene 08%, p-cymene 15%, terpinen-4-ol 12.5%, α-terpinol 13%) and those of Kétou are: gamma-Terpinene 47%, beta-Caryophyllene 28%, 1,8-cineol 13.50%, Germacrene-D 4.84 %, α-pinene 4.65%, β-Myrcene 1.92%, α-Pinene 4.64%. These results explain many fluctuations in the values of the physicochemical properties of the various essential oils obtained. Oxygen compounds represent respectively (99.8% for the plant of Djidia, 93.34% for the Kétou plant, 76.8% for the Savalou plant and 31.37% for the Mono plant) of the chemical composition essential oil of *Lippia multiflora*. The level of these compounds is relatively important in the essential oil Djidja, Kétou and Savalou, as well as in the other Mono with a respective difference ≈ 68.43, 61.97 and 45.43%. Linalool was found in the oil of *Lippia multiflora* of Djidja with a significant percentage (91%) than in *Lippia multiflora* of Savalou (0.3%), the difference is 90.7%. So study can concludes that the region of Djidja favors this compound in the set of essential oil.

Same thing for the germacrene-D, (E) - nerolidol and δ-cadinene respective percentage (2.9%, 0.5% and 0.4%) found in the essential oil Djidja and Respective

Aure (2 , 2%, 0.1% and 0.2%) found in the essential oil of Savalou but with a very small difference of the order of (0.7%, 0.4% and 0.2%).Terpinen-4-ol was found in *Lippia multiflora* oil of Mono with a significant percentage (12.50%) than in *Lippia multiflora* from Savalou (0.3%), the difference was 12.2%. Myrcene is found in *Lippia multiflora* Kétou oil with a percentage (4.84%) than in *Lippia multiflora* Savalou (2.2%) whose difference was (2.64%). 1,8-Cineol was found in *Lippia multiflora* oil of Kétou with a percentage (13.50%) that in *Lippia multiflora* of Djidja (0,2%) whose difference was (13,3%). Caryophyllene oxide (10.25%) was found in the oil of *Lippia multiflora* of Mono and aure (0.1%) found in the essential oil of Djidja but with a difference of (10.15 %. Gamma-Terpinene was found in Ketu *Lippia multiflora* oil with a significant percentage (47%) as compared to Mono *Lippia multiflora* (13.65%), the difference being 33.35%. So it can we reported that the Kétou region favors this compound in the essential oil complex. Similar results were found with α-pinene. On the other hand note that some compounds are present in the essential oil of *Lippia multiflora* Savalou, with a much higher percentage than the essential oil of Djidja, Mono and Kétou with a more or less different rates. In this case, study can conclude the Savalou region of Djidja, Mono and Kétou has been a risk of having compounds well present but with very low percentages. For β-Myrcene (1.92% -1.98%) and α-pinene (4.50%-4.65%), it was found that they remained almost stable, did not undergo large changes. The variation detected in the chemical composition of the essential oil from the region of Savalou, Djidja and Aure Mono and Kétou, were related to several parameters such as: the environmental factor, the nature of the soil, climatic and geographical conditions that change from one region to another, and at the time of harvest [19]. As for the total number of compounds found in *Lippia multiflora* Kétou was five compounds less than the other Savalou, Djidja and Mono which contain respectively nineteen, fourteen and ten compounds. The essential oil of *Lippia multiflora* is generally composed of molecules that the pharmaceutical industry often uses. Indeed, the presence of α-pinene compounds, the most common molecule-marking terpene in nature, was an effective molecule for the treatment of respiratory conditions such as colds, coughs or bronchitis. Moreover, thanks to the similarity of its smell with that of fir, it was commonly used in deodorants and cleaning products. On the other hand, the presence of the compound (Z)-β-ocimene was known for its properties and its stabilities notably preventing its oxidation. This composition was different from that previously reported by[1,7,13].

CONCLUSION

The chemical composition of the essential oil extracted from the leaves of *Lippia multiflora* harvested in the regions of Kétou, Djidja, Savalou and Mono was studied by CPG and CPG coupled with mass spectrometry. Djidja oil is mainly composed of linalo, germacrene D and α-caryophyllene, the Savalou oil was made up of 1,8-cineol, sabinene, α-terpinol, the one of Mono composed of gamma-terpinene, -cymene, Terpinene, Caryophyllene oxide, Carvacrol, Methanethiol, Alpha-terpinene, Beta Pinene and Ketu Oils consist of sesquiterpenes including gamma-Terpinene, Cineole, beta-Caryophyllene, caryophyllene oxide. The different essential oils investigated are composed of molecules with terpene structures that act as isolation or synergistic effect of the majority and minority compounds they contain on the different bacteria. The different sensitivities determined on each of them, essential oils as well as their compositions (Table 3) act differentially on the isolated microbial strains and in the present study, it appears that the Gram + are more sensitive to their effect than the Gram. Through these works, the essential oil of *Lippia multiflora* offers hope. Indeed, this oil, commonly called "Gambia tea" is an aromatic plant very little known in Benin and used in the treatment of several pathologies in traditional medicine. Bibliographic research has also shown that this plant has several therapeutic virtues. The results of present work confirm these effects and show that this plant has excellent antimicrobial properties.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

All the authors' participle in writing, giving feedback on this manuscript, have read and approved the final manuscript.

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Table 1: Synoptic review of methods for detection, isolation and identification of microbial strains tested.

Microbial strains	Origin	Detection Method	Isolation	Identification
Escherichia coli	Tomato sauce	ISO 7251. NPP (Sept.1994)	Confirmation Kovacs Reagent Indole Test	API 20E
Klebsiella pneumoniae	Cow milk	ISO 4832 NPP (Jul.1991)	Isolation on EMB	API 20E
Staphylococcus aureus	Cooked corn dough	SO 6888. Baird-Parker, Nutritive Agar (Jun.2003)	Coagulase test	API Staph

Table 2: Yields of essential oils of the studied plants

Lippia multiflora	Part analyzed	Yield
Bohicon *(*Djidja)	Leaf	2.18±0.04
Savalou (Chetti)	Leaf	1.07±0.03
Kétou *(Ikpinlè)*	Leaf	0.58±0.02
Mono *(Houéyogbé)*	Leaf	1.70±0.04

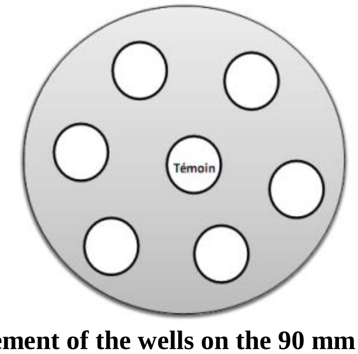

Figure 1: Arrangement of the wells on the 90 mm diameter petri dish

Table 3: Chemical compounds of the oil extracted from the leaves of *Lippia multiflora* harvested in the area of Savalou, Bohicon, Mono and Kétou.

Chemical composition	Savalou (Tchetti)	Bohicon (Djidja)	Mono (Houéyogbé)	Kétou (Ikpinlè)
camphèn-6-ol	0.1			
α-cubebène	0.1			
(E)- nerolidol	0.1	0.5		
terpinolène	0.2			
Linalool	0.3	91		
terpinen-4-ol	0.3		12,50	
β-pinène	1.9			
γ-terpinène	0.3			
α-thujène	0.3			
Pinocarvone	0.2			
δ-cadinene	0.2	0.4		
Isoledene	0.2			
germacrene – D	2.2	2.9		4.84
myrcène	2.9	0.1		
α-pinène	5.7		4.50	4.65
Aromadendrène	6.2			
α-terpinol	13.8		13	
sabinène	14.4	0.1		
1,8-cinéol	50.6	0.2		13.50
Romadendrène		0.1		
oxyde de caryophyllène		0.1	10.25	
α-muurolène		0.1		
α-copaène		0.2		
trans-β-ocimène		0.3		
allo-aromadendrène		1.9		
α-caryophyllène		2.1		
β-Myrcene			1.98	1,92
beta-Caryophyllene				28
gamma-Terpinène			13.65	47
Methanethiol			08.12	
Beta Pinene			0 8	
carvacrol			13	
p-cymène			15	

Table 4: Standard used for reading the results of the antibiogram tests of plant extracts[17].

Inhibition zone diameter	Degree of sensitivity of the germ	Symbol
Δ<7mm	Insensible	-
7mm ≤Δ<8mm	Sensible	+
8mm ≤ Δ<9mm	Assez sensible	++
Δ ≥9mm	Très sensible	+++

Table 5: Inhibition zone diameters (mm) of the different bacterial strains

	Inhibition zone diameter (mm)			
Bacterial strains	**LD**	**LS**	**LK**	**LM**
Staphylococcus aureus ATCC 25923	40	34	32	30
Escherichia coli ATCC 25922	30	29	30	27
Proteus mirabilis A24974	23	23	27	26
Staphylococcus aureus	34	32	00	33
Escherichia coli	30	27	30	60
Klebsiella pneumoniae	29	30	29	32
Pseudomonas aeruginosa	09	07	10	14