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Journal of Medicinal Plants Research

Full Length Research Paper

Chemical composition and antimicrobial activity of essential oils from *Aframomum citratum*, *Aframomum daniellii*, *Piper capense* and *Monodora myristica*

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This study was initiated to evaluate the chemical composition and *in vitro* antimicrobial activity of essential oils from four Cameroonian spices and to determine the therapeutic effect of a cream based on essential oil from *Aframomum citratum*. Essential oils were extracted from seeds by hydrodistillation and analyzed by gas chromatography coupled with mass spectrometry (GC/MS). The broth microdilution method was used for the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) determinations. The therapeutic effect of a cosmetic cream based on essential oil from *A. citratum* seed (1.25, 2.5 and 5% w/w) was evaluated against dermatosis induced with a Methicillin-Resistant *Staphylococcus aureus* (MRSA) in rats. The main identified compounds in the essential oils are geraniol for *A. citratum*; eucalyptol, α-terpineol and geraniol for *Aframomum daniellii*; β-pinene, germacrene D, trans-β-caryophyllene, α-pinene, naphthalene and sabinene for *Piper capense*; α-phellandrene, germacradienol and δ-cadinene for *Monodora myristica.* Essential oil of *A. citratum* (MIC = 8-4096 µg/ml) was the most active against bacteria and fungi, following in decreasing order by those of *A. daniellii, P. capense* and *M. myristica.* The antibacterial activity of the essential oil of *A. citratum* against MRSA and *Escherichia coli* S2(1) (MIC = 8 µg/ml) was higher than that of amoxicillin used as reference drug (MIC = 128- 256 µg/ml). The combination of essential oils of *A. citratum* and *A. daniellii* (1:1) displayed a synergistic effect. The cream based on essential oil of *A. citratum* (5%) and Baneocin (reference drug) eradicated the dermatosis induced with MRSA in rat after two weeks of treatment. These results indicate that the tested essential oils possess antimicrobial activities which could be a function of either the individual or the additive effects of the identified phytoconstituents.

Key words: Spices, hydrodistillation, essential oils, gas chromatography/mass spectrometry (GC/MS), antibacterial, antifungal, synergistic effect.

INTRODUCTION

The skin can be infected by different types of microorganisms, most often by Gram-positive bacteria such as *Staphylococcus* species. Bacterial skin infections are widespread all over the world and many are caused by *Staphylococcus aureus*. The treatment of *S. aureus* infection, particularly Methicillin-Resistant *S. aureus*

(MRSA), is a challenge in clinical practice (Malachowa et al., 2013; Song et al., 2017).

In developing countries, the main difficulties that accompany their treatments with conventional medicines are the high cost and toxic effects of the common antibiotics, as well as the development of multi-resistant pathogenic microorganisms to the treatments (Yang et al., 2016). Complementary and alternative medicines (CAMs) are used by 60-80% of developing countries and are also the most widespread sources of medicines in the world (Lee et al., 2012). Indeed, among all the CAMs, essential oils represent the most popular choices for the treatment of fungal skin infections (Millikan, 2002) and one of their main applications is their use in dermatology (Reichling et al., 2009).

Essential oils are primarily used as natural preservatives, flavorings and fragrances in cosmetic products (Fernandes et al., 2013). They are potential sources of antioxidants and natural antimicrobials, in addition to their multiple properties such as antiparasitic, analgesic and cytotoxic properties (Mith et al., 2014).

Among the potential sources of essential oils, spices have long been investigated because they contain volatile bioactive compounds that can be of interest in therapy and nutrition. Indeed, essential oils from medicinal spices and vegetables are important sources of antimicrobial agents in addition to their ability to stimulate the digestive system (Rahman et al., 2011). Medicinal spices and vegetables have traditionally been used as food additives, coloring, flavoring and preservative agents as well as antiparasitic, antihelmintic, analgesic, expectorant, sedative, antiseptic and antidiabetic substances in many parts of the world (Rahman et al., 2011; Dzoyem et al., 2014).

Aframomum daniellii (Hook.f.) K. Schum belonging to Zingiberaceae family is a large, robust, perennial plant that is about 3-4 m tall and is usually found under shades in plantations near riverine areas. Its seeds are used for flavouring traditional dishes as well as food additives, laxative, anti-helmintic, antibacterial and antifungal agents. The rhizome juice of this plant is effective in the treatment of body odor and toothache (Pamela et al., 2016). The leaves and seeds are used in the treatment of internal and external piles (Focho et al., 2009). This plant is also used in traditional food preparations for its flavoring, coloring and preservative properties (Tajkarimi et al., 2010), as well as to cure malaria, dysentery, dysmenorrhea, infertility, rubella, leprosy and cancers (Titanji et al., 2008).

Aframomum citratum (Zingiberaceae) K. Schum is a perennial herbaceous producing leafy stems up to 3 meters tall from a rhizomatous rootstock (Burkil, 2000). Young shoots are eaten as a vegetable while the seeds are eaten as a spice in Cameroon. The plant is traditionally used as an aphrodisiac and also to treat bacterial infections, malaria and cancers (Titanji et al., 2008; Kuete et al., 2011).

Piper capense Lin. f (Piperaceae), known as long black pepper, is an endemic plant of East Africa found in wet highlands where it is produced traditionally for human consumption and medical uses (Kokwaro, 1976; Van Wyk and Gericke, 2000).

M. myristica (Gaertn) Dunal is a perennial edible plant of the Annonaceae family. It is mostly found in the humid tropical forests of West and Central Africa and commonly known as African nutmeg and calabash nutmeg. The seeds of this plant are used to cure constipation, uterine hemorrhage, diuretic and fever (Dzoyem et al., 2014). Some biological activities such as cytotoxic, antiprotozoal, antibacterial, anti-inflammatory and antioxidant activities have been documented for *A. citratum, A. daniellii, P. capense* and *M. myristica* (Kuete et al., 2011; Dzoyem et al., 2014).

However, from literature search, no scientific investigations have been conducted till date to verify the *in vivo* antimicrobial activities of the above plant species and there is a paucity of data on their essential oil composition. This work was therefore carried out to evaluate the chemical composition and *in vitro* antimicrobial activity of essential oils from the seeds of *A. citratum*, *A. daniellii*, *P. capense* and *M. myristica* as well as to determine the therapeutic effect of a cream based on essential oil from *A. citratum*.

MATERIALS AND METHODS

Plant materials

Dried seeds of *A. citratum, A. daniellii, P. capense* and *M. myristica* were purchased in February 2016 at market “B” in Bafoussam, situated in the Western region of Cameroon. The plant species were identified using their seeds by Mr. Fulbert TADJOUTEU, a Botanist of the National Herbarium of Cameroon by comparison with specimens whose voucher numbers were 37736/HNC, 43130/HNC, 6018/SRF/NHC and 2949/SRF/NHC for *A. citratum, A. daniellii, P. capense* and *M. myristica*, respectively.

Extraction of essential oils

The essential oils were obtained by hydrodistillation using a Clevenger-type apparatus. 500 ml of distilled water was separately added to seeds of *A. citratum* (100 g), *A. daniellii* (100 g), *P. capense* (200 g) and *M. myristica* (200 g). A magnetic stirrer was introduced in the apparatus and the mixture was heated on a hot plate. Hydrodistillation was carried out for 6 h with *A. citratum* and *A. daniellii* and for 1 h with *P. capense* and *M. myristica*. The essential oils were dried over a column of anhydrous sodium sulphate (Sigma-Aldrich, St. Louis, MO, USA) and then stored in

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amber tubes at 4°C until analyses.

Determination of the chemical composition of essential oils

The essential oils were analyzed by gas chromatography coupled with mass spectrometry (GC/MS), using an Agilent apparatus (6890 N series), fitted with a HP-5MS fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm) and coated with 5% phenyl 95% dimethylpolysilosane. The initial temperature was set at 50°C and the oven was heated up to 110°C at a rate of 3°C/min, then from 110 to 300°C at a rate of 10°C/min. The carrier gas was pure helium at a flow rate of 1.2 ml/min. The injector temperature was 250°C, applying the split ratio of 1:5. Mass spectra were obtained using electron ionization source at 70 eV. Ion source temperature was maintained at 230°C and the mass range was m/z 40-400 u. A scan interval of 0.5 s and fragments from 40 to 550 Da were maintained. The essential oil was solubilized in pentane at concentration of 2 mg/ml and 1 µl was injected on the chromatographic system. The relative quantity of the compounds present in the essential oils was expressed as a percentage based on the peak area produced in the chromatogram. From the obtained chromatograms, retention indices (RI) of components were determined relatively to the retention times of a series of *n*-alkanes (C_8-C_{40}) with linear interpolation. Compounds were identified by comparing their retention indices and their mass spectra with those of Wiley Library data 2009.

Microorganisms

The used microorganisms in this study included fungal and bacterial species involved in skin infections and wound contaminations. These microorganisms included reference strains from American Type Culture Collection and clinical isolates. Fungal strains were made of two dermatophytes: *Microsporum gypseum* E1420 and *Trichophyton violaceum* obtained from *Ecole Nationale Vétérinaire d'Alfort* in France; seven yeast strains: *Candida albicans* ATCC 1663, *C. albicans* ATCC 9002, *C. albicans* IS1*, Candida parapsilosis* ATCC 22019*, Candida tropicalis* ATCC 750*, Candida krusei* ATCC6258, *Cryptococcus neoformans* IP90526; four amphotericine B and nystatin sensitive *Candida spp* isolates: *Candida krusei, Candida parapsilosis*, *Candida lipolithica* and *Candida haemophilus*; twenty amphotericine B and nystatin resistant isolates of *C. albicans*: *Ca* Da11, *Ca* E01, *Ca* F021, *Ca* F045, *Ca* K42, *Ca* D10, *Ca* F066, *Ca* F005, *Ca* F017, *Ca* F057, *Ca* K14, *Ca* F015, *Ca* F023, *Ca* F040, *Ca* F002, *Ca* F041, *Ca* K072, *Ca* F049, *Ca* F026, *Ca* K22 and eight nystatin resistant isolates of *C. neoformans*: CN, CN169, CN173, CN047, CN091, CN046, CN 096, CN158, obtained from Pasteur Institute (IP, Paris-France). Bacteria were constituted of eight Gram-positive species: *S. aureus* ATCC 25923, methicillin resistant *S. aureus* MRSA03, methicillin resistant *S. aureus* MRSA04, methicillin sensitive *S. aureus* MSSA01, *Staphylococcus aureus* ST120, *Enterococcus aerogenes* ATCC 13048, *Enterococcus aerogenes* and *Enterococcus adecarboxylate* and eight Gram-negative bacteria: *Escherichia coli* ATCC 10536, *Escherichia* coli, entero-aggregative *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* PA01, *Bacillus subtilis, Klebsiella pneumoniae* ATCC2513883, *Shigella flexneri* obtained from our laboratory collection. The bacteria and yeasts were maintained at +4°C on agar slants.

Experimental animals

In this study, 42 *Wistar albino* rats (21 males and 21 females; 10-12 weeks old; 150-200 g) were used. They were bred in the animal house of the Department of Biochemistry, University of Dschang, Cameroon. The animals were fed with a standard diet. Food and water were given *ad libitum* to all animals used for the experiments. Animals were maintained at room temperature (22 ± 2°C). The study was conducted according to the ethical guidelines of Committee for Control and Supervision of Experiments on Animals (Registration no. 173/CPCSEA, dated 28 January, 2000), Government of India, on the use of animals for scientific research.

Determination of the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC)

MIC was determined by broth micro dilution method as previously described (Tamokou et al., 2009; Fogue et al., 2012) with slight modifications. The microbial inocula were prepared from 24 h, 48 h and 7 days old broth cultures from bacteria, yeasts and dermatophytes, respectively. The absorbance was read at 600 nm (for bacteria), 530 nm (for yeasts) and 450 nm (for dermatophytes) using a spectrophotometer (Jenway™ 6305 UV/Visible Spectrophotometer, Fisher scientific, UK).

From the prepared microbial solutions, other dilutions with sterile physiological solution were prepared to give a final concentration of 2 $\times 10^5$ colony-forming units (CFU)/ml for bacteria and 2×10^5 spores/ml for yeasts and dermatophytes. Stock solutions of essential oils were prepared in 5% tween 80 at concentrations of 16.38 (for essential oils) and 2.04 mg/ml (for pure reference drugs). The antimicrobial susceptibility test was performed in a 96-well microplate. The twofold serial dilutions of test samples were made in Mueller Hinton Broth (MHB) (Conda, Madrid, Spain) for bacteria and Sabouraud Dextrose Broth (SDB) (Conda, Madrid, Spain) for yeasts and dermatophytes.

The final concentrations ranged from 4.096 to 0.032 mg/ml for the essential oil and from 128 to 0.50 µg/ml for the reference drugs. For every experiment, a sterility check (5% tween 80 and medium), negative control (5% tween 80, medium and inoculum) and positive control (5% tween 80, medium, inoculum and reference drug) were included. The plates were covered with the sterile sealer and incubated at 35°C for 24 h (for bacteria) and 48 h (for yeasts). Dermatophytes were incubated at 27°C for 5 days. Bacterial growth was monitored colorimetrically using iodotetrazolium chloride (INT). Viable bacteria change the yellow dye of p-iodonitrotetrazolium violet to a pink color. Yeasts and dermatophytes growth in each well was determined by observing and comparing the test wells with the positive and negative controls. The absence of microbial growth was interpreted as the antibacterial or antifungal activities. The MIC was the lowest concentration of the essential oil that prevented change in color or visible growth of micro-organisms.

Minimum Bactericidal Concentrations (MBCs) and Minimum Fungicidal Concentrations (MFCs) were determined by adding 50 µl aliquots of the well (without INT), which did not show any microbial growth after incubation during MIC assays, into 150 µl of essential oil-free Mueller Hinton Agar (for bacteria) and Sabouraud Dextrose Agar (for yeasts and dermatophytes). MBCs or MFCs were defined as the lowest concentration yielding negative growth. All the experiments were performed in triplicate. Amoxicillin, nystatin and griseofulvin were used as positive controls for bacteria, yeasts and dermatophytes, respectively.

***In vivo* antibacterial assay**

The *in vivo* antibacterial activity was determined with the essential oil of *A. citratum* seed which displayed the most *in vitro* antimicrobial activity.

Formulation of cosmetic cream

Cosmetic cream was made using the modified formula of Banker

and Rhodes (1995). Two mixtures were prepared separately: mixture A made up of water (97.4%), glycerol (1.7%) and hydroxyl-propyl-methyl-cellulose (HPMC) (0.9%) and mixture B made up of wax (15.1%), kernel oil (82.9%) and shea butter (2.0%). The two mixtures were mixed at 70°C in a water bath for 4 min and then cooled on ice bath for 2 min, followed by addition of sodium benzoate (1%) as cream preservative. Finally, 1.25, 2.5 and 5 g of essential oil of *A. citratum* were added to 100 g of cream to yield the final concentrations of oil in the cream of 1.25, 2.5 and 5% respectively. Control cream was made up of cream without any antibacterial and without essential oil.

Bacterial infection induced with *S. aureus* MRSA03

Prior to infection, rats were starved for 12 h and anesthetized using ketamine (100 mg kg^{-1} body weight) under sterile conditions. The dorsal fur of the animals was shaved with an electric clipper and the site of the infection (3 cm diameter) was outlined on the back of the animals using a marker pen, then disinfected with ethanol 95° and abraded with sandpaper (N°120) 1 min before inoculation. Then, rats were inoculated at the site of infection with 10^8 CFU/ml of Methicillin-Resistant *S. aureus* (MRSA) suspension prepared from an overnight culture (Kugelberg et al., 2005). One group was not infected and not treated (uninfected group). Infected rats were divided into six groups of three animals each (three control groups and three test groups). The first control group was not treated (untreated group), the second and third control groups received cream without essential oil (blank group) and Baneocin® (2%) 250 UI/5000 UI (Baneocin group), respectively. The three other groups were treated with cream-based essential oil from seed of *A. citratum* at 5, 2.5 and 1.25% (w/w), respectively. Treatment started 24 h after the establishment of the infection by dermal application of 0.1 g of cream-based on essential oil and Baneocin® once per day for 14 consecutive days.

Evaluation of *in vivo* antibacterial activity

The efficacy of the treatment was evaluated on a clinical and mycological basis. Clinical efficacy was based on changes observed at the site of infection during the test. These observations were based on measurements taken at the site of infection from two perpendicular lines drawn on the site and measuring on the one hand, the evolution of the inflammation (inflammation percentage) and on the other hand, the evolution of epithelialization (epithelialization percentage). These parameters were noted every three days.

$$\text{Inflammation } \% = \frac{\text{Horizontal diameter} + \text{Vertical diameter}}{2} \quad (1)$$

$$Epithelialization\ \% = \frac{\text{Horizontal diameter} + \text{Vertical diameter}}{2} \quad (2)$$

The epithelialization time, that is, the number of days required for the scar to fall off without residual gross injury, was determined as the epithelialization period (Ameri et al., 2013). For the mycological efficacy, animals were anesthetized with chloroform vapors at the end of the treatment and a skin sample (4 g) was taken, ground in a porcelain mortar in the presence of 4 ml of physiological saline (NaCl) 0.09%) and the ground product obtained was centrifuged at 3000 rpm for 15 min. The supernatant obtained after centrifugation was decanted and used for culture on Mannitol Salt Agar medium in order to count the number of Colony Forming Units (CFU) of bacteria per gram of skin. Body weights of animals were measured before sacrifice. The organs (liver, kidneys, lungs, heart and spleen) were carefully dissected out, blotted, observed macroscopically and weighed immediately using a Sartorius electronic balance. The relative organ weight (ROW) of each animal was then calculated as follows:

$$\text{ROW} = \frac{\text{Absolute organ weight (g)x } 100}{\text{Body weight of rat on day of sacrifice (g)}} \quad (3)$$

Statistical analysis

Data were subjected to the one-way analysis of variance (ANOVA) and recorded as mean ± standard deviation (SD) and where differences exist, means were compared using Waller Duncan test at 0.05 significant levels. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 12.0) software.

Ethics

The experiments were carried out observing the welfare of animals as recommended by World Health Organization (WHO). Moreover, all procedures involving animals were carried out in strict compliance with the rules and regulations of local Ethics Committee.

RESULTS

Yield of extraction and chemical composition of essential oils

The essential oils of *A. citratum*, *A. daniellii* and *M. myristica* were translucent with extraction yields of 1.0 ± 0.1%, 1.1 ± 0.1% and 2.4 ± 0.5%, respectively whereas that of *P. capense* seed was green with the extraction yield of 1.6 ± 0.3%. A total of 6 compounds were identified in the essential oil of *A. citratum* seeds (Table 1). These compounds were mainly composed of monoterpenes with a marked predominance of oxygenated monoterpenes (geraniol, 96.80%) and the absence of sesquiterpenes. Eighteen compounds were identified in the essential oils of *A. daniellii* belonging mainly to oxygenated monoterpenes (Table 1). The essential oil of *A. daniellii* seed was predominantly constituted of eucalyptol (48.8 ± 9.9%), α-terpineol (21.7 ± 2.6%) and geraniol (10.5 ± 1.2%). A total of 25 compounds were identified in the essential oil of *P. capense*. The essential oil of this plant species was slightly more monoterpenic (56.2 ± 4.4%) than sesquiterpenic (41.4 ± 4.8%) and was mostly composed of hydrocarbonated compounds (90.7 ± 5.9%) than oxygenated compounds (6.8 ± 5.8%). The major compounds found in the essential oils of *P. capense* are β-pinene (37.3 ± 1.7%), germacrene D (9.8 ± 4.1%), trans-β-caryophyllene (8.8 ± 3.8%), α-pinene (8.6 ±

Table 1. Qualitative and quantitative compositions of essential oils from the studied spices.

Compound	RI	*A. citratum*	*A. daniellii*	*P. capense*	*M. myristica*
Monoterpenes		**99.3 ± 0.1**	**93.3 ± 2.4**	**56.3 ± 4.3**	**79.3 ± 2.8**
Monoterpene hydrocarbons		**0.3 ± 0.2**	**3.2 ± 1.1**	**52.3 ± 4.4**	**74.6 ± 3.0**
Ethylether	689	-	-	-	0.9
α-Thujene	921	-	-	-	1.1 ± 0.2
α-Pinene	927	-	0.5	8.1 ± 0.9	3.1 ± 0.4
Sabinene	968	-	-	4.7 ± 1.3	-
β-Pinene	971	-	2.2 ± 0.3	36.5 ± 1.9	-
Myrcene	990	-	-	-	1.0 ± 0.2
β-Myrcene	995	0.2±0.0	-	0.7 ± 0.1	2.0 ± 0.2
α-Phellandrene	1002	-	-	-	61.5 ± 5.1
δ-3-Carene	1006	-	-	1.4 ± 0.1	-
p-Cymene	1020	-	-	-	1 ± 0.1
Limonene	1024	-	1.3 ± 0.2	1.5 ± 0.5	5 ± 0.6
1,8-Cineole	1027	0.3	-	-	-
Oxygen-containing monoterpenes		**99.0 ± 0.1**	**90.1 ± 3.4**	**4 ± 1.5**	**4.7** ± 1.0
Eucalyptol	1026	-	**48.8 ± 9.9**	-	-
Linalool	1100	1.3 ± 0.2	2.6 ± 0.7	1.3 ± 0.4	2.8 ± 0.2
Linalyl propionate	1164	-	0.7	-	-
4-Terpineol	1174	-	2.9 ± 1.9	1.5 ± 1.2	-
α-Terpineol	1188	-	**21.7 ± 2.6**	-	1 ± 0.1
Sabinol	1200	-	**-**	-	1.1 ± 0.3
Geraniol	1257	**96.8 ± 0.3**	**10.5 ± 1.2**	**-**	**-**
Geranial	1274	0.3 ± 0.05	0.4	-	-
Bornyl acetate	1288	-	-	1.8 ± 0.3	-
Geranyl acetate	1388	0.5 ± 0.1	3.0 ± 1.0	-	-
Sesquiterpenes		**-**	**3.8 ± 2.1**	**41.2 ± .2**	**18.9** ± 3.3
Sesquiterpene hydrocarbons		**-**	**2.6 ± 1.1**	**34.3 ± 4.2**	**8.3** ± 2.6
α-Cubebene	1357	-	-	0.5 ± 0.05	-
α-Copaene	1380	-	-	0.6 ± 0.1	-
β-Cubebene	1393	-	-	2 ± 0.6	-
Trans-β-caryophyllene	1424	-	1.6 ± 0.5	6.5 ± 1	-
Santalen	1425	-	-	-	1.3 ± 0.3
β-Selimene	1460	-	-	1.1 ± 0.1	-
α-Amorphene	1482	-	-	0.9 ± 0.0	-
Germacrene D	1488	-	-	7.4 ± 1.7	-
α-Amorphene	1500	-	-	2 ± 0.7	0.5
α-Muurolene	1506	-	-	-	1.0 ± 0.3
γ –Cadinene	1522	-	1.5 ± 0.2	-	1.9 ± 0.4
Naphthalene	1524	-	-	10.7 ± 0.4	-
δ-Cadinene	1532	-	0.4	1.5 ± 0.5	4.2 ± 1.0
Germacrene B	1570	-	0.7	2.4 ± 0.05	-
Oxygen-containing sesquiterpenes		**-**	**1.7 ± 0.4**	**6.9 ± 4.1**	**10.6 ± 0.8**
Octabicyclooctanol	1350	-	0.4	-	-
Germacradienol	1588	-	-	-	7.9 ± 0.6
Caryophyllene oxide	1598	-	1.2 ± 0.2	1.2 ± 0.2	-
Guaicol	1609	-	-	2.4 ± 0.8	-
t-Muurolol	1659	-	0.7	-	1.4 ± 0.1
α-Amorphene	1661	-	-	1.5	-
t-Muurolol	1672	-	-	0.9 ± 0.4	1.3 ± 0.2
Azulene methanol	1684	-	-	1.2 ± 0.6	-

RI: Retention indice.

0.9%), naphthalene (8.3 ± 3.5%) and sabinene (4.8 ± 1.1%). Twenty compounds were identified in the essential oil of *M. myristica* belonging to monoterpenes (84.8±6.3%) especially monoterpene hydrocarbons (81.8±8.2%). The main compounds are α-phellandrene (61.5±5.1%), germacradienol (7.9 ± 0.6%) and δ-cadinene (4.2 ± 1.1%).

Antimicrobial activity of essential oils

In this study, the antibacterial and antifungal activities of essential oils from the studied spices were evaluated using broth micro dilution method against pathogenic microorganisms including yeasts, dermatophytes, Gram-positive and Gram-negative bacteria. The results summarized in Tables 2 to 4 showed that essential oils were active against the tested microorganisms with MIC values varying from 8 to 4096 µg/ml. Essential oil of *A. citratum* was active against all the tested bacteria (100%), while essential oils of *A. daniellii, P. capense* and *M. myristica* were active against only thirteen of the seventeen tested bacteria (76.5%) (Table 2). Interestingly, the antibacterial activity of *A. citratum* was significant particularly against *S. aureus* MSSA01, *B. subtilis* and *E. coli* S2 (MIC = 8 µg/ml and MBC = 32 µg/ml). *E. coli* EC136 and *Klebsiella spp* (clinical isolates) were the most resistant bacteria to the essential oils. The bactericidal effect of essential oils was observed with *A. citratum*, *A. daniellii*, *P. capense* and *M. myristica* essential oils on 11/17 (64.70%), 8/17 (47.05%), 7/17 (41.17%) and 6/17 (35.29%) of tested bacteria, respectively.

The results also showed that essential oils were active against the tested fungi with MIC values ranging between 32 and 4096 µg/ml (Table 3). Essential oils of *A. citratum*, *P. capense*, *A. daniellii* and *M. myristica* were active against all the fungal species (100%), while essential oil of *A. citratum* was the most active. *C. albicans* ATCC 9002 was the most sensitive yeast (MIC = 256 - 512 µg/ml), whereas *C. lipolithica* (MIC = 512 - 4096 µg/ml) was the most resistant yeast. The essential oils from the studied spices displayed different degrees of antifungal activity against *C. albicans* and *Cryptococcus neoformans* resistant isolates with MIC values ranging between 128 and 4096 µg/ml (Table 4).

The essential oils of *A. citratum*: 23/28 (82.14%) were the most active followed in a decreasing order by those of *A. daniellii*: 13/28 (46.42%), *P. capense*: 6/28 (21.42%) and *M. myristica*: 6/28 (21.42%). The combination of essential oils of *A. citratum* and *A. daniellii* (1:1) was active against 100% of the tested isolates, while the combination of essential oils of *P. capense* and *M. myritica* (1:1) was only active against 3/28 (10.71%) of the tested isolates (Table 4). Moreover, the antifungal activity of the combination of essential oils of *A. citratum* and *A. daniellii* (1:1) was greater than that of these essential oils used alone. However, the combination of essential oils of *P. capense* and *M. myritica* (1:1) reduced their antifungal activities compared to those of these essential oils used alone. In general, the MFC and MBC values are fourfold lesser than the MIC values on the corresponding microorganism; suggesting that the tested essential oils have fungicidal / bactericidal effects.

***In vivo* antibacterial activity**

The therapeutic effect of a cream based on essential of *A. citratum*, which was found to be the most active essential oil, was evaluated against dermatosis induced with a Methicillin-Resistant *S. aureus* (MRSA) in rats. Animals infected with *S. aureus* showed visible inflammation 24 h post infection, characterized by the redness and swelling of the skin at the sites of inoculation. The percentage of inflammation reduces progressively during the treatment untill it reaches at 0% after 15 days of treatment in male and female rats (Figure 1).

In male rats, the epithelialization times were 9 days with 1.25 and 5% of cream and uninfected group and 12 days for the other groups (Figure 2). In female rats, the epithelialization times were 6 days for the groups treated with 1.25 and 5% of cream; 9 days for the untreated group and 12 days for the other groups (Figure 2). These results indicate that rats treated with 1.25 and 5% of cream and Baneocin exhibited shorter epithelialization times than controls (untreated, uninfected and blank groups) and those treated with 2.5% of cream.

The results also show that treatment significantly ($p < 0.05$) reduced the number of Colony Forming Units (CFU) of bacteria at the infection site (Figure 3). In male rats, the number of CFU of bacteria at the infection site comparable with those of uninfected group were noted with 5, 2.5 and 1.25% of cream based on essential oil from *A. citratum* and Baneocin after 14 days of treatment (Figure 3). In female rats, the number of CFU of bacteria at the infection site in the groups treated with 5% of cream based on essential of *A. citratum* and Baneocin were lower than those obtained in the uninfected group. These results suggest that 5% of cream based on essential of *A. citratum* and Baneocin may be used to treat methicillin resistant *S. aureus* dermatosis induced in rats. Cream without essential oil (blank treatment) has a number of CFU of bacteria at the infection site comparable to that of untreated group. Organ-to-body weight ratio, an index often used in toxicological evaluations, was not significantly altered by the treatments (Table 5).

DISCUSSION

Differences in the extraction yields were noted between

Table 2. Antibacterial activities (MIC and MBC in µg/ml) of essential oils from the tested spices.

Bacteria	*A. citratum*			*A. daniellii*			*P. capense*			*M. myristica*			AMOX	
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC
S. aureus ATCC25923	4096	>4096	-	8	>4096	-	32	>4096	-	4096	>4096	-	1	2
S. aureus ATCC25923	1024	>4096	-	>4096	-	-	2048	>4096	-	512	>4096	-	0.5	2
S. aureus	32	32	1	128	512	4	2048	4096	2	2048	>4096	-	2	2
S. aureus MSSA01	8	32	4	32	128	4	256	256	1	512	4096	8	128	128
S. aureus MRSA03	64	512	8	512	512	1	1024	2048	2	1024	2048	2	32	32
S. aureus MRSA04	32	256	8	32	64	2	512	2048	4	512	>4096	-	32	32
S. aureus ST120	2048	>4096	-	2048	>4096	-	>4096	>4096	-	>4096	-	-	0.50	1
B. subtilis	8	32	4	512	4096	8	8	64	4	1024	4096	4	4	4
E. coli ATCC10536	1024	>4096	-	1024	>4096	-	1024	>4096	-	512	>4096	-	0.5	1
E. coli EC136	2048	>4096	-	>4096	-	-	>4096	-	-	>4096	-	-	4	4
E. coli S2(1)	8	32	4	8	64	8	2048	>4096	-	8	128	16	256	256
Entero-aggregative E. coli	256	512	2	1024	>4096	-	512	>4096	-	>4096	-	-	16	16
E. adecarboxylate	1024	2048	2	>4096	-	-	>4096	-	-	1024	>4096	-	32	32
E. aerogenes	512	2048	4	512	>4096	-	512	>4096	-	2048	2048	1	2	4
P. aeruginosa ATCC27853	32	256	8	32	256	8	32	512	16	32	1024	32	4	4
Klebsiella spp	512	>4096	-	>4096	-	-	>4096	-	-	>4096	-	-	32	32
Shigella flexneri	256	1024	4	512	2048	4	1024	1024	1	512	>4096	-	16	16

MIC: Minimum inhibitory concentrations; MBC: Minimum bactericidal concentrations; R = MBC/MIC; AMOX: amoxicilline.

Table 3. Antifungal activities (MIC and MFC) of essential oils from the tested spices.

Yeast	*A. citratum*			*A. daniellii*			*P. capense*			*M. myristica*			Reference*		
	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R
C. albicans ATCC 9002	256	256	1	512	512	1	512	512	1	512	512	1	0.50	1	2
C. albicans ATCC 1663	1024	1024	1	1024	1024	1	512	512	1	1024	1024	1	2	2	1
C. albicans IS1	256	512	2	512	1024	2	512	1024	2	256	1024	4	4	4	1
C. parapsilosis	512	1024	2	512	1024	1	512	1024	2	512	1024	2	0.50	1	2
C. parapsilosis ATCC 22019	1024	1024	1	1024	1024	1	512	1024	2	1024	1024	1	1	1	1
C. krusei ATCC 6258	512	1024	2	2048	2048	1	1024	1024	1	1024	1024	1	0.50	1	2
C. krusei	1024	1024	1	256	1024	4	1024	1024	1	512	1024	1	32	32	1
C. tropicalis ATCC 750	1024	2048	2	2048	2048	1	1024	1024	1	1024	1024	1	0.50	1	2
C. lipolithica	2048	2048	1	4096	4096	1	512	1024	2	1024	1024	1	0.50	1	2
C. haemophilus	256	1024	4	4096	4096	1	512	1024	2	512	1024	1	0.50	1	2
Dermatophytes															
M. gypseum E1420	256	4096	16	4096	4096	1	512	512	1	2048	4096	2	0.50	1	2

Table 3. Cont.

T. violaceum	32	1024	32	128	256	2	512	512	1	2048	4096	2	1	1	1				

MIC: Minimum Inhibitory Concentrations; MFC: Minimum Fungicidal Concentrations; - : not determined; R = MFC/MIC; *Nystatin for yeast and griseofulvin for dermatophytes

Table 4. Antifungal activities (MIC and MFC in µg/ml) of essential oils from the tested spices against *Candida albicans* and *Cryptococcus neoformans* resistant isolates.

Yeasts	*A. citratum*			*A. danielli*			*P. capense*			*M. myristica*			*Ac/Ad*			*Pc/Mm*			*Nystatin*		
	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R
Amphotericin B and nystatin resistant *C. albicans*																					
Ca Da11	128	512	4	1024	1024	1	>4096	-		>4096	-	-	4096	4096	1	>4096	-	-	16	32	2
Ca E01	512	512	1	1024	1024	1	>4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	32	32	1
Ca F021	1024	1024	1	2048	2048	1	>4096	-	-	1024	1024	1	4096	4096	1	2048	-	-	32	128	4
Ca F045	1024	1024	1	2048	2048	1	>4096	-		>4096	-	-	4096	4096	1	>4096	-	-	32	128	4
Ca K42	2048	2048	1	2048	2048	1	>4096		-	2048	2048	1	4096	4096	1	>4096	-	-	32	32	1
Ca D10	4096	4096	1	4096	4096	1	4096	4096	1	4096	4096	1	2048	4096	2	2048	2048	1	16	64	4
Ca F066	4096	>4096	-	2048	-	-	4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	-	-	-
Ca F005	4096	4096	1	>4096	-	-	>4096	-	-	>4096	-	-	4096	4096-	1	>4096	-	-	256	256	1
Ca F017	4096	4096	1	>4096	-	-	>4096	-	-	>4096	-	-	4096	4096	1	>4096			32	64	2
Ca F057	4096	4096	1	4096	4096	1	2048	2048	1	>4096	-	-	2048	4096	2	>4096	-	-	128	128	1
Ca K14	512	512	1	1024	2048	2	>4096	-	-	>4096	-	-	512	1024	2	>4096	-	-	64	64	1
Ca F015	1024	1024	1	2048	2048	1	>4096	-	-	>4096	-	-	2048	2048	1	>4096	-		64	64	1
Ca F023	>4096	-	-	>4096	-	-	>4096	-	-	>4096	-		4096	4096	1	>4096	-	-	256	256	1
Ca F040	>4096	-	-	>4096	-	-	>4096	-	-	>4096	-		2048	4096	2	>4096	-	-	256	256	1
Ca F002	>4096	-	-	>4096	-	-	>4096	-	-	>4096	-		4096	4096	1	>4096	-	-	128	256	2
Ca F041	512	512	1	1024	1024	1	4096	4096	1	>4096	-	-	2048	2048	1	>4096	-	-	32	128	4
Ca K072	2048	2048	1	>4096	-	-	>4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	128	256	2
Ca F049	2048	-	-	4096	-	-	>4096	-	-	>4096	-	-	2048	2048	1	>4096	-		128	256	2
Ca F026	2048	4096	2	>4096	-	-	>4096	-	-	>4096		-	4096	4096	1	>4096	-	-	256	256	1
Ca K22	4096	-	-	>4096	-	-	>4096		-	>4096	-	-	4096	>4096	-	>4096	-	-	128	256	2
Nystatin resistant *C. neoformans*																					
CN	4096	4096	1	>4096	-	-	4096	-	-	4096	-	-	2048	>4096	-	2048	-	-	256	256	1
CN 169	4096	4096	1	>4096	-	-	>4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	256	256	1
CN 047	1024	4096	4	>4096	--	-	>4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	128	256	2
CN 091	4096	4096	1	>4096	-	-	>4096	-	-	>4096	-	-	4096	>4096	-	>4096	-	-	256	256	1
CN 046	>4096	-	-	>4096	-	-	>4096	-	-	4096	-	-	512	512	1	>4096	-	-	32	32	1
CN 158	4096	-	-	4096	-	-	1024	-	-	4096	4096	1	2048	4096	2	>4096	-	-	128	256	2
CN 173	>4096	-	-	>4096	-	-	>4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	256	256	1
CN 096	4096	4096	1	>4096	-	-	>4096	-	-	>4096	-	-	4096	4096	1	>4096	-	-	128	128	1

MIC: Minimum Inhibitory Concentrations; MFC: Minimum Fungicidal Concentrations; - : not determined; R = MFC/MIC; Ac/Ad: combination of essential oils of *A. citratum* and *A. daniellii* (1:1), Pc/Mm : combination of essential oils of *P. capense* and *M. myristica* (1:1).

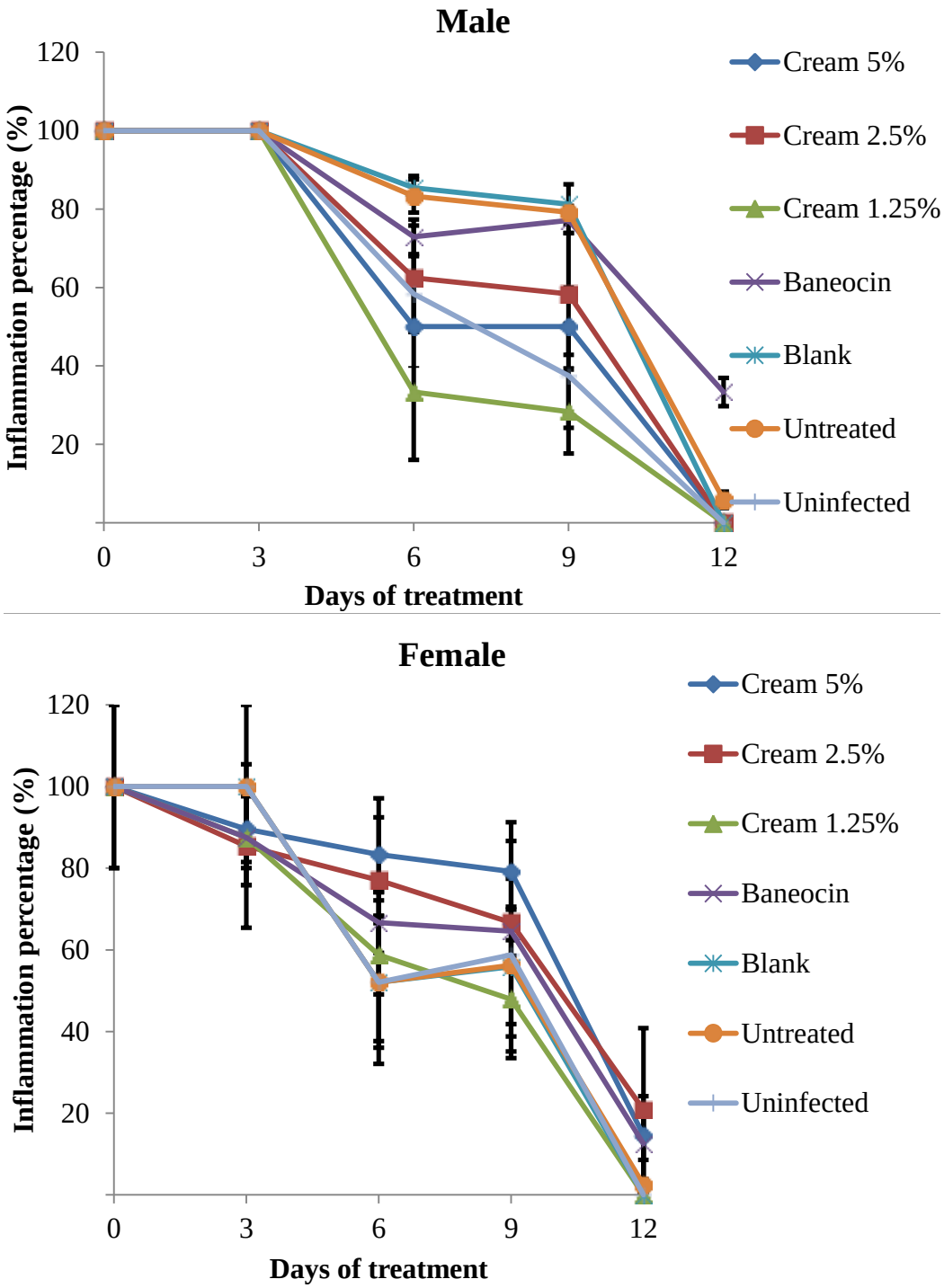

Figure 1. Evolution of the inflammation in male and female rats during the treatment.

the studied essential oils. These differences may be due to the different plant species used. The extraction yield of the essential oil from *A. citratum* seeds is lower than that obtained after 4 h of hydrodistillation from *A. citratum* seeds (2.8%) bought at Kribi in Cameroon (Amvam Zollo et al., 2002). The extraction yield of the essential oil from *A. daniellii* dried seeds is comparable to that obtained from the fresh seeds of this plant species (1.3%) collected from Nigeria (Essiens et al., 2017).

The extraction yield from *P. capense* seed (1.6%) is slightly similar to that obtained in the literature (1.98%) after 3 h of hydrodistillation from dried ground seeds

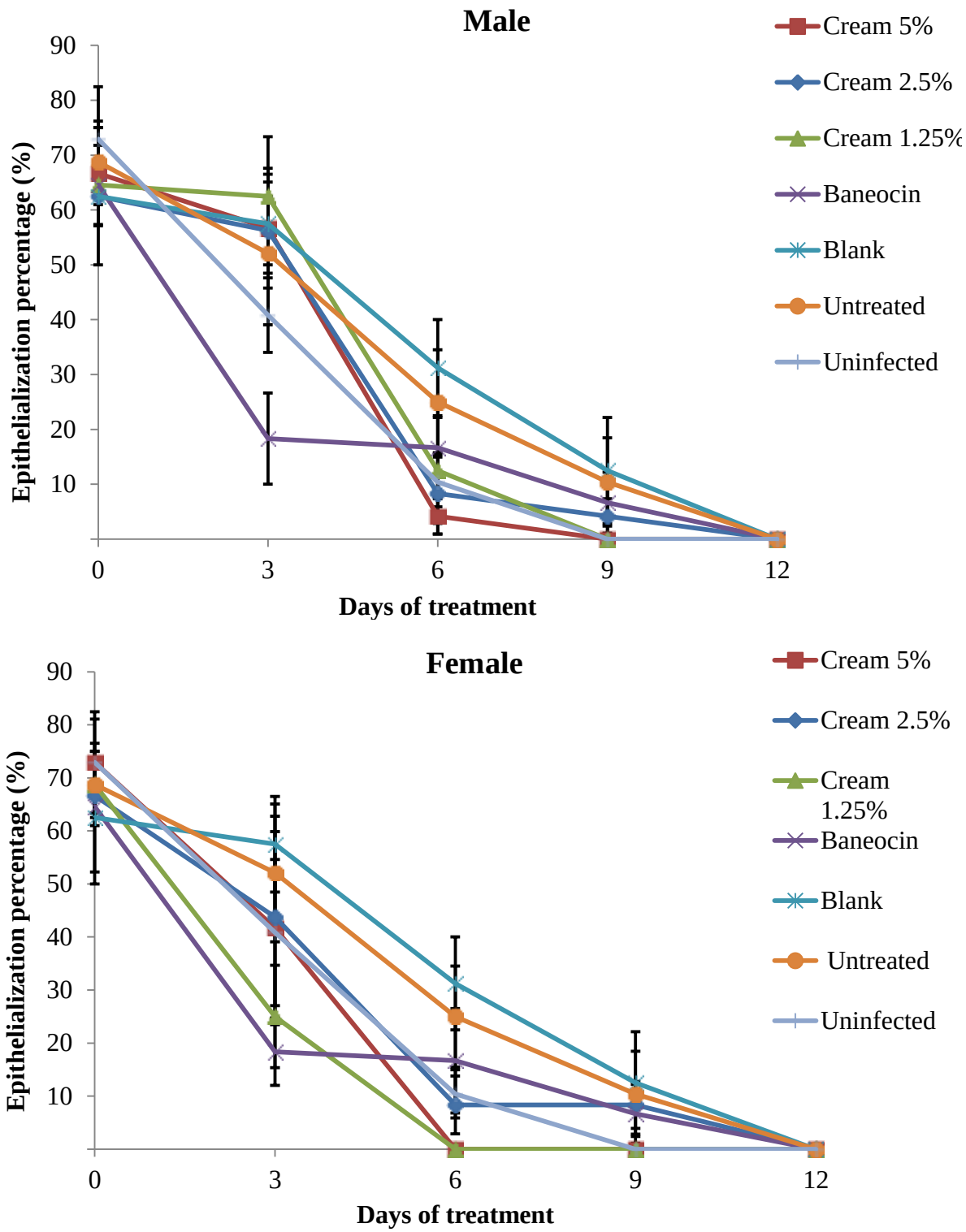

Figure 2. Evolution of epithelialization percentages in male and female rats during the treatment. Bars represent the mean ± SD of three independent experiments carried out.

(Woguem et al., 2013). This result was also slightly similar with that obtained by Tchoumbougnang et al. (2009), after 5 h of hydrodistillation (1.51%). The extraction yield of the essential oil from *M. myristica* seed (2.4%) was higher than that obtained after 7 h of hydrodistillation of this plant species (0.21%), in

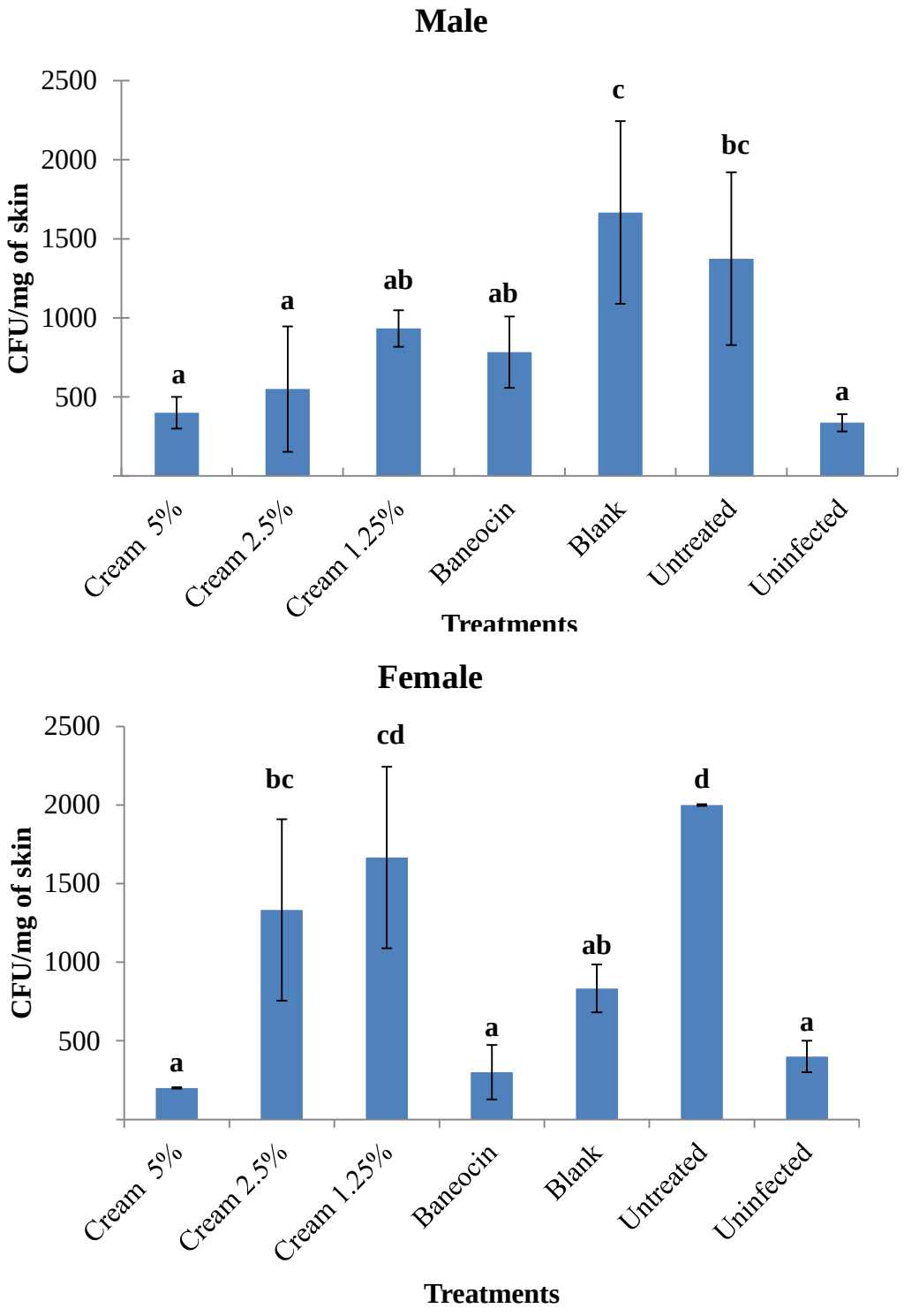

Figure 3. Effect of cosmetic cream based on essential oil of *A. citratum* on bacterial load (CFU/mg) on male and female rats after 14 days of treatment. Bars represent the mean ± SD of three independent experiments. Letters a-d indicate significant differences between samples according to one way ANOVA and Waller Duncan test; $p<0.05$.

Democratic Republic of Congo (Cimanga et al., 2002). However, this result was comparable to those obtained after 5 h of hydrodistillation from the same plant species collected in Kribi-Cameroon (2.72%) and Gore-Chad

Table 5. Relative organ weight in male and female rats at the end of the treatment.

Sex	Treatment	Relative organ weight (g/100 g b.w.)				
		Heart	Lung	Liver	Kidney	Spleen
Male	Cream 5%	0.53 ± 0.05^{a}	0.94 ± 0.14^{a}	5.78 ± 0.62^{a}	0.88 ± 0.14^{a}	0.57 ± 0.3^{a}
	Cream 2.5%	0.62 ± 0.11^{a}	1.03 ± 0.08^{a}	6.52 ± 0.48^{a}	1.00 ± 0.1^{ab}	0.63 ± 0.11^{ab}
	Cream 1.25%	0.52 ± 0.06^{a}	0.99 ± 0.11^{a}	5.99 ± 0.78^{a}	1.05 ± 0.14^{abc}	0.31 ± 0.07^{a}
	Baneocin	0.57 ± 0.08^{a}	1.03 ± 0.17^{a}	6.38 ± 0.13^{a}	1.08 ± 0.1^{abc}	0.46 ± 0.19^{a}
	Blank	0.62 ± 0.06^{a}	1.09 ± 0.15^{a}	7.05 ± 0.84^{a}	1.19 ± 0.1^{bc}	0.98 ± 0.23^{bc}
	Untreated	0.56 ± 0.02^{a}	0.96 ± 0.21^{a}	6.40 ± 0.57^{a}	1.08 ± 0.14^{abc}	1.09 ± 0.19^{cd}
	Uninfected	0.63 ± 0.02^{a}	0.86 ± 0.02^{a}	6.64 ± 0.65^{a}	1.23 ± 0.01^{c}	1.41 ± 0.01^{d}
Female	Cream 5%	0.41± 0.02^{ab}	0.84 ± 0.13^{a}	5.19 ± 0.35^{abc}	0.92 ± 0.05^{a}	0.53 ± 0.1^{abc}
	Cream 2.5%	0.41 ± 0.03^{ab}	0.80 ± 0.06^{a}	4.68 ± 0.8^{abc}	0.90 ± 0.06^{a}	0.46 ± 0.06^{ab}
	Cream 1.25%	0.47 ± 0.03^{abc}	0.98 ± 0.21^{a}	5.00 ± 0.49^{ab}	1.00 ± 0.08^{a}	0.51 ± 0.07^{abc}
	Baneocin	0.32 ±0.23^{a}	0.60 ± 0.43^{a}	3.92 ± 2.89^{a}	0.81± 0.6^{a}	0.41 ± 0.31^{a}
	Blank	0.61 ± 0.08^{c}	1.11 ± 0.36^{a}	6.52 ± 0.71^{bc}	1.21 ± 0.12^{a}	1.00 ± 0.12^{bc}
	Untreated	0.59 ± 0.01^{bc}	1.02 ± 0.02^{a}	7.32 ± 0.81^{c}	1.29 ± 0.15^{a}	1.03 ± 0.59^{c}
	Uninfected	0.57 ± 0.02^{bc}	1.00 ± 0.05^{a}	6.24 ± 0.04^{abc}	1.18 ± 0.03^{a}	0.44 ± 0.13^{a}

Data represent the mean ± SD of three independent experiments. For the same organ and sex, letters a-d indicate significant differences between samples according to one way ANOVA and Waller Duncan test; $p<0.05$.

(1.87%) (Bakarnga-Via et al., 2014). The differences in the extraction yields and those in the literature may be due to the place and period of harvesting of the plant, the variety of plant species used, the duration of extraction, the environmental variations (Zheljazkov et al., 2015).

The findings of the present study showed that the essential oil of *A. citratum* seeds was mainly composed of monoterpene with a predominance of oxygenated monoterpenes (geraniol, 96.80%) and the absence of sesquiterpenes. These results are in agreement with those of Amvam Zollo et al. (2002) who obtained an essential oil consisting mainly of oxygenated monoterpenes with a predominance of geraniol (70%). The results of the chemical composition of essential oils from *A. daniellii* seeds were in agreement with those of the early reports. Indeed, Essien et al. (2017) reported a high content of 1,8-cineole (53.4%), α-terpineol (12.2%) and β-pinene (9.1%) in the essential oil from *A. danielli* seeds whereas the main compounds identified from the essential oils of this plant collected in Sao Tome were 1,8-cineole (25.5-34.4%), β-pinene (14.1-15.2%) and α-terpineol (9.9-12.1%) (Martins et al., 2001). Moreover, Adegoke et al. (1998) recorded 1,8-cineole (59.8%), β-pinene (13.2%) and α-terpineol (9.3%) in high proportions in the same essential oil from Nigeria, while 1,8-cineole (48.9%) was the main compound of *A. danielli* seeds collected in Cameroon (Menut et al., 1991).

The chemical composition of the essential oil of *P. capense* seed was comparable to those reported in the literature (Woguem et al., 2013, Amvam Zollo et al., 1998, Tchounbougnang et al., 2009). Indeed, previous study showed that essential oil obtained from *P. capense* seed is mainly constituted by monoterpenes (64.7%) with predominance of hydrocarbons (56.5%). Major constituents were monoterpene hydrocarbons: α-pinene (8.9%), sabinene (10.0%), β-pinene (33.2%) and sesquiterpene hydrocarbons: α-caryophyllène (6.3%) and germacrene D (3.8%) (Woguem et al., 2013). Other studies also reported that major volatile compounds of fruits from Western Cameroon were monoterpene hydrocarbons: α-pinene (10.5-14.4%), sabinene (14.7-17.4%), β-pinene (46.8-59.3%) and sesquiterpene hydrocarbons: (E)-caryoplyllene (3.4-4.0%) and germacrene D (2.5-5.2%) (Amvam Zollo et al., 1998; Tchoumbougnang et al., 2009).

However, the extraction yields were slightly different with respect to those obtained in this study. The results of the chemical composition of essential oils of *M. myristica* seeds are comparable to those of Bakarnga-Via et al. (2014), who found 67.1% of α-phellandrene and 4.2% of α-pinene in a sample collected from Kribi (Cameroon) and 52.7% of α-phellandrene and 14.9% of limonene in a sample collected from Gore (Chad). Our results are also in agreement with those of Lamaty et al. (1987), who found 48.8% of α-phellandrene in a sample collected in Yaounde (Cameroon); however, the extraction yields were slightly different.

Differences in antimicrobial activity were noted between the studied essential oils. These differences may be due to the different phytoconstituents identified in these essential oils. Indeed, the antimicrobial activities of medicinal plants are correlated with the presence in their extracts of one or more classes of bioactive secondary metabolites (Reuben et al., 2008).

According to established criteria, MIC values in the range of 100- 1000 µg/ml are indications that botanicals

have antimicrobial activities (Simoes et al., 2009). Also antimicrobial activity of edible plant extracts or extracts from edible parts of plants is considered highly active if MIC values are below 100 µg/ml, significantly active if 100 ≤ MIC ≤ 512 µg/ml, moderately active if 512 < MIC ≤ 2048 µg/ml, low activity if MIC > 2048 µg/ml and not active if MIC > 10 000 µg/ml (Tamokou et al., 2017).

Amongst essential oils that showed the highest activities (MICs < 100 µg/ml), there are essential oil of *A. citratum* against *S. aureus*, *S. aureus* MSSA01, *S. aureus* MRSA03, *S. aureus* MRSA04, *B. subtilis*, *E. coli* S2(1), *entero-aggregative E. coli* and *P. aeruginosa* ATCC 27853; essential oil of *A. daniellii* against *S. aureus* ATCC 25923, *S. aureus* MSSA01, *S. aureus* MRSA04, *E. coli* S2(1) and *P. aeruginosa* ATCC 27853; essential oil of *P. capense* against *S. aureus* ATCC 25923, *B. subtilis* and *P. aeruginosa* ATCC 27853 and essential oil of *M. myristica* against *E. coli* S2(1) and *P. aeruginosa* ATCC 27853.

The results of antimicrobial activities of *A. citratum*, *P. capense*, *A. daniellii* and *M. myristica* clearly indicate that the essential oils from these plants have antibacterial and antifungal properties. These data corroborate those of the previous works (Tatsadjieu et al., 2003; Fasoyiro and Adegoke, 2007; Steenkamp et al., 2007; Samie et al., 2010). Collectively, the present study showed that the tested essential oils have antimicrobial activities and are effective against methicillin resistant *S. aureus*, amphotericin B and nystatin resistant *Candida albicans* and *Cryptococcus neoformans*. The overall results of the present investigation confirmed the traditional uses of the studied spices in the treatment of microbial infections. Taking into account the medical importance of the tested microorganisms, this result can be considered as promising in the perspective of developing new antimicrobial agents from plant origin. During the MIC and MMB determination, we have noted that MMC values are in general fourfold lesser than the MIC values on the corresponding microorganism; suggesting that the studied essential oils have a microbicidal effect on the sensitive microorganisms (Mims et al., 1993).

Combinations of antibiotics can lead to synergistic effects especially during the therapy of fungal infections. These combinations have been recognized as being able to delay the emergence of resistant strains of microorganisms (Aiyegoro and Okoh, 2009). The effect of synergy between plant-derived essential oils makes it possible to use essential oils when their efficacy alone is reduced (Nascimento et al., 2000). These observations could explain the evaluation of the antifungal activity of the combination of essential oils of the studied plants, because in addition to substances having direct antifungal activity, it has been demonstrated that within plants, other substances can act as adjuvants by modulating the activity of antifungal agents (Veras et al., 2012). The antifungal activities of the combination of essential oils of *A. citratum* and *A. daniellii* (1:1) were greater than those of these essential oils used alone. However, the combination of essential oils of *P. capense* and *M. myritica* (1:1) reduced their antifungal activities compared to those of these essential oils used alone. The above findings suggest that the combination of essential oils of *A. citratum* and *A. daniellii* (1:1) has synergistic effect, whereas the combination of essential oils of *P. capense* and *M. myritica* (1:1) has an antagonistic effect. The monoterpene and sesquiterpene compounds found in these essential oils would be responsible for the observed effects with respect to certain *C. albicans* and *C. neoformans* resistant isolates.

The results of the therapeutic effect of the cream based on essential oil of *A. citratum* seeds against dermatosis induced with methicillin resistant *S. aureus* in rats revealed that epithelialization time was significantly shorter in animals treated with cream based on essential oil of *A. citratum* compared to negative control groups. Indeed, epithelialization involves the proliferation and migration of epithelial cells through the wound bed (Sanwal and Chaudhary, 2011). Therefore, a shorter epithelialization time could be due to facilitated epithelial cell proliferation and/or increased viability of epithelial cells (Mulisa et al., 2015).

Thus, the shorter epithelialization time in the animals treated with the essential oil reinforce the hypothesis according to which the essential oil of *A. citratum* has a potential application as an antibacterial healing agent. Moreover, the fact that the cream based on essential oil of *A. citratum* significantly reduced the number of Colony Forming Units (CFU) of bacteria at the infection site compared to the negative controls also supports the *in vivo* antibacterial properties of *A. citratum* essential oil. To the best of our knowledge, this is the first report on the therapeutic effect of the essential oil from *A. citratum.*

Conclusion

The overall results of the present investigation indicated that the main compounds identified in the essential oils are geraniol (98%) for *A. citratum*; eucalyptol (48.8%), α-terpineol (21.7%) and geraniol (10.5%) for *A. daniellii*; β-pinene (37.3%), germacrene D (9.8%), trans-β-caryophyllene (8.8%), α-pinene (8.6%), naphthalene (8.3%) and sabinene (4.8%) for *P. capense*; α-phellandrene (61.5%), germacradienol (7.9%) and δ-cadinene (4.2%) for *M. myristica.* The tested essential oils possess antimicrobial activities which could be a function of either the individual or the additive effects of the identified volatile components. The cream based on essential oil of *A. citratum* (5%) can be used in the treatment of dermatosis induced with MRSA subject to further toxicological and pre-clinical studies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Cytotoxicity of selected Ethiopian medicinal plants used in traditional breast cancer treatment against breast-derived cell lines

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Traditional medicine is widely practiced in Ethiopia. Here we investigate the toxicity of extracts of seven medicinal plants traditionally used to treat breast cancer in Ethiopia. These plants, *Sideroxylon oxyacanthum*, *Zanthoxylum chalybeum*, *Clematis simensis*, *Clematis longicauda*, *Dovyalis abyssinica*, *Vernonia leopoldi,* and *Clerodendrum myricoides*, were selected based on recommendations by traditional healers and on the frequency of use. After harvesting the plant material, the water content was determined and the powder was subjected to methanol extraction resulting in crude extracts which were tested for cytotoxicity in dose response assay. Then the methanol extract of the most toxic plants was subjected to further solvent-solvent fractionation to gain petroleum ether, hexane, chloroform, ethyl acetate, and water fractions and these were also tested for cytotoxicity in dose response assays. Extracts of *Z. chalybeum* and *C. myricoides* were not toxic. The crude extracts of *S. oxyacanthum, C. simensis*, and *D. abyssinica* showed cytotoxicity with half maximal inhibitory concentration 50% (IC50) below 1 µg/ml in the human breast cancer cell lines JIMT-1, MCF-7, and HCC1937. The ethyl acetate fraction of *V. leopoldi* was the most cytotoxic fraction of all fractions tested with an IC50 of 0.87 µg/ml in JIMT-1 cells. The aqueous fraction of *S. oxyacanthum* and the chloroform fraction of *C. simensis* were also cytotoxic. In conclusion, our data show a wide difference in in vitro toxicity of medicinal plants used to treat breast cancer patients, which may guide the use of traditional medicine and the choice of plants for isolation of new compounds for cancer treatment.

Key words: Cancer, Ethiopia, *in vitro* cytotoxicity, 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), traditional medicine.

INTRODUCTION

Cancer is a group of diseases comprising a combination of genetic, metabolic, and signaling aberrations (Long and Ryan, 2012). It constitutes an enormous burden globally despite the progress of medicine over the years (Font-Burgada et al., 2016). As per the estimate by GLOBOCAN, about 18.1 million new cancer cases and 9.6 million deaths occurred in 2018 (Bray et al., 2018). Lung cancer and breast cancer accounted for 2.09 million cases each and 1.76 and 0.63 million deaths, respectively (Bray et al., 2018). Overall, 57% of new

cancer cases, 65% of cancer-associated deaths, and 48% of the total number of diagnosed cancer cases occurred in the less developed regions of the world (Torre et al., 2016).

Traditional medicine (TM) has a long history of use in human ailment management systems globally and the World Health Organization promotes it as a source of less expensive and comprehensive medical care especially in developing countries (WHO, 2013). Accessibility, affordability, and acceptance by the community are core reasons for wider use of TM practices in Africa (Abdullahi, 2011). Ethiopia is a hub for different cultures and biodiversity and the medico-religious and historical accounts substantiate age-long TM usage (Kibebew, 2001).

Various sources reported that more than 70% of Ethiopians frequently use TM for their healthcare, where more than 95% of the TMs are sourced from plants (Kibebew, 2001; Teklay et al., 2013). The importance of identifying the active biological fractions and subsequently the active components in plants used in TM is substantiated by various reports and is a wide-open field of research nowadays (Fabricant and Farnsworth, 2001; Yuan et al., 2016).

The present study was carried out to assess the cytotoxicity of selected Ethiopian medicinal plants used in traditional breast cancer treatment. Using a dose response assay, we investigated the toxicity of crude and solvent-solvent fractions of selected plants in three human breast cancer cell lines and one normal-like human breast epithelial cell line.

MATERIALS AND METHODS

Selection of anticancer medicinal plants

Ethnobotanical reports from Ethiopia and judicious *in situ* investigations were used to select anti-cancer medicinal plants used for cancer treatment by traditional healers. Accordingly, medicinal plants widely used in different part of the country with special reference to breast cancer treatment were selected following analytical ethnobotanical tools (Tuasha et al., 2018a,b). The plant specimens were collected from Dalle district (Sidama Zone, Southern Nations, Nationalities and Peoples Regional State, southern Ethiopia) and from the Yayu forest biosphere (Illubabor Zone, Oromia Regional State, southwestern Ethiopia) in the month of June, 2015. Identification and authentication were done at the National Herbarium, Addis Ababa University, Addis Ababa, Ethiopia. The medicinal plants investigated are *Sideroxylon oxyacanthum* (Baill.) (Family: Sapotaceae), *Zanthoxylum chalybeum* Engl. (Family: Rutaceae), *Clematis simensis* Fresen. (Family: Ranunculaceae), *Clematis longicauda* Steud. ex A. Rich (Family: Ranunculaceae), *Dovyalis abyssinica* (A. Rich.) Warb. (Family: Flacourbitaceae), *Vernonia leopoldi* (Sch. Bip. ex Walp.) Vatke (Family: Asteraceae), and *Clerodendrum myricoides* (Hochst.) Vatke (Family: Lamiaceae). The voucher specimens were deposited at the herbarium (NT014, NT012, NT037, NT072, NT017, NT073, and NT006, respectively). Except for the species *D. abyssinica* (stem bark) and the genus Clematis (whole aerial part), the leaf part of the medicinal plants was used. The two species, *S. oxyacanthum* and *C. longicauda* are endemic to the Ethiopian flora (Dagne, 2011), whereas, outside of Ethiopia, *V. leopoldi* is found only in Yemen (Marzouk and Abd Elhalim, 2016). Figure 1 shows images of some of the plants taken during specimen collection.

Determination of the water content of the medicinal plants

The water content of the medicinal plants was determined by estimating the weight loss up on drying in an air-ventilated open space at ambient temperature. The initial weight of the plant material was determined by weighing in the field during collection using a portable beam balance. After recording the initial weight, the plant material was thoroughly washed with tap water and rinsed with distilled water to remove adulterants and contaminants. The plant material was then dried in a shaded and ventilated open space. When dry, it was pounded and ground to finer powder. The fine powder was weighed and once again subjected to further drying. After repeated checking that a weight loss of not more than 0.25% took place between measurements, the final weight loss was determined by comparing the initial weight with the final weight. The drying process took approximately 15 days.

Extraction and fractionation of the medicinal plants

Fine powder (500 g) of each medicinal plant was subjected to extraction. Accordingly, 100 g of the powder was suspended in 500 ml of 80% methanol (MeOH) in H_2O in an Erlenmeyer flask. The suspension was macerated by shaking on a rotary water bath shaker (DZK-2, Shanghai, China) (120 routes per minute) for 72 h at ambient temperature. Thereafter, the liquid and solid phases were separated by filtration, initially using cotton cloth while squeezing gently. Subsequently, three consecutive filtrations were performed using Whatman filter paper №1 (Whatman LTD, England) at ambient temperature. Removal of the solvent and concentration was performed using a rotary vacuum evaporator (BÜCHI-Germany) under reduced pressure at 45°C. The concentrated extract was then freeze-dried by lyophilization (CHRIST, Alpha 2-4 LDplus, *Osterode, Germany*).

The crude extract was then further fractionated according to the following procedure. Solvent-solvent (1:1, v/v) fractionation was performed based on the polarity of the solvents. Accordingly, the dried crude MeOH extract was weighed and allowed to completely dissolve in 250 ml of 10% MeOH in Millipore H_2O in an Erlenmeyer flask. A separation funnel was used for the partitioning and 250 ml of n-hexane (100%) was added. It was then sealed with a stopper and the stopcock was tightly closed. The solution was then gently mixed in the funnel before letting it partition for 1 h which resulted in an aqueous and an n-hexane layer. The n-hexane phase was carefully collected. The volume of the aqueous phase was determined and an equal volume of chloroform (100%) was added. The same procedure as described above was followed, resulting in the collection of a chloroform phase. The volume of the aqueous phase was determined and mixed with an equal volume of ethyl acetate (100%). The same procedure was followed, resulting in the collection of an ethyl acetate phase and an aqueous phase. All the fractions were

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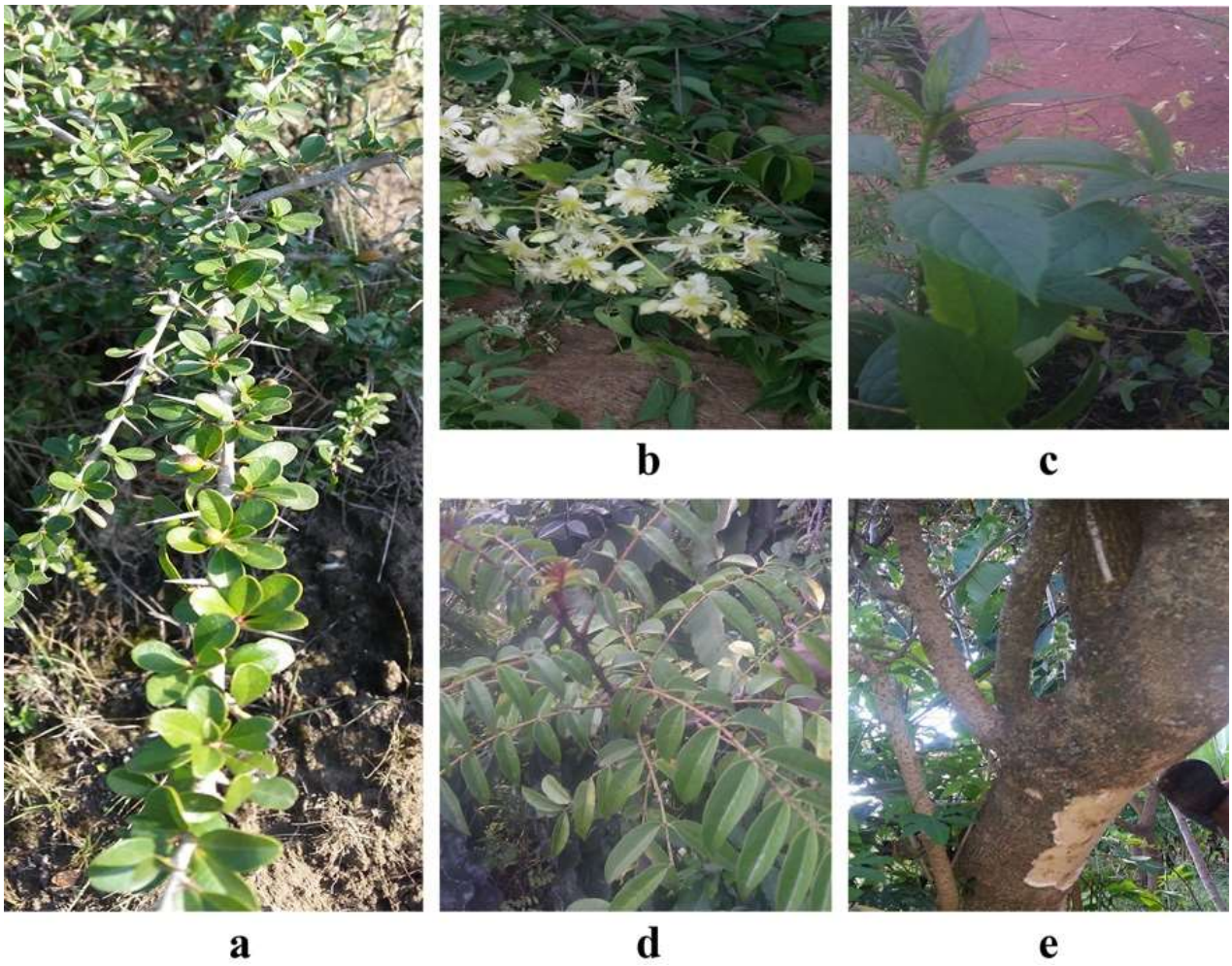

Figure 1. Images of some of the medicinal plants taken during specimen collection (a - leaves of *S. oxyacanthum*, b - aerial part of *C. simensis*, c - leaves of *C. myricoides*, d – leaves of Z. *chalybeum,* and e - debarking of *D. abyssinica)*.

concentrated and freeze-dried as described above.

A slightly modified partitioning procedure was followed to obtain fractions of *C. longicauda* and *V. leopoldi.* Briefly, 100 g of fine powder of each plant was dissolved in 500 ml of 90% MeOH in Millipore H_2O and then the solution was subjected to maceration in a shaking water bath (DZK-2, Shanghai, China) for 12 h at ambient temperature. Filtration and concentration of the crude extract was performed as described above. The dried crude extract was weighed and fractioned with petroleum ether and then by ethyl acetate to give petroleum ether and ethyl acetate fractions, respectively. The fractions were freeze dried as described above and stored at -20°C until use. All chemicals used for the extraction and partitioning process were purchased from Sigma-Aldrich (St. Louise, MO, USA).

Cell lines and culturing conditions

The human breast cancer cell lines JIMT-1 (population doubling time (PDT) ≈ 24 h), HCC1937 (PDT ≈ 35 h), MCF-7 (PDT ≈ 35 h), and one normal-like cell line (MCF-10A, PDT ≈ 15 h) were used for the cytotoxicity experiments. The JIMT-1 human ductal breast carcinoma cell line, was established from a pleural metastasis of a 62-year old patient with breast cancer who was clinically resistant to trastuzumab (Tanner et al., 2004). The human ductal breast carcinoma cell line HCC1937, was sourced from a 24 years old patient (Tomlinson et al., 1998). The MCF-7 cell line was derived from a pleural effusion of a 69 years old female with an epithelial breast adenocarcinoma (Soule et al., 1973). The cell lines JIMT-1, MCF-7, and HCC1937 represent HER2 positive, luminal A, and basal-like breast cancer sub-groups, respectively. The MCF-7 (HTB-22) and HCC1937 (CRL-2336) cancer cell lines as well as the human normal-like breast epithelial cell line MCF-10A (CRL-10317) were purchased from American Type Culture Collection (Manassas, VA, USA). The JIMT-1 cell line (ACC589) was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

JIMT-1 cells were routinely cultured in DMEM/Ham's F-12 medium (VWR, Lund, Sweden) supplemented with 10% fetal bovine serum (FBS) (VWR), 1 mM non-essential amino acids (VWR), 10 µg/ml insulin (Sigma-Aldrich, Stockholm, Sweden), 1 mM L-glutamine (VWR), and 100 U/ml penicillin/100 µg/ml streptomycin (VWR). The MCF-7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM non-essential amino acids, 10 µg/ml insulin, 1 mM L-glutamine, and 100 U/ml penicillin/100 µg/ml streptomycin. The HCC1937 cells were

Table 1. Estimation of water content of the medicinal plants.

Plant	Part used	Weight at collection (g)	Final dry weight (g)	Water content (%)
S. oxyacanthum	Leaf	1200	720	40.0
Z. chalybeum	Leaf	1500	820	45.3
C. simensis	Whole part	1200	610	49.2
C. longicauda	Whole part	850	445	47.6
D. abyssinica	Stem bark	900	550	38.9
V. leopoldi	Leaf	1000	415	58.5
C. myricoides	Leaf	750	290	61.3

cultured in RPMI 1640 medium (VWR) supplemented with 10% heat-inactivated FBS, 1 mM non-essential amino acids, 10 µg/ml insulin, 20 ng/ml epidermal growth factor (Sigma-Aldrich), and 100 U/ml penicillin/-100 µg/ml streptomycin. The MCF-10A cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM non-essential amino acids, 10 µg/ml insulin, 20 ng/ml epidermal growth factor, 50 ng/ml cholera toxin (Sigma-Aldrich), 250 ng/ml hydrocortisone (Sigma-Aldrich), and 100 U/ml penicillin/100 µg/ml streptomycin. All cell lines were kept at 37°C in a humidified incubator with 5% CO_2. The cells were seeded at different densities. Accordingly, JIMT-1 cells were seeded at 1.5×10^4 cells/cm^2, both MCF-7 and HCC1937 at 2×10^4 cells/cm^2, and MCF-10A cells were seeded at 10^4 cells/cm^2. Tissue culture vessels of the appropriate size were used with the volume of medium about 0.2-0.3 ml per cm^2.

MTT dose response assay

The yellow soluble tetrazolium salt 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) is reduced by metabolically active cells to insoluble formazan crystals (Mosmann, 1983) and this *in vitro* assay is a widely accepted model in a 96-well format to obtain dose response curves and half maximal inhibitory concentration 50% (IC_{50}). Briefly, confluent cells were detached by trypsinization and counted in a hemocytometer. Cells of the different cell lines were seeded at the recommended densities (described above) in 180 µl of medium into the wells of 96-well plates and then allowed to attach for 24 h before addition of the extracts/fractions.

For the treatments, stock solutions of 1 mg/ml (crude methanol extracts) and 250 mg/ml or 500 mg/ml (fractionated extracts) were prepared in 100% MeOH and were allowed to dissolve completely. Then, the highest used concentration of 20 µg/ml with 4% MeOH in PBS (crude extracts) and 5 mg/ml with 2% MeOH in PBS (fractionated extracts) were prepared and sterile-filtered using 0.22 µm filters followed by serial dilutions to the lowest concentration of 0.01 µg/ml (crude extracts) and 0.001 mg/ml (fractionated extracts). Then, 20 µl of the serially-diluted methanolic crude extracts or fractionated extracts were added to obtain the desired concentrations in the wells of the 96-well plates. The controls received 20 µl 4% MeOH in PBS (crude extracts) or 2% MeOH in PBS (fractionated extracts). Thus, the final MeOH concentration in all wells was 0.4% (crude extracts) or 0.2% (fractionated extracts). The plates were then incubated for 72 h before MTT addition.

MTT (Sigma-Aldrich) was dissolved in PBS to a concentration of 5 mg/ml in PBS, the solution was sterile-filtered, and stored at -20°C wrapped with aluminum foil to protect from light exposure. After 72 h of incubation, 20 µl of the MTT solution was added to the wells and the 96-well plates were wrapped with aluminum foil and returned to the CO_2 incubator for 1 h. The formazan crystals were dissolved by adding 100 µl of 100% DMSO to each well. To dissolve the precipitates, the plates were allowed to gently swirl at room temperature for 10-15 minutes. Then the absorbance was read at 540 nm using a Labsystems iEMS Reader MF (Labsystems Oy, Helsinki, Finland) and using DeltaSoft II v.4.14 software (Biometallics Inc., Princeton, NJ, USA). The percent of control was calculated as absorbance units in the presence of the extracts/fractions as percentage of that in the control and thus the dose response curves were drawn and IC_{50} values were obtained using the GraphPad Prism software (San Diego, CA, USA) version 7.02. The dose-response experiments were performed several times (3 to 6) for each extract/fraction, and the mean IC_{50} ± SD was calculated.

RESULTS

Water content and estimation of extract yield

Based on the weight measurements before and after drying, we determined the weight loss and obtained a measure of water content to get an estimate of how much plant material would be needed for future use (Table 1). The water content was highest in the leaves of *C. myricoides* (61.3%) and lowest in the stem bark of *D. abyssinica* (38.9%).

In Table 2, we present the percent methanolic crude extract and solvent-solvent fraction yield for the most cytotoxic anticancer medicinal plants. Accordingly, high yield was obtained from solvent with high polarity (that is, aqueous phase) for *S. oxyacanthum* and *C. simensis*. Similarly, proportionally high yield was obtained from the more polar solvent for the medicinal plant *V. leopoldi* (Table 2).

Traditionally used anticancer medicinal plants show dose dependent cytotoxicity

We initiated our study by investigating the toxicity of crude methanolic extracts to identify plants for further analysis. An MTT assay was used to determine the overall basal toxicity profiles of the crude MeOH extracts in the breast-derived cell lines. Thus, IC_{50} values were obtained after treating the cell lines for 72 h with different methanolic crude extracts (Figure 2 and Table 3). The crude extract of *S. oxyacanthum* was found to be the

Table 2. Percent yield of the crude and solvent fractions of the three most cytotoxic medicinal plants.

The extract/solvent fraction	Net yield (%) ‡
S. oxyacanthum (MeOH crude)	20.6
hexane	2.3
chloroform	5.1
ethyl acetate	10.1
aqueous	67.9
C. simensis (MeOH crude)	19.2
hexane	11.7
chloroform	17.0
ethyl acetate	3.2
aqueous	26.0
V. leopoldi (MeOH crude)	29.4
petroleum ether	23.5
ethyl acetate	46.3

‡ The crude yield was calculated from the initial dried plant material used for extraction as described in Methods section (i.e., 500 g for *S. oxyacanthum* and *C. simensis*; and 100 g for *V. leopoldi).*

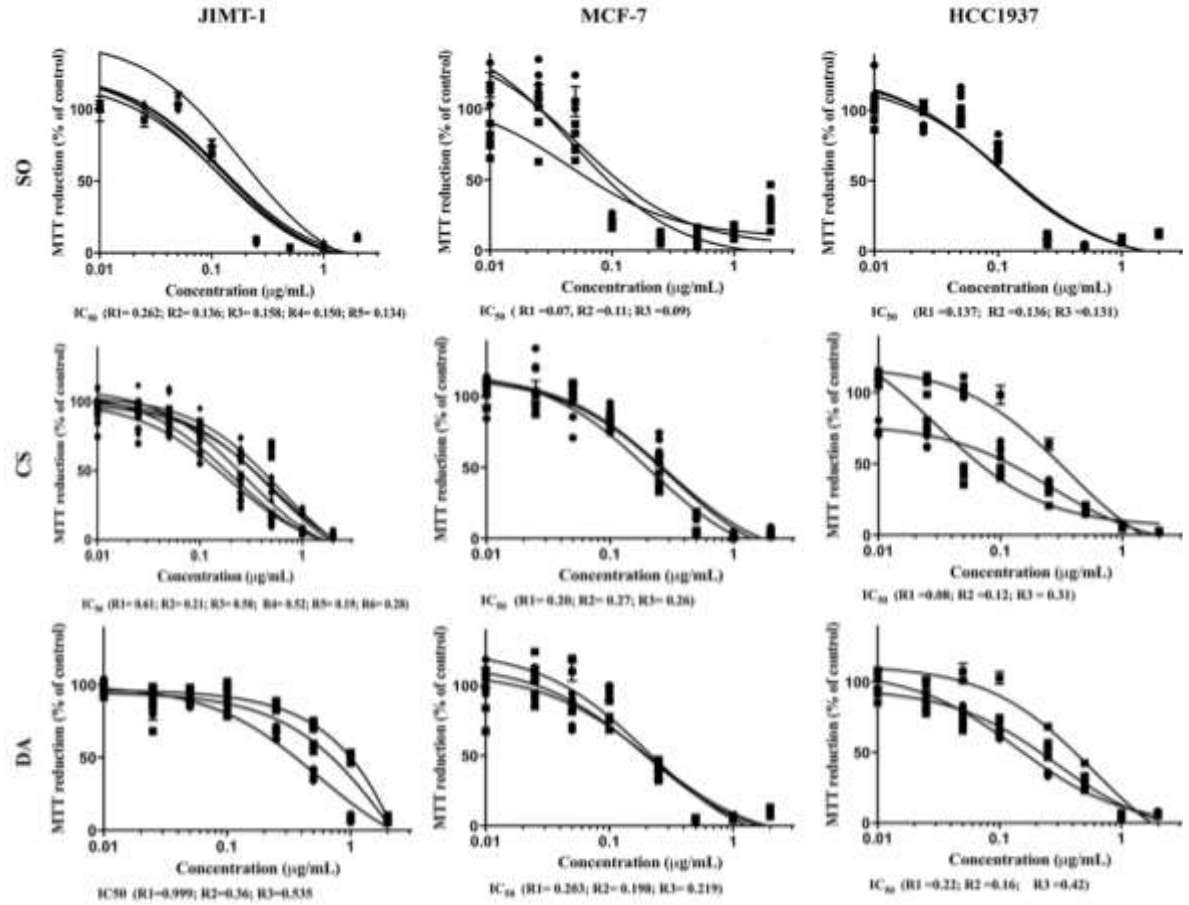

Figure 2. Dose response curves obtained after treatment of the three breast cancer cell lines JIMT-1, MCF-7, and HCC1937 with methanolic extracts of Ethiopian medicinal plants. SO, Methanolic crude extract of *S. oxyacanthum*; CS, methanolic crude extract of *C. simensis*; DA, methanolic crude extract of *D. abyssinica*. Note: Each curve represents one experiment with n=6 wells in each point. The values in parenthesis show IC_{50} values in the different repeats (R). The cells were treated for 72 hours before evaluation using an MTT assay.

Table 3. The IC_{50} (µg/ml) values obtained using an MTT assay after treating breast cancer cell lines with crude MeOH extracts of selected Ethiopian medicinal plants used in traditional breast cancer treatment.

Crude MeOH extract	Cell line		
	JIMT-1	MCF-7	HCC1937
S. oxyacanthum	0.17 ± 0.05[a]	0.09 ± 0.02	0.13 ± 0.003
C. simensis	0.27 ± 0.01	0.24 ± 0.04	0.17 ± 0.12
D. abyssinica	0.63 ± 0.33	0.21 ± 0.01	0.27 ± 0.14
C. myricoides	NA[b]	0.74[c]	NA[d]
Z. chalybeum	NA[b]	NA[b]	NA[d]

[a]The number of 96-well assays run for each plant extract is found in Figure 1 as each assay generates one curve. Unless specified, replicates are ≥ 3 96-well plate independent assays with n = 6 wells in each assay. The data are presented as mean ± SD. [b]NA = not applicable as no IC_{50} was found and no toxicity at the highest concentration of 2 µg/ml. [c]Mean of two replicates. [d]The MTT test was not carried out in this cell line since no toxicity was found in the other two.

Table 4. The IC_{50} (µg/ml) values obtained using an MTT assay after treating two breast cancer cell lines (JIMT-1 and MCF-7) and one normal-like breast epithelial cell line (MCF-10A) with solvent-solvent fractions of Ethiopian traditional medicinal plants.

Solvent fraction	Cell line		
	JIMT-1	MCF-7	MCF-10A
S. oxyacanthum (aqueous)	69 ± 2[a]	49 ± 2.6	80 ± 2
S. oxyacanthum (hexane)	NA[b]	NA[c]	NA[c]
S. oxyacanthum (chloroform)	694 ± 20	NA[b]	NA[c]
S. oxyacanthum (ethyl acetate)	660 ± 44	240 ± 16	NA[c]
C. simensis (chloroform)	80 ± 19	190 ± 70	97 ± 9
C. simensis (hexane)	NA[b]	NA[c]	NA[c]
C. simensis (ethyl acetate)	NA[b]	NA[b]	NA[c]
C. simensis (aqueous)	858 ± 190	NA[b]	NA[c]
V. leopoldi (ethyl acetate)	0.87 ± 0.2	3.5[d]	1.72[d]
V. leopoldi (petroleum ether)	80[d]	NA[b]	NA[c]
C. longicauda (ethyl acetate)	70[d]	NA[b]	NA[c]
C. longicauda (petroleum ether)	170[d]	NA[b]	NA[c]

[a]The number of 96-well assays run for each fraction is found in Figure 2 as each assay generates one curve. Unless specified, replicates are ≥ 3 96-well plate independent assays with n = 6 wells in each assay. The data are presented as mean ± SD. [b]NA = not applicable as no IC_{50} was found and no toxicity at the highest concentration of 1 mg/mL. [c]The MTT test was not carried out in this cell line since no desirable level of toxicity was found in the other two. [d]Mean of two replicates.

most cytotoxic against all the cell lines used where the lowest concentration of 0.09 µg/ml was recorded in the MCF-7 cell line. MeOH extracts of *C. simensis* and *D. abyssinica* were also highly cytotoxic. Extracts from *Z. chalybeum* and *C. myricoides* (except against MCF-7; 0.74 µg/ml, N=2) did not give IC_{50} values at the maximum concentration of 2 µg/ml (Table 3).

After the IC_{50} values of the crude extracts were determined, *S. oxyacanthum and C. simensis* were selected for solvent-solvent fractionation for further testing. Based on reports of wider use among the herbal medicine practitioners, the plants, *V. leopoldi* and *C. longicauda* were included for the solvent-solvent fractionation. For this further study, we decided to include the normal-like MCF-10A and omitted the HCC1937 breast cancer cell line since the MCF-7 and JIMT-1 cells resulted in quite similar IC_{50} in the crude extract testing (Table 3).

Table 4 shows the obtained IC_{50} values and Figure 3 the dose response curves of fractions that resulted in the lowest IC_{50} values. Table 4 shows that not all fractions were toxic and that specific fractions were toxic in the respective plants. Also, it is clear that the fractions are less toxic than the crude MeOH extract. Nevertheless, the aqueous fraction of *S. oxyacanthum*, the chloroform fraction of *C. simensis*, and the ethyl acetate fraction of *V. leopoldi* were found to be toxic at low concentrations. The ethyl acetate fraction of *V. leopoldi* was the most cytotoxic fraction with an IC_{50} value at the concentration of 0.87 µg/ml in JIMT-1 breast cancer cell (Table 4). Notably, the IC_{50} values of MCF-10A cells were higher than those of the JIMT-1 cells, which is not as clear when

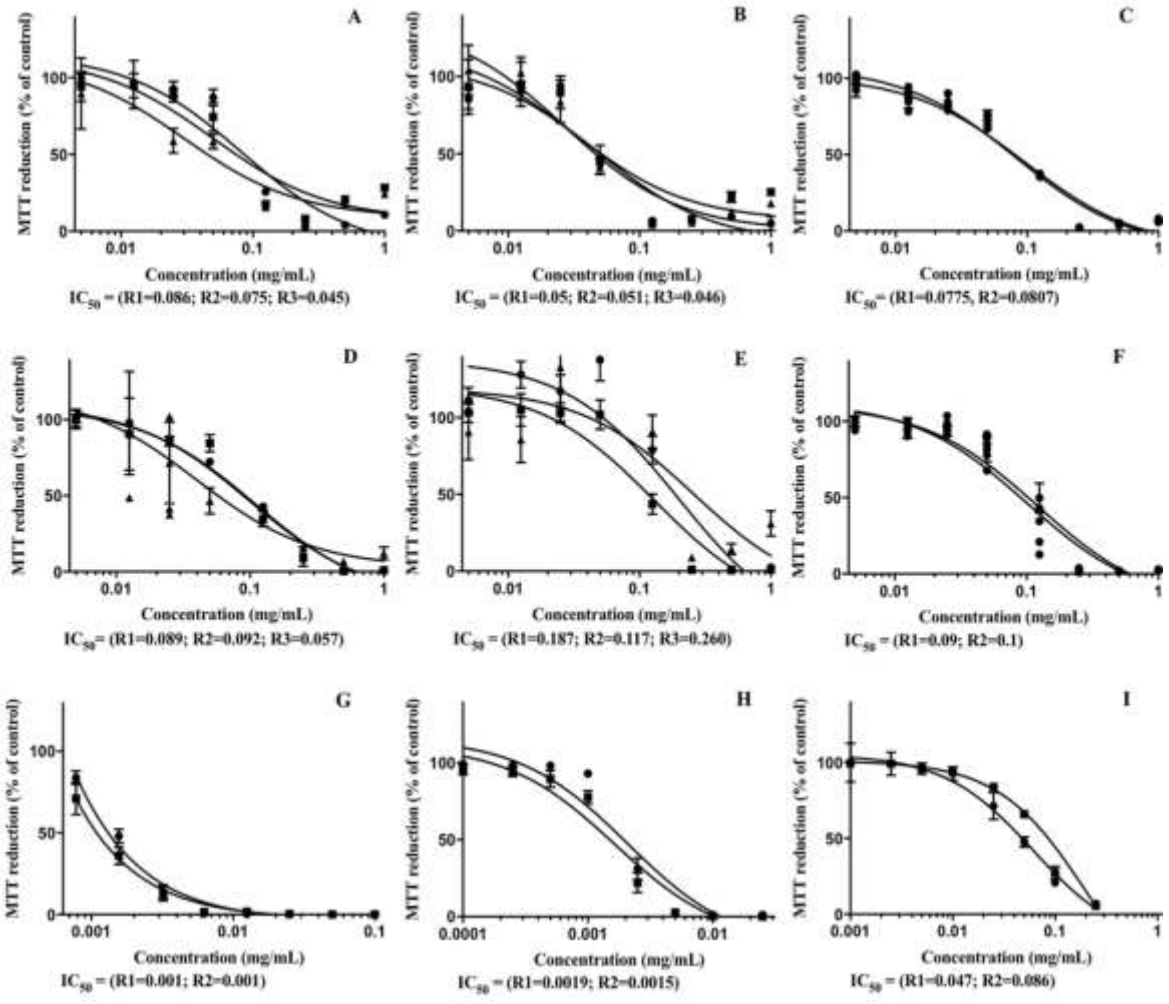

Figure 3. Dose response curves obtained after treatment of the breast cancer cell lines JIMT-1 and MCF-7 and the normal-like breast epithelial cell line MCF-10A with solvent-solvent fractions of Ethiopian traditional medicinal plants. The images show the following dose response curves: the aqueous fraction of S. oxyacanthum of JIMT-1 (A), MCF-7 (B), and MCF-10A (C) cell lines; the chloroform fraction of C. simensis of JIMT-1 (D), MCF-7 (E), and MCF-10A (F) cell lines; the ethyl acetate fraction of V. leopoldi of JIMT-1 (G) and MCF-10A (H); and the ethyl acetate fraction of C. longicauda of the JIMT-1 cell line (I). Note: Each curve represents one experiment with n=6 wells in each point. The x-axis is mg/mL. The values in parenthesis show IC50 values in the different repeats (R). The cells were treated for 72 hours before evaluation using an MTT assay.

comparing the IC_{50} values of MCF-10A and MCF-7. This may be caused by differences in gene expression between JIMT-1 cells and MCF-7 cells (Figure 3).

DISCUSSION

Global inequity in primary healthcare coverage has forced mankind to use ancient alternative medical practices in order to save lives. Of note is the use of plant-based traditional healing practices sustained for millennia in different parts of the world. Ethiopia is one of the ancient civilizations in Africa and the traditional herbal medicine is widely practiced across the country (Tuasha et al., 2018b). Cancer patients prefer to visit traditional healers over conventional therapeutics for a number of reasons. Mainly, lack of awareness among the majority in rural regions results in stigma and cancer patients avoid public presentations. In addition, rising drug costs and economic insufficiency are other driving forces towards the use of TM (WHO, 2013). These days, the chemotherapeutics employed to treat cancer are challenged by cancer recurrence (Ahmed et al., 2017). New strategies are, therefore, needed to acquire lasting cure against cancer. To this end, plant-based TM practices and applications are dependable sources for the search for new lead materials for preclinical work with the goal to be used in clinical medicine today

(Fabricant and Farnsworth, 2001). Extraction and fractionation are the starting steps in research involving traditional medicinal plants aiming at isolation and purification of chemical constituents.

The TM is commonly used by cancer patients and breast cancer patients are the most likely users (Vardy et al., 2013). The assumptions for its wide use lies in that it is safe, causes less complications, and is less likely to cause dependency (Olaku and White, 2011). Most of the users usually combine TM with the conventional drugs hoping it boosts the effects (Richardson et al., 2004). Various reports have shown that the clinical use of traditional herbal medicine improved disease symptoms and quality of life, reduced chemo/radiotherapy-induced side effects, and resulted in tumor size reduction (Molassiotis et al., 2005). In addition, anti-angiogenesis effects, prevention of tumor recurrence, and assisting the body's immune system to battle cancer have been documented in conjunction with TM (Shahid, 2013; Levitsky and Dembitsky, 2015).

In the present study, selected Ethiopian medicinal plants implicated for use in traditional breast cancer treatment were investigated. According to various reports in Ethiopia, these plants are traditionally used against a range of human ailments in addition to cancer. For instance, *Z. chalybeum* is used to treat malaria, sickle cell disease, tuberculosis, pneumonia, colds, ulcers, sore throat, tonsillitis, urticaria (hives), measles, abdominal pain, diarrhea, intestinal worms, bilharzia, amoebiasis, general body pain, female sterility, venereal diseases, uterine fibroids, fainting, dizziness, and headache (http://www.combonimissionaries.co.uk, 2017). Some species from the genus Clematis are used for the treatment of leprosy, fever, various skin diseases, headache, common cold, hemorrhoids, and eczema (Wubetu et al., 2017). *D. abyssinica* was reported for its use in the treatment of hemorrhoids (Chekole et al., 2015) and the use of *C. myricoides* include casting out an evil spirit (Araya et al., 2015; Chekole et al., 2015), treating snake bites (Teklay et al., 2013), malaria (Asnake et al., 2016), diarrhea (Kefalew et al., 2015), arthritis/rheumatism, conjunctivitis, and trachoma (Araya et al., 2015), '*Almaz-balechira'* (herpes zoster) (Teklehaymanot et al., 2007) coughs, headaches, and abdominal pains. For various human ailments, different parts of the medicinal plants with various modes of preparation are employed.

Though this should be interpreted cautiously, the results of the present study indicate that some medicinal plants selected for breast cancer treatment by TM practitioners may not have the sought efficacy. For instance, the species *Z. chalybeum* is one of the frequently cited medicinal plants from different part of Ethiopia and it is also identified as a choice of traditional healers during the in situ investigation (Regassa, 2013; Kewessa et al., 2015; Tuasha et al., 2018a). In cell culture, the MeOH extract was not cytotoxic up to the maximum concentration of 2 µg/ml, although it is possible that its metabolites may exert toxicity in the traditional medical practices. In the used cell culture systems there is no biotransformation. On the other hand, the species S. oxyacanthum (IC50 as low as 0.09 µg/ml in MCF-7 cells of crude methanol extract) was not widely reported across the country and yet selected based upon the recommendation of TM practitioners during the in situ investigation (Tuasha et al., 2018a). Also, the results reported underline the need to consider many factors during the selection phase of the medicinal plants for experimental investigation.

The traditional healers, use fresh plant material for the remedy preparation because of the perception that the freshly collected material is more efficacious than material in dried forms. They use water as the main solvent for the extraction. The amount of the plant material initially collected is not consistent across the traditional healers and the amount used for extraction in water varies considerably. Nevertheless, practitioners consider different factors before deciding the dose given to the patient (Teklay et al., 2013). The water content is usually high for harvested medicinal plant materials and this is in agreement with the present study where as high as 61.3% water content was recorded for *C. myricoides* (Poós and Varju, 2017). There is variation in the water content between different plants and also between different parts of a plant. This implies that for medicinal plants with high water content, a relatively large amount of the plant material has to be harvested. Collection of large amounts of plant materials, especially the root and the bark of a plant, poses unequivocal threat to the sustainability of the medicinal plants and thus needs conservational utilization (Teklay et al., 2013).

In our study, the cytotoxicity of the fractions was not directly related with the yield of the extract. For instance, though the material obtained from the aqueous phase of *C. simensis* was the most abundant, the most cytotoxic fraction was the chloroform fraction indicating that the activity would be related to biological constituent(s) soluble in less polar solvents. In the case of *S. oxyacanthum*, however, disproportionately large quantity yield is obtained in the aqueous fraction which is also the most cytotoxic fraction. In this case, the activity in the aqueous phase is very closely related with the traditional medicine preparation. This finding supports the claim of traditional healers that the plant *S. oxyacanthum* is considered to be the most effective remedy. Generally, since the crude extraction mimicked the way traditional remedies were prepared, it is justifiable that bioactive fractions are contained in their preparations.

Based on the solvent-solvent fractionation, the ethyl acetate fraction of V. leopoldi was found to be the most

cytotoxic fraction across the cell lines used (JIMT-1 = 0.87 µg/ml, MCF-7 = 3.5 µg/ml and MCF-10A = 1.72 µg/ml). To our knowledge, this is the first in vitro cytotoxicity report of its kind for the solvent fractions of *V. leopoldi*. A number of studies with other species from the genera reported cytotoxicity at varying concentrations. For instance, the crude MeOH extract of the stem of *V. divaricata* yielded the IC50 values of 10.1, 12.6, and 9.9 µg/ml in HL-60, MCF-7, and PC-3 cells, respectively (Lowe et al., 2014). In addition, dose- and time-dependent cytotoxicity (ranging 9-26 mg/ml) against various cancer cells was reported for the extracts of V. condensate (Thomas et al., 2016). *Vernonia cinerea* was also reported to show potent cytotoxicity against colon adenocarcinoma cells (HT29) and hepatoma cells (HepG2) (Khay et al., 2012). The acetone extract of V. guineensis was also reported to show high toxicity against ten different cell lines with the IC50 values ranging 4-26 mg/mL (Toyang et al., 2013). These findings indicate the potential of the plants in the genus Vernonia to have anticancer activities. Therefore, our report of cytotoxicity at low concentrations suggests the use of this species as a source for the isolation of defined compounds for investigation of bioactivity against cancer cells.

The genus Clematis has received large interest in conventional medicine and investigations on different species have revealed various biological activities (Hao et al., 2013; Kırmızıbekmez et al., 2018). We here report the chloroform fraction of *C. simensis* to be cytotoxic against all the breast cancer cell lines tested. Literature sources show that the genus Clematis is rich in triterpene saponins, alkaloids, flavonoids, lignans, steroids, coumarins, macrocyclic compounds, phenolic glycosides, anemonin, and volatile oils as major classes of chemical constituents (Sun and Yang, 2009; Chawla et al., 2012). Phytochemistry of the chloroform fraction within the genus was reported to be rich in carbohydrates and flavonoids and thus the cytotoxic activities of this fraction could be related to these chemical constituents (Karimi et al., 2017).

Generally, the crude methanol extracts showed higher toxicity than the individual fractions since it contains all the cytotoxic components. This is in line with previous reports where cytotoxicity of crude extracts and fractions have been evaluated (Rasoanaivo et al., 2011; Tantengco and Jacinto, 2015; Mtunzi et al., 2017).

Conclusion

According to the present study, the crude extracts/ fractions of some traditionally used medicinal plants have shown desired cytotoxic effects against various breast cancer-derived cell lines. The ethyl acetate fraction of *V. leopoldi* was found to be the most cytotoxic of those tested. In addition, the aqueous fraction of *S. oxyacanthum* and the chloroform fraction of *C. simensis* were found to be highly cytotoxic at low concentrations against all the cell lines used. The slightly higher IC_{50} values found for MCF-10A cells (especially in comparison to JIMT-1 cells) should be exploited in further studies. One of the limitations of the present study is that we were unable to exactly relate the cytotoxicity findings to the use of these plants in TM, since it is not possible to obtain accurate information of how much is used in the preparation of remedies by traditional healers. However, the fact that toxicity was found at low concentrations of some fractions, suggests cautionary TM dosage and guides isolation and characterization of biologically active compounds.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of the College of Natural and Computational Science (CNS-IRB), Addis Ababa University (IRB/022/2016). Additionally, Armauer Hansen Research Institute/All Africa Leprosy Rehabilitation and Training Hospital (AHRI/ALERT) Ethics Review Committee granted an approval (Project Registration №: PO19/16). The material transfer agreement (MTA) was granted from the Ethiopian Biodiversity Institute (EBI) to ship the genetic material and conduct scientific research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

DMSO, Dimethyl sulfoxide; **FBS,** fetal bovine serum; **IC_{50},** half maximal inhibitory concentration 50%; **MTT,** 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; **PDT,** population doubling time; **TM,** traditional medicine.

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Ethnobotanical survey and phytogeographical study of plants species from genus *Acacia* in Bénin

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The genus *Acacia*, mainly distributed in the tropical and subtropical regions, has been used in traditional medicines for the treatment of microbial infections, malaria, diarrhea, oedema and inflammation. The present study aims to provide a comprehensive data on the distribution and medicinal use of *Acacia* species. Ethnobotanical survey and phytogeographical study were undertook using field interviews. Informant consensus factor (ICF), frequency of citation (Fc), fidelity level (FL) and use value (UV) were also assessed. A total of 16 species belonging to genus *Acacia* were inventoried in Benin. A total of 108 informants were interviewed during ethnobotanical survey. Except *Acacia auriculiformis*, all inventoried species cited by informants were traditionally used to treat various ailments. This species were mostly used as medicinal treatments (93.75 % of the species) and to produce wood and fibers (6.25 %). Leaves, bark and roots were the most used parts and decoction was the most cited method of preparation. *A. macrostachya* (UV = 1.94), *A. nilotica* (UV = 1.21), *A. hockii* (UV = 1.20) and *A. ataxacantha* (UV = 1.17) and *A. sieberiana* (UV = 1.15) were the most used species. *A. nilotica* (75%), *A. dudgeon*ii (67%) and *A. seyal* (50%). *A. nilotica* (FL = 75%) and *A. dudgeonii* (FL = 67%) were the most species cited by informants to treat Digestive System Disorder whereas *A. Hockii* (67%), *A. Senegal* (67%), *A. ataxacantha* (60%), *A. erythrocalyx* (50%) and *A. gerrardii* (50%) were cited for Infectious diseases. The extensive literature survey reveals 16 *Acacia* species distributed in 10 phytogeographic districts in Bénin. These species where mostly used in traditional medicine to treat infectious diseases and Digestive System Disorder. The results of this study open new research perspectives on *Acacia* species not yet studied.

Key words: *Acacia*, ethnobotany, phytogeography, medicinal plants.

INTRODUCTION

Herbal remedies used in traditional medicine are the primary health care resource in many rural communities around the world. In low- and middle-income countries where the number of practitioners of modern medicine may not be enough to meet the health care needs of the country, traditional medicine and its practitioners are

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considered an important resource for population health (Oyinlola et al., 2016).

Traditional medicine is defined as the sum of all knowledge, skills and practices based on the theories, beliefs and experiences of different cultures, whether explicable or not, and which are used in the preservation of health, as well as in the prevention, diagnosis, improvement or treatment of physical or mental illnesses (WHO, 2013). Contrary to traditional medicine, the modern medicine which is defined as a medical system based on western scientific principles, often perceived as a symbol of modernity, development and globalization in the non-Western world, alters care-seeking behaviour and often dismiss traditional medicines (Teixidor-Toneu et al., 2017). However, traditional medicine continues to be used because of people beliefs about the intrinsic efficacy and effectiveness of traditional medicines for many illnesses, the inadequacy of the modern health system, the unavailability of medicines, unaffordable medical bills, the cheap cost, the distance to public health center or a combination of these factors (Mwaka et al., 2015; Thomas, 2013).

The West African savannahs and especially those of Bénin are populated by species belonging to several botanical families including Fabaceae which gathers several genus such as acacia. Several studies reported that *Acacia* species play an important role in the rural health system. They are traditionally used for the treatment of febrile convulsions, tooth decay, bronchitis , cough, dysentery, pneumonia, pneumonia, malaria, primary infection of syphilis, sterility and stomach-ache, cold, congestion, fever, hemorrhoids, leucorrhoea, intestinal pains and acute diarrhea (Amoussa et al., 2014; Kabbashi et al., 2016).

Previous study on *Acacia ataxacantha* in 2013 showed that acacias species are subject to confusion in the market because of their large morphological similarity. The sellers of medicinal plants do not always distinguish these species which are all called "acacia" in the markets. This confusion among *Acacia* species may be one of the reasons for the lack of research data on some *Acacia species*. Indeed, several challenges need to be addressed for the successful implementation of medicinal plant research on *Acacia* species. Thus, this study was undertaken to document the distribution, identification and traditional use of *Acacia* species. Specifically, *Acacia* species were listed, phytogeographic districts of each species were identified and an ethnobotanical survey was conducted to collect data on their vernacular names, their traditional use and the diseases for which they are used.

METHODOLOGY

Study area and demography

The study area covered 77 town belonging to the 12 departments, situated in West Africa between latitudes 6°15' N - 12°25' North and longitudes 0°40 E - 3°45 East (Akoègninou et al., 2006). It covers a total land area of 112.622 km^2 with a population estimated to about 10 million (INSAE, 2013). The profile of the country is an undulating plateau except for a few scattered hills in the center and the north. The altitude varies from sea level to 400-650 m in the northwest, where the Atacora mountain chain is the outstanding feature and a region of great ecological and species diversity in the country (Agbani et al., 2018; Adéoti et al., 2009). The mean annual rainfall varies from 900 to 1300 mm. The mean annual temperature ranges from 26 to 28°C and can exceptionally reach 35 to 40^{o}C in some northern localities (Adéoti et al., 2009).

Phytogeographical study of *Acacia* species in Bénin

In order to provide a better knowledge, to list *Acacia* species in Bénin and identify their phytogeographical district, exhaustive review of literature (Akouehou et al., 2011; Arbonnier, 2000, Akoègninou et al., 2006 ; Alexiades and Sheldon, 1996) available at the National Herbarium of University of Abomey-Calavi were consulted. The major environmental factors such as soil types, plant formation and climate were recorded.

Ethnobotanical survey and consent

Ethnobotanical surveys were conducted from March to august 2015 using field interviews. To collect data on traditional uses of *Acacia* species in study area, a questionnaire was developed to facilitate interviews with informants. They were between the ages of 17 to 72, with the average age of 45. Local dialect such as Dendi, Ditammari, Yoruba, Nagot and Fon were used to conduct interviews. In each area of survey, a local assistant was recruited to facilitate the conversation and avoid any misunderstanding during interview. Demographic data of informants such as gender, age, occupation and education level were documented. The local names of species, medicinal and parts used, mode of preparation and administration and the availability in the area were noted.

Plant collection

Specimens of plants reported by informants were collected immediately with the help of informants or their assistants. At least, the specimens were deposited at National Herbarium of Abomey-Calavi University (Bénin) where the botanical identification was done.

Data processing

A list of species cited by informant was established in Excel. The local and scientific names, family names, ailments treated, part used, preparation and administration mode, and areas in which species grow were also recorded. In order to identify the first disease traditionally treated by *Acacia* species, all listed disease during survey were classified into 13 categories according to Camara-Leret et al. (2012).

Quantitative data analysis

Frequency of citation (Fc)

The most important species in a study area was identified by calculating the frequency of citation (Fc) (Ahmad et al., 2014). Which is the ratio between the number of informants who mentioned a specie and the total number of informants (Tardio and Santayana, 2008).

Factor of informant consensus

Agreement among informants about specie for a particular remedy was determined by calculating the informant consensus factor (ICF) and fidelity level (FL). In this study, the informant consensus factor (ICF) was used to determine the level of similarity among information delivered by various informants. FIC is also explained as the importance of each medicinal plant use category depending on the homogeneity of informant's answer (Trotter and Logan, 1986). The FIC was calculated using the following formula (Heinrich et al., 1998):

$$FIC \frac{Nur - Nt}{Nur - 1}$$

Where Nur = number of use reports for a specific category; Nt = number of species used for the disease category. This factor range from 0 to 1. A higher value of ICF (close to 1) indicates a greater consensus on the use of a given plants to treat a particular ailment category. A low value of ICF (close to 0) indicates that the informants disagree with the category of use of a plant (Andrade-Cetto and Heinrich, 2011).

Fidelity level (FL)

The fidelity level (FL) was also calculated as a tool to get the percentage of informants claiming the use of a certain plant for the same major purpose. It is defined as the ratio between the number of informants who independently claimed a use of a plant species to treat a particular disease (Np) and the total number of informants who mentioned the plants as a medicine to treat any given disease (N) (Friedman et al., 1986):

$$FL = \frac{Np}{N \times 100}$$

Plant species with high fidelity level is important to local people to treat ailments. It is noted that the number of times mentioned for a given plant by all of the informants for a specific disease was considered for this factor.

Use value (UV)

The most important medicinal uses of plants were assessed by calculating the use value (UV) which was used to calculate the citation of plants during interviews (De Albuquerque et al., 2009).

$$UV = \frac{\sum Uis}{ns}$$

ΣUis is the sum of the total number of use citations by all informants for a species; ns is the total number of informants.

RESULTS

Social status of informants

A total of 108 informants composed of sixty men (55.6%) and forty-eight women (44.4%) were interviewed during the study. They were aged between 17 and 72 years. Eighty three were above 40 year old and twenty five were under 40. Informants have a low education level, 82% have not been to school or have primary education while 18% reached secondary school. Belonging to several ethnic speaking Ditammari, Dendi and Fon, informants were spiritual healers, traditional midwives or traditional healers. In addition to traditional medicine, most of informants are farmers or breeders.

Phytogeographic study of *Acacia* species in Bénin

The bibliographic review carried out on species from genus *Acacia* in Bénin, allowed to list sixteen species distributed in all phytogeographic districts. Climate and soil types appeared as the main determinants of phytogeographic districts in which species are identified. The lists of species are presented in Figure 1. The *Acacia* species were mainly distributed in three phytogeographic districts. In the "Ouémé-Valley" district characterized by Hydromorphic soil in southern Benin, 7 species have been identified. In Northern Bénin, two districts, "Borgou-North" and "Mekrou-Pendjari" characterized by ferruginous soil, eight and ten species of *Acacia* were respectively identified. The distribution of species, the types of soil, the major plants formation and exclusive species of each phytogeographical district were documented and summarized in Table 1.

Description and geographical distribution of *Acacia* species in Bénin

Sixteen species from genus *Acacia* were listed in Bénin after bibliographic review (Arbonnier, 2000, Akoègninou et al., 2006; Alexiades and Sheldon, 1996). The description of each *Acacia* species is summarized in Table 2 and the geographical distribution is presented in Figure 2.

Ethnomedicinal data of *Acacia* species

The ethnobotanical survey allowed collecting 117 presumed species from *Acacia* genus. The collected specimen deposited to the National Herbarium of the University of Abomey-Calavi in Bénin allowed identifying 108 specimens grouped into sixteen species (*A. auriculiformis, A. ataxacantha, A. erythrocalyx, A. farnesiana, A. macrostachya, A. nilotica, A. polyacantha, A. ehrenbergiana, A. sieberiana, A. dudgeonii, A. amythethophylla, A. gerrardii, A. hockii, A. senegal, A. seyal, A. gourmaensis*) belonging to genus *Acacia* (Fabaceae) and nine specimens not belonging to the *Genus* acacia. The traditional used of collected species, vernacular name and parts used were summarized in Table 3.

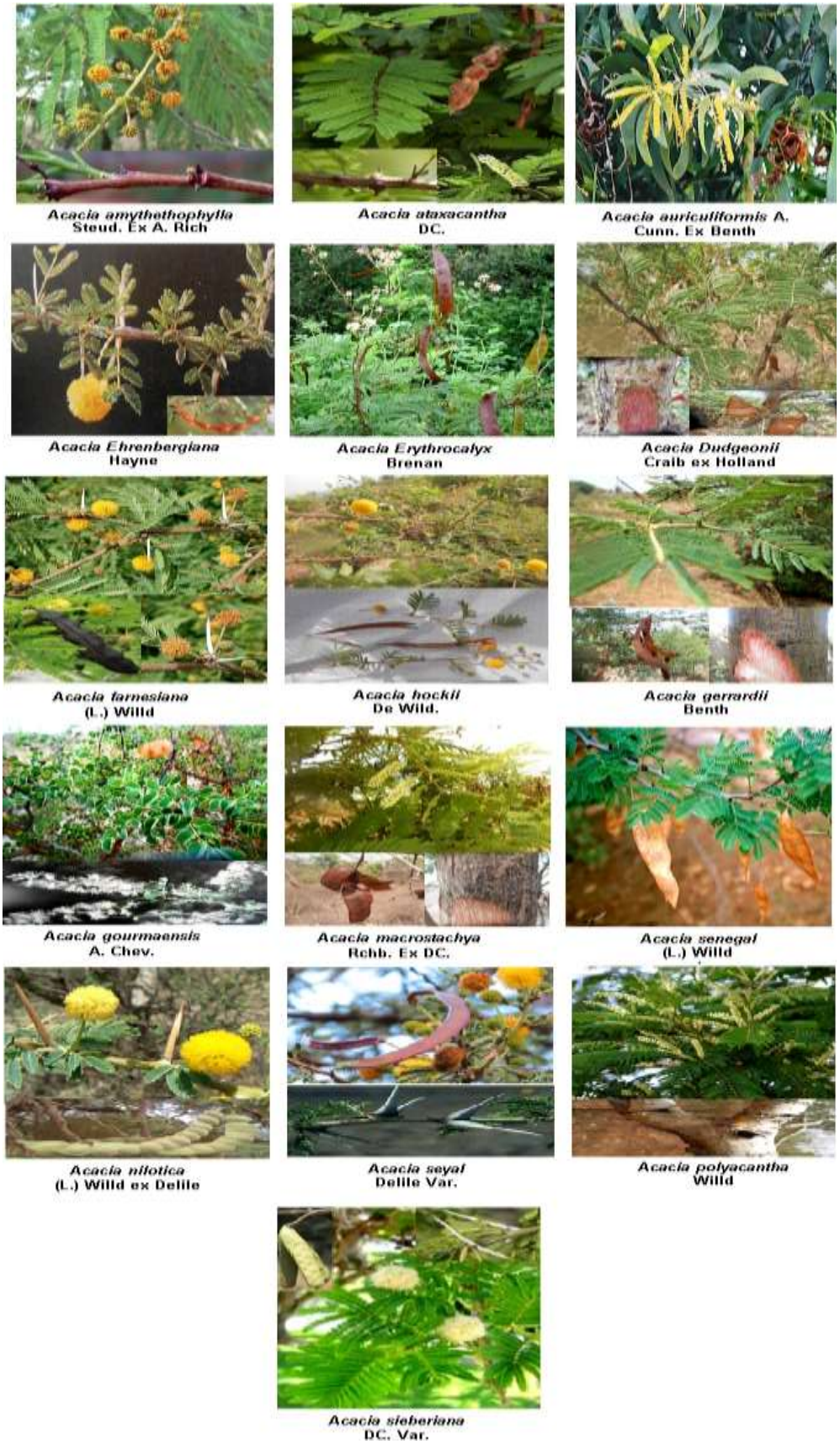

Figure 1. Pictorial description of *Acacia* species inventoried in Bénin.

Table 1. Phytochorological zones based on major soil types, major plant formation and species exclusive.

Phytogeographic districts	Species exclusive to the phytogeographical district	Major plant formation	Major soil type
Coast	*A. auriculiformis*	Coastal forest and derived thickets, Mangrove	Sandy + Hydromorphic and halomorphic soils
Pobè	None found	Semi-deciduous forest	Ferrallitic soils and without concretions
Ouémé-Valley	*A. auriculiformis, A. ataxacantha, A. erythrocalyx, A. farnesiana, A. macrostachya, A. nilotica, A. polyacantha*	Swamp and semi-deciduous forest	Hydromorphic soils
Kouffo	*A. ataxacantha, A. polyacantha, A. sieberiana*	Semi-deciduous forest	Ferrallitic soils with, vertisoils
Zou	*A. macrostachya, A. sieberiana*	Dry forest, woodland, and riparian forest	Ferruginous soils on and crystalline rocks
Borgou-South	*A. gourmaensis, A. hockii, A. macrostachya*	*A. gourmaensis, A. hockii, A. macrostachya*	Ferruginous soils on and crystalline rocks
Borgou-North	*A. ataxacantha, A. ehrenbergiana, A. erythrocalyx, A. macrostachya, A. nilotica, A. polyacantha, A. sieberiana, A. dudgeonii*	Dry forest, woodland, and riparian forest	Ferruginous soils and crystalline rocks
Atacora Chain	*A. farnesiana, A. gourmaensis, A. hockii, A. seyal*	Riparian forest, dry forest, and woodland	Poorly evolved and mineral soils
Mékrou-Pendjari	*A. amythethophylla, A. ehrenbergiana, A. erythrocalyx, A. dudgeonii, A. gerrardii, A. nilotica, A. polyacantha, A. hockii, A. senegal, A. seyal*	Tree and shrub savannahs, dry forest and riparian forest	Ferruginous soils, with concretions and sedimentary rocks
W du Niger	*A. macrostachya, A. nilotica, A. sieberiana*	Tree and shrub savannahs, dry forest and riparian forest	Ferruginous soils, with concretions and sedimentary rocks

Table 2. Botanical description of *Acacia* species inventoried in Bénin.

Species	Height	Leaves	Flowers	Bark	Pods	Spines	Efflorescence
Acacia amythethophylla Steud. ex. A. Rich.	Tree up to 15 m, fairly branched	20 pairs of pinnae each with 36-48 leaflets	Yellow, drying red, 8-13 mm in diameter	Rough, scaly	Pods ± oblong, about 11.5 x 1.7 cm	Stipular, 2 per node, 3-10 mm	Dry season
Acacia ataxacantha DC.	Climbing shrub or liana	5-12 pairs of pinnae each with 30-40 leaflets	4-8 cm long, dense, subsessile in axillary white spikes	Yellow-brown	6 - 7 seeds thin, flattened, 6-10 cm long, purplish brown mature	Curved spines	Flowers in august - November, fruits in October- November
Acacia auriculiformis A. Cunn. ex Benth.	False phyllodes tree, 15 - 30 m	Dense foliage open, spreading crown, 10-16 and 1.5-2.5 cm	8 cm long, creamy yellow and sweet scented.	Vertically fissured	Flat (6.5 x 1.5 cm), Initial : straight Mature : twisted irregular spirals	None	flowering occurs June – July; pods ripen august - October

Table 2. Contd.

Acacia dudgeonii Craib. ex Holl.	Spiny shrub, 2.5- 7 m height	3-7 cm, 20-30 pairs of leaflets with 20 pairs of pinnae	White, 2.5-6.0 cm long, shorter than the leaves	Fissured, flaking	Oblong, flattened, glabrous, 3.0-8.0 x 1.5-2.5 cm, pale brown	Stipular, 2 per node, 3-9 mm	Flowers (March to June), fruits (July to September)
Acacia ehrenbergiana Hayne	Branched shrub up to 6 m	1-2 pairs of pinnae, 6-12 pairs of leaflets	Yellow with 1.0 to 1.5 cm in diameter	Brown, shiny, peeling	Sickle pods, 12 x 0.5 cm approx. red-brown in maturity.	Stipular, 1 cm, sometimes 6 cm	Dry season, after the rainy season
Acacia erythrocalyx Brenan	Creeping shrub up to 3 m	7-15 pairs of pinnules with 17-38 pairs of leaflets	White, globular, 9 mm in diameter approx	Smooth, brownish-grey, with papery scales	Flattened, 9-15 x 1.3-5 cm, glabrous, brown-red, seeds (10-12)	Spines non-stipular, curved, 1-5 mm	Flowers in August, fruits in November, December
Acacia farnesiana (L.) Willd.	Thorny shrub 1.5-4 m	4-8 pairs of pinnae, 10-12 leaflets	Yellow; fragrant; 1-1,5 cm in diameter	Brown, black, scaly at maturity	Pods ± spindle-shaped, 5-8 x 0.5-0.8 cm with 7-8 seeds	Stipular thorns straight and slender	Flowers août, October, November
Acacia hockii De Wild.	Small tree, 5 m approx	6-8 pairs of pinnules with 15-30 leaflets	Globular, 2 cm diameter approx., yellow-orange	orange-red, dark spots, often scaly	Linear, falcate, puberulous, 5-7.5x0.5 cm	Stipular spines 2 per node, 1-3 cm approx	Dry season beginning
Acacia gerrardii Benth	Tree or shrub 3-15 m height	5–10 pairs of pinnae; 12-28 leaflets	Cream-white heads of 7-12 mm diameter	Cloves falcates (curved), ± 7-16 cm.	Flattened, curved or rarely straight	Paired, mostly straight, 1.5 cm, sometime long and/or recurve	Flowers and pods November
Acacia gourmaensis A. Chev.	Shrub or small tree up to 4.5 m	3-5 pinnules to 1 pair of leaflets	Inflorescence in loose ears 4 cm long	Cracked or scaly, orange-brown	Pods ± oval, 3.7 x 1.5 cm approx. with 1-3 seeds	Non-stipulated spurs. 2 per node	Rain season
Acacia macrostachya Reichenb. ex DC.	Shrub or small tree up to 4.5 m	17-27 pairs of pinnules with 21-56 pairs of leaflets	Cream-colored with 5-10	Cracked brown-red	Flat, ± oval, 9.3 x 2.3 cm, at 2-5 seeds	Shaped curved claws, red-brown	During the first rains and in the dry season after foliage
Acacia nilotica (L.) Willd. ex Delile	Small tree from 2.5 to 14 m high	2-11 pairs; leaflets 7-25 pairs, 1.5-7 mm	Bright yellow, in axillary heads 6-15 mm in diam	Thin, rough, cracked, dark red-brown	Pods especially variable, linear, indehiscent, 8-17 cm	Gray-pubescent, slightly curved, 3 cm long	Flower (october-december); fruit (march-june)

Ailments treated with *Acacia* species

Acacia species cited by informants were used to treat 61 various ailments classified in 13 categories such as: Blood and cardio-vascular problem (BCVP), cranial system (CS), dental health (DH), digestive system disorder (DSD), general ailments (GA), infectious diseases (ID), muscular skeletal system (MSS), nervous

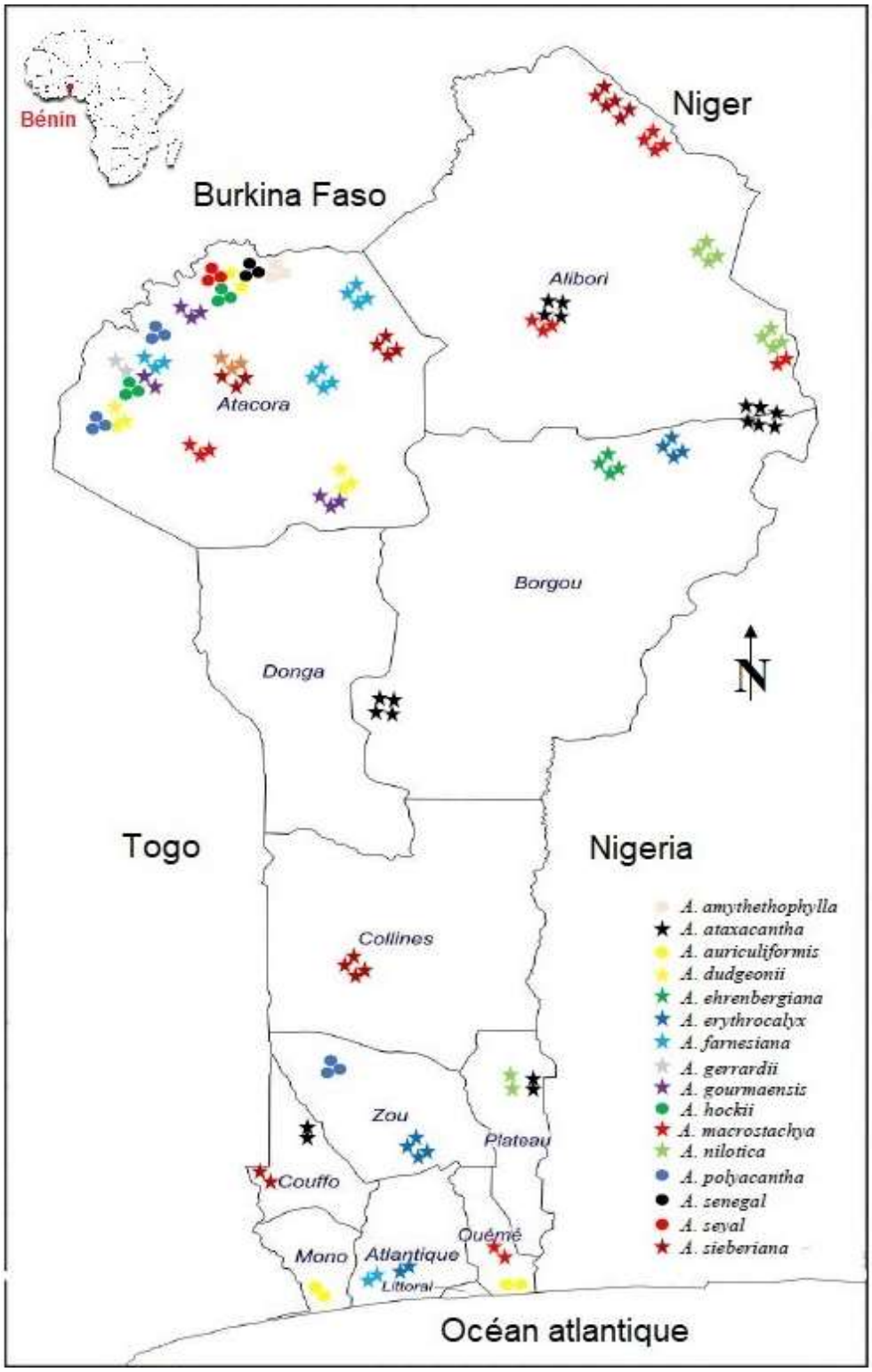

Figure 2. Phytogeographical distribution of species from genus *Acacia* in Bénin.

system (NS), pregnancy, birth and puerperium (PBP), reproductive system (ReprS), respiratory system (RespS), sensory system (SS) and veterinary (Vet). Frequencies of citation of disease categories range from 0.93 to 37.96%. ID (37.96%) and DSD (35.18%) were the most cited category (Table 4). BCVP, CS, DH, GA, MSS, NS and SS

Table 3. Name, traditional uses of *Acacia species* in Bénin.

Botanical name	Voucher number	Vernacular name	Part(s) and traditional used	UV
A. amythethophylla Steud. ex A. Rich	YH 284/HNB	Acacia	Entire plant extract drunk to treat diarrhea, wounds, cutaneous infections; root bark extract drunk as uterine sedative, psychosis	0.97
A. *ataxacantha* DC.	YH290/HNB	vèwunkan (f, g); pofi (g); èwun, èwunagogo, èwunadele, èlèèwon (y, n); gairi (ba); Buisson de rocher (fr), moraré (pl)	Dough of the leaves used as abscess medicine; leaves decoction: used as convulsions medicine, drunk to treat dysentery, headache, infections and pneumonias	1.17
A. auriculiformis A.Cunn. ex Benth	YH291/HNB	None found	None found	-
A. Dudgeonii Craib ex Holland	YH 292/ HNB	Pattuki yanorgo (pl)	Whole plant extract drunk as antiseptic; root is used to treat the snakebite; peels extract drunk as dysenteries and enteritis medicine	0.71
A. Ehrenbergiana Hayne	**	Bakanichili, Djilukii (pl), Karamnaga (pl)	Peel: anti-inflammatory, flatulences and diuretic medicine properties; Pods taken as emollient; Leaves and fruits are also edible; wood used as paralysis problems	0.49
A. Erythrocalyx Brenan	YH 293/ HNB	Olusoèlèèwon, èwon, èwan, èwanadele (y, n).	Entire plant used as snakebite medicine; roots are taken as aphrodisiac; leaves: as emetics and febrifuges, angina, injury and dermatose medicine (Arbonier, 2000).	1.04
A. farnesiana (L.) Willd	YH 294/ HNB	boni (y); ban (ba)	Whole plant used to treat diarrhea, liver problems and inflammation	0.81
A. hockii De Wild.	AA 6615/HNB	Dandanechi (pl)	Leaves: Used to treat malaria and abscess; Root: taken as gastritises and hookworm problems; Plant taken as emollient in north Africa	1.20
A. gerrardii Benth.	YH 285/ HNB	Gonponyalehi (pl)	Peel decoction drunk as diarrhea, emetic medicine, vomitive, cough, and asthma medicine. Root and Leaves for stomach ache. Root: as bilharzia (schistomiasis) medicine and pain	0.87
A. gourmaensis A. Chev.	AA6614/ HNB	Koukounkoumbou (ba), PƆN yara (So).	Root is used against the cough; peels are used as icterus, malaria and renal affection and purgative medicine	0.46
A. macrostachya Rchb. ex DC.	AA 6616/HNB	Onaré, Tschildi pl)	Root used as gonorrhea^{+} and syphilis; Peels as aphrodisiac, gastritis, disinfecting, anthelmintic; to treat gastro-intestinal, diarrhea, vomiting, cholera, flatulences, tooth decay and gingivitis; Leaves are used as angina and antidote of snakebite	1.94
A. nilotica (L.) Willd. ex	YH 295/ HNB	Gabaruwa (wa); bani (ba); kaara(So)	Whole plant: to treat digestive candidiasis; pods and seeds: to treat hemorrhoid, diarrhea, cough, vomiting, stomach aches, dysenteric and scorbutic. In Mali whole plant is taken as gastric ulcer, oral wounds, rates and amoebic.	1.21

Table 3. Contd.

A. nilotica (L.) Willd. ex	YH 295/ HNB	Gabaruwa (wa); bani (ba); kaara(So)	Whole plant: To treat digestive candidiasis; pods and seeds: to treat hemorrhoid, diarrhea, cough, vomiting, stomach aches, dysenteric and scorbutic. In Mali whole plant is taken as gastric ulcer, oral wounds, rates and amoebic	1.21
A. polyacantha Willd.	AA6617/ HNB	Hilikan, dènwi (t); dégà (g); èdè, ègè-èdè (y, n); gaja (a); bokosaka ba); maarukokobè (d).	Plant: used as unrests gastrointestinal and astringent. Root: used as œdema, tooth decay, asthma, gonorrhea, malaria. Peel: used as œdema, hemorrhoids, syphilis; Peel, pods and leaves are taken as dysentery medicine; Pods: as sore throats	1.07
A. senegal (L.) Willd	YH 296/ HNB	Gommier blanc (fr)	Plant is applied as pains of heart in Niger. In Mali, leaves: as the bilharzia, abdominal pains and sore throats; eraser: as pain of chest and otitis; root: as wounds	0.63
A. seyal Delile var.	YH 297/ HNB	menèn (d), puwituani (So);	Plant is used as peritonitis, stomach aches and œdema medicine; peel to treat dysentery, bacterial infections, leprosy and rheumatism	0.65
A. sieberiana DC. var.	YH 298/ HNB	kukumbu, sagunu kpika, sakiburo kpika, lepusia (ba) ; sihe (y, n); Konkompieli, aduwè, caga (f).	Peels of root and leaves are used to treat snakebite, osteoarthritises and rheumatism. Stem leafed against hyperyretique convulsions or infantile, syncopates, tetanus, œdema and sickle cell disease	1.15

Bariba (ba), Dendi (d), Fon (f), Nago (n), Somba (So), Yoruba (y), Waama (wa), Peul (pl) plants in Red list (**).

Table 4. Frequency and informant consensus factor of each category of ailments

Category of ailments (list of diseases)	Number of plant cited	Number of informants citing the category	Frequency of citation (%)	Informant consensus factor (IFC)
Blood and Cardio-Vascular System (BCVS): Cardiac problems in children, low blood pressure	2	2	1.85	0
Cranial System (CS): Early and late closing of baby's fontanel	1	1	0.93	0
Dental Health (DH): Caries, causes teeth nerves insensitivity, dental abscess syndesmotome	2	4	3.70	0.67
Digestive System Disorder (DSD): Carminative, colic, diarrhea, constipation, anti-emetic, indigestion, liver disorders, intoxication from meat eating, laryngitis, gastric ulcer, intestinal ulcer, orexigenic after diarrhea, intestinal pain, dysentery	14	38	35.18	0.65
General ailments (GA): Weakness, headache, fever, side stitch, yellow fever	2	2	1.85	0
Infectious Diseases (ID): Malaria, measles, scabies, tetanus, infected and syphilitic wounds, bilharzia	15	41	37.96	0.65
Muscular-Skeletal System (MSS): Twists, fractures, low back pain, muscle aches, sprains, broken member	4	5	4.63	0.25

Table 4. Contd.

Nervous System (NS): Calming nerves, epilepsy, nerves swelling	1	2	1.85	1
Pregnancy, Birth and Puerperium (PBP): Menstrual pain, contraception, infertility treatment, pain and dizziness during pregnancy, prenatal care, induce labor, post partum recovery, healing wound after delivery, post partum hemorrhage, remove rest of placenta in uterus, promote lactation	0	0	0.00	0
Reproductive System (ReprS): Painful menstruation, sexually transmitted diseases (syphilis and gonorrhea), aphrodisiacs, contraceptive	5	7	5.55	0.32
Respiratory System (RespS): Flu, cold, bronchitis, asthma, pulmonary infection, bronchitis, cough	3	7	5.55	0.67
Sensory System (SS): Eye infections, conjunctivitis, mouth infection, boils, eye stye	2	3	2.77	0.50
Veterinary (Vet): Treatment of cattle's diseases	0	0	0.00	0

were the least mentioned by informants, with less than 5% of citation (Figure 3A). The ICF values ranged from 0 to 0.67. High consensus was obtained for DSD, ID, DH and RS. The highest ICF value (0.67) was obtained with RS and DH categories treatment followed by species used to treat DSD and ID (0.65). However, Informants Consensus factor was low (ICF < 0.50) for plants used as remedies for RS and MSS. Frequency and Informant Consensus Factor of each category of illness were summarized in Table 4.

Part used and mode of preparation

Medicinal plants in the study area were prepared in many different ways depending on the species, part used and the ailments treated. Parts of the plants mainly used for the preparation of herbal remedies were leaves (24%), followed by bark (22%), roots (21%), whole plant (19%) and fruits (14%) (Figure 3B). Decoction (56%) was the most common process of preparation. The species were also dried, powdered (22%) and used directly in food. Sometimes the fresh part of plant was chewed (20%) or used as cold infusion (2%) (Figure 3C).

Use value of *Acacia* species

In this study, use values (UV) have demonstrated the importance of medicinal uses of *Acacia* species in Benin. UV values ranged from 0.46 to 1.94 (Table 3). *A. macrostachya* (UV = 1.94), A*. nilotica* (UV = 1.21), *A. hockii* (UV = 1.20), *A. ataxacantha* (UV = 1.17) and *A. sieberiana* (UV = 1.15) were the most used species. *A. gourmaensis* have the lowest Use Value (UV = 0.46) and *A. auriculiformis* was not cited by informants.

Fidelity level (FL) of *Acacia* species

The fidelity level index (FL) is used to identify the most commonly species used by populations for the treatment of a certain diseases. The most used species have a maximum FL. In the present study, the fidelity level varied from 6 to 75% in all categories of ailments. The highest value of FL was obtained for *A. nilotica* (75 %), *A. dudgeon*ii (67 %) and *A. seyal* (50 %) used to treat DSD. In the treatment of ID, the most cited species included *A. Hockii* (67%), *A. Senegal (*67%), *A. ataxacantha (*60%), *A. erythrocalyx* (50%) and *A. gerrardii* (50%). Indeed, five species had the highest fidelity level (≥ 50%) for ID and three species (≥ 50%) for DSD.

DISCUSSION

Many species of plants are used in traditional medicine in several forms by the populations for the management of diseases. In previous studies on *Acacia ataxacantha*, it was found that several species have the *Acacia* name in markets and these species are not always differentiated by

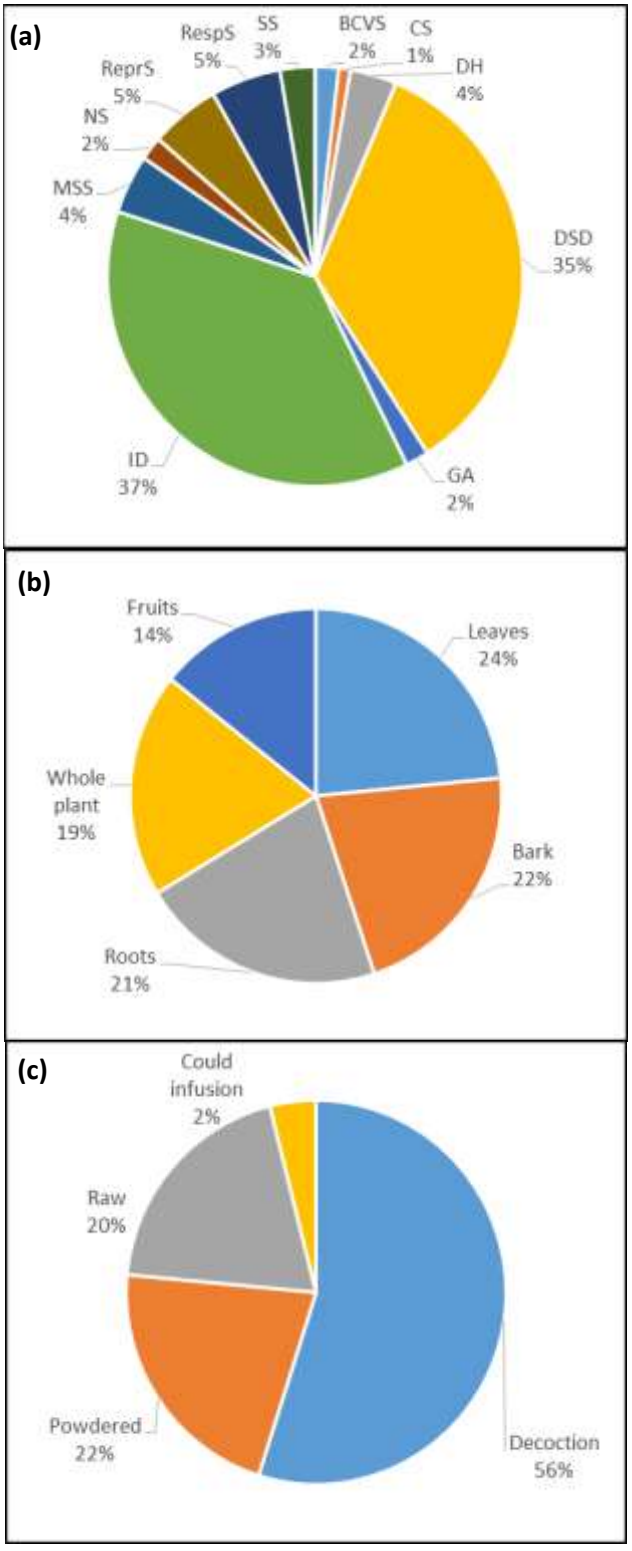

Figure 3. Ailments treated (**A**), parts used (**B**) and preparation methods (**C**). Blood and Cardio-Vascular System (BCVS), Cranial System (CS), Dental Health (DH), Digestive System Disorder (DSD), General ailments (GA), Infectious Diseases (ID), Muscular-Skeletal System (MSS), Nervous System (NS), Pregnancy, Birth and Puerperium (PBP), Reproductive System (ReprS), Respiratory System (RespS), Sensory System (SS), Veterinary (Vet).

sellers of medicinal plants. Indeed, the botanical description of some species of the *Acacia* genus is very close. This leads to this confusion in the markets. The objective of this study was to identify the species of *Acacia* listed in Benin in the literature, and carry out a phytogeographical study as well as an ethnobotanical survey on the medicinal use of different species from genus acacia.

In the present study, a total of sixteen (16) *Acacia* species belonging to the family of Fabaceae were reported in Benin. Except *Acacia auriculiformis*, all inventoried species were used in traditional medicine. These results show that almost all species from genus *Acacia* are used in traditional medicine and most ailments treated were ID and DSD. All over the world, the use of *Acacia* species was also reported in traditional medicine (Zahoor et al., 2017; Kefalew, 2015; Tahani et al., 2018; Teklehaymanot, 2017). Among these species, eight (8) are essentially distributed in the Sudanese region (Northern Benin): *A. amythetophylla*, *A. dudgeoni, A. ehrenbergiana*, *A. gerrardii, A. gourmaensis, A. hockii, A. senegal* and *A. seyal;* one (1) in the Congolese zone (south Bénin): *A. auriculiformis* and four (4) in the whole of the country: *A. ataxacantha, A. erythrocalyx, A. macrostachya and A. sieberiana*. Except *A. ataxacantha, A. auriculiformis* and *A. ehrenbergiana*, the limits of the areas of distribution of the reported species seems to cross North Benin.

Similar species were found in Burkina Faso and Niger with the exception of *A. laeta, A. albida* and *A. tortilis* (Wittig et al., 2004; Guinko, 1997). This distribution suggests that *Acacia* species have a distribution area characterized by a Sudanese climate. During the ethnobotanical survey, a total of 108 informants were interviewed, 32 female and 76 male, ranging from 17 to 72 years old. Informants possessing a high knowledge on plants and their uses were targeted and were selected by resource persons. The majorly used parts for the preparation of herbal remedies were leaves, bark, roots, whole plant and fruits. Leaves were the most used part. Many previous studies showed the use of leaves in the preparation of various recipes in traditional medicine (Akinwunmi and Amadi, 2019; Zahoor et al., 2017; Odewo and Adeyemo, 2018; Balcha, 2014).

Conversely, other studies reported roots as the most used part in preparing drugs (Chalabra et al., 1993). Generally, when the roots are used, the whole plant is torn off. It is then unlikely to survive. Indeed, the use of leaves in the preparation of traditional remedies offers a certain advantage to the preservation of biodiversity unlike the devastating effects caused by the use of roots and barks. The use of leaves is less dangerous than to the use of underground parts or the use of whole plants (Giday et al., 2003; Zheng and Xing, 2009).

Medicinal remedies were usually used as a decoction, infusion, powder or chew. These results are in accordance with previous investigations (Bulut et al., 2017; Demie et al., 2018; Palheta et al., 2017). It is well documented that african traditional medicine is a form of holistic health care system organized into three levels of specialty, namely divination, spiritualism, and herbalism. The traditional healer provides health care services based on culture, religious background, knowledge, attitudes, and beliefs that are prevalent in his community (Ozioma and Chinwe, 2019).

Indeed, the informants met during the study confirmed that traditional treatment methods are usually accompanied by halucinogenic rites and evocations of spirits (according to each local healer). These practices are essential for effective treatments. As previous reports on medicinal plants in Benin, this study revealed that ID and DSD (like diarrhea and dysentery) were the most cited by informants whereas pregnancy, PBP and veterinary were not cited by informants (Arbonnier, 2000). Blood and cardio-vascular system, dental health, general ailments, muscular-skeletal system, reproductive system, respiratory system, nervous system and sensory system were rarely cited by local people as they do not seem to suffer or do not know the symptoms of these categories of ailments. Fifteen and fourteen *Acacia* species were cited respectively to cure infectious diseases and digestive system disorder which are the most important diseases in Bénin according to Arbonier (2000). More than 37 and 35% of informants respectively agreed on the use of *Acacia* to treat ID and DSD.

The species with high FL, *A. nilotica*, *A. dudgeonii* and *A. hockii, A. senegal,* and *A. ataxacantha* used respectively for DSD and ID treatment were good candidates for further pharmacological prospection. In fact, data collected during the study showed men and women have very good knowledge on medicinal plants in all categories of diseases. The reason is that women learn about medicinal plants during their young age in order to take care of their household when they become adults. Young people and men are responsible for collecting these medicinal plants. It was also found that people of different ages had comparable knowledge of the *Acacia* species. This is justified by the fact that young people work with their parents and are responsible for harvesting the plants. This role played by young people facilitated the sharing of knowledge between seniors and the younger generation. In contrast, studies undertaken in Ethiopia and China showed an interest loss on the use of medicinal plants among young people caused by the influence of modernization (Giday et al., 2009; Hong et al., 2015).

Conclusion

This study showed that sixteen species from genus *Acacia* are found in Benin. Ethnobotanical survey results allowed highlighting the similarities between *Acacia* species and the difficulties related to the knowledge and

identification of these species by sellers and traditional healers. Ethnobotanical survey showed that infectious diseases and digestive system disorders were the most treated using *Acacia* species. Indeed, the number of *Acacia* species used in traditional medicine for the treatment of infectious diseases is a good indicator of the potential that exists locally. These results could help to identify new research topics especially regarding the discovery of new compounds to fight infectious diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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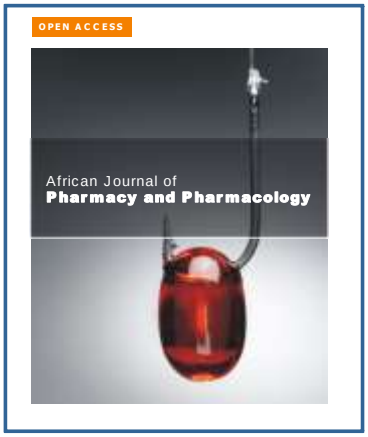

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