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Ethno-botanical survey, Chemical Composition and *in vitro* Antimicrobial activity of Essential oil from the root bark of *hazomalania voyronii* (jum.) Capuron (hernandiaceae)

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ABSTRACT

Microbial infectious diseases constitute a major public health problem worldwide. The emergence of multidrug resistance among several pathogenic organisms to drugs commonly used to treat such diseases is a great challenge today. The renewed interest that is given to indigenous medicinal plants as alternative sources of antimicrobials is due to the enormous chemical and structural diversity of plant derived secondary metabolites. *Hazomalania voyronii* is widely used in Malagasy Traditional Medicine as an herbal remedy to treat incurable wounds and bacterial infections. The Aim of the study was to evaluate the chemical composition and antimicrobial activity of essential oil from the root bark of *H. voyronii.* An ethno-botanical survey was conducted in the south of Madagascar from Novembre to December 2011 according to the principles laid out in the Declaration of Helsinki and the Nagoya protocol. Twenty five traditional healers were interviewed about medicinal plants used in folk medicine to treat wounds and bacterial infections. The most cited plant *H. voyronii* in this category (use value= 0.59; Informant consensus factor= 0.35) was collected and identified at the Department of Botany, Tsimbazaza Zoological and Botanical Park, Antananarivo, Madagascar. Essential oil was isolated from the root bark of this plant species by hydro-distillation. Antimicrobial activities were carried out using agar disc diffusion and micro-dilution broth methods with Gram-positive and Gram-negative bacteria as models. The chemical compositions of the essential oils were determined by Gas chromatography-Flame Ionization Detector (GC-FID) and Gas chromatography-Mass spectrometry (GC/MS).

Analysis of the root barks essential oil of *H. voyronii* (Jum.) Capuron by GC-FID and GC/MS enabled the identification of 28 compounds corresponding to 92.2% of the total essential oil composition. Based on functional groups classification, the essential oil from the root bark of the *H. voyronii* contained 13.4% of monoterpens hydrocarbons, 0.08% of sesquiterpens hydrocarbons, 36.6% of oxygenated monoterpens and 42.2% of oxygenated sesquiterpens. The major compounds were (-) Spathunelol (42.3%), Eucalyptol (22.0%), Limonene (10.3%), Borneol (10.2%), Myrtenal (2.0%), Perrylaldehyde (1.3%) and α-pinene (1.4%). The essential oil from the root bark of *H. voyronii* originated from Madagascar showed bactericidal activity (MBC/MIC ≤ 4).

The ability of essential oils in this study to display antimicrobial activity may represent a rational explanation for the use of this

plant species for wounds healing and treatment of bacterial infections by Malagasy traditional healers. Further studies involving the evaluation of both cytotoxicity of this oil toward human cells and the role of the lead molecules in the antimicrobial activity of the essential oil and combinational studies with conventional drugs are in progress.

Keyword: Medicinal plant, *Hazomalania voyronii*, essential oil, antimicrobial activity, Madagascar.

INTRODUCTION

Microbial diseases constitute a major public health problem worldwide, and are currently the world's leading cause of death because of the emergence of multidrug resistance among several pathogenic agents to drugs commonly used to treat such diseases [1, 2].

Despite the efforts in drugs R & D, few new antimicrobial drugs have emerged. The renewed interest that is given to medicinal plants as alternative sources of antimicrobials is due to the enormous chemical and structural diversity of plant derived secondary metabolites [3]. Madagascar as well as Democratic Republic of the Congo constitutes each one a rich reservoir of medicinal plants (hotspot of biodiversity) and the first line of treatment for poor people in such countries is the use of herbal medicines at home [4-6].

In order to preserve the ethno-medical cultural heritage of these countries, a multidisciplinary research program aiming at the identification, the validation and the sustainable use and conservation of medicinal plant species with pharmacological properties established a database for public purpose [2, 4-6, 7].

Several essential oils have been reported to possess interesting antimicrobial activity suggesting the possibility of using them as alternative of synthetic antimicrobials in order to overcome the increasing resistance of some pathogens to the conventional antibiotics drugs [8]. During an ethno-botanical survey conducted in the South of Madagascar, it was reported that *Hazomalania voyroniii (*Jum.*)* Capuron (syn. *Harnandia voyroniii*) known under the vernacular name of "Hazomalagny" or "Hazomaimbo" [Malagasy name] is traditionally used by the local communities to treat malaria, hypertension, bacterial infections and for wound-healing. Since wounds are generally infected by microbial pathogenic agents, it can therefore, be hypothesized that *H. voyronii* essential oil could be effective in the management of diseases caused by microbial agents. This plant species belongs to the family of Hernandiaceae [9]. Hernandiaceae family is widely distributed in the subtropical and tropical regions, with about 5 genus and 54 species [10]. 10 species exist in America, 42 in Malaysia and two species are originated from Madagascar.

The chemical composition and antimicrobial activity of essential oils from *Hazomalania voyronii* has not been studied yet. They could be promising sources of antimicrobial secondary metabolites. In order to support the folk use of *H. voyronii* as anti-malarial agent in Madagascar, Ratsimamanga and co-workers [11] isolated three isoquinoline alkaloid dimmers with pavine and benzyl tetrahydroisoquinoline moieties from an alkaloid extract of *H. voyronii.* These secondary metabolites were found to have chloroquine-potentiating action against *P. falciparum* FcM29 strains.

The aim of the present study was to determine the chemical composition of essential oils extracted from the root of *H. voyronii* and to investigate the *in vitro* antimicrobial activity of these essential oils against bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*) in order to validate scientifically the traditional use of this plant species. This is the first report involving the chemical composition and antimicrobial activity of essential oil from the root of *H. voyronii* originated from Madagascar.

MATERIALS AND METHODS

Ethno-botanical survey

Ethno-botanical information about the plant species selected for this study was obtained by interviewing traditional healers during field work which was conducted in the South of Madagascar. Surveys were conducted from November to December 2011 in five villages of the South of Madagascar. These villages are Beravy, Nosy-Mbositra, Mareanano, Sakaraha and Bemokarana. There are six different ethnic groups (tribes) inhabiting the South of Madagascar: Antandroy, Antanosy, Antaisaka, Bara, Mahafaly and Sakalava. They all share a common language Malagasy, which is the unique characteristic of this island.

A total of 25 traditional healers were interviewed. Informants were selected for their authentic knowledge on the utilization of medicinal plants. Malagasy, the national language of Madagascar was used during anthropological interviews. Traditional healers were interviewed on a voluntary basis. The study followed principles laid out in the Declaration of Helsinki as previously reported [12]. The questionnaires were divided into three sections: (i) personal information such as name, age, sex, marital status and studies level; (ii) traditional medicine practice (including knowledge of diseases and symptoms); (iii) plant vernacular names, plant part used, preparation methods, and administration route of remedies. Informed consent was obtained from both the Government of Madagascar to collect plant samples and to conduct non-commercial research on Malagasy medicinal plants and the respondents to divulge information. A benefit-sharing agreement on mutually agreed terms were also established between Congo DR (University of Kinshasa), Madagascar (Malagasy Institute of Applied Research and University of Toliara) and France (Pharmacognosy Laboratory, Paul Sabatier University) according to the principles laid out in the Nagoya protocol [13-15].

Selection and collection of plant material

The plant species *H. voyroniii* was selected based on its relative citation frequency (use value= 0.59) and the informant consensus factor value (0.35). Plant root samples were collected in Mareanano village, district of Manja (Southwest of Madagascar) on December 2011. The plant sample was identified by comparison with reference specimens available at the Department of Botany, Tsimbazaza Zoological and Botanical Park, Antananarivo. Voucher specimens with assigned sample number FR-01was deposited at the Herbarium of the Laboratory of

Applied Chemistry, Layflaylle Street, University of Toliara, Madagascar.

Essential oils isolation

Four hundred grams of *H. voyronii* were extracted for four hours by hydro-distillation using a Clevenger-type apparatus. Briefly, the plant roots were completely immersed in water (1000 mL) and heated to boiling after which the essential oil was evaporated together with water vapor and finally collected after decantation. The oil obtained was dried over anhydrous sodium sulphate and then stored under argon in a sealed vial, and kept at -10 °C until the time of further analysis. The percent yield was calculated relative to the dried mass of the initial sample.

Chemical analysis of essential oils and identification of the constituents

The quantification and analysis of the essential oil sample from the root of *H. voyronii* were carried out using gas chromatography (GC) and gas chromatography coupled to a mass spectroscopy (GC/MS).

The quantification of oil was performed in a Varian model 3400 gas chromatograph/flame ionization detector (GC-FID), under the following conditions: one-column injector, a DB-Wax fused silica capillary column (60 m x 0.32 mm i.d x 0.25 µm film). The temperature of the oven increased from 50 °C to 200 °C in a rate of 5 °C/min; up there it was held during 30 min. The injector and detector temperature was 260 °C. The carrier gas was helium maintained at 2.0 ml/min, dual FID; split ratio 1:70. The response factors were taken as 1.0 for all compounds, with reference of n-hexanol as internal standard. The linear retention indices were calculated with reference of C_5-C_{22}. Concentrations are given as the average of triplicate analysis.

GC-MS analysis of volatile compounds was performed on an AGILENT technologies AutoSystem XL GC-SM system operating in the EI mode at 70 eV, equipped with a split/splitless injector. Two columns were used: a HP 6890 GC column 1 (30 m x 250 µm i.d., film thickness 0.25 µm) and an HP 6890 GC column 2 (25 m x 250 µm i.d., film thickness 0.25 µm). Analytic conditions were: injector and transfer line temperature, 220 °C and 240 °C, respectively; Oven temperature was programmed as following: isothermal at 70 °C for 5 min, then increased to 180 °C, at a rate of 5 °C/min and subsequently held isothermal for 15 min (HP 6890 GC column 2); isothermal at 70 °C for 1 min, then increased to 200 °C, at a rate of 4 °C/min and held isothermal for 15 min (for HP 6890 GC column 1). Analytic conditions were: injector and transfer line temperature, 250 °C and 280 °C, respectively; carried gas used was helium at a flow rate of 1.3 mL/min; and the injection volume of 10 µL.. The identification of the oil constituents were based on their retention index (RI), and by comparison of their mass spectra with those of Wiley and NIST (National Institute of Standards and Technology) libraries and literature data [16-18].

Biological evaluation

Microbial strains

The activity of the essential oils samples was tested toward 9 different microorganisms: Gram positive bacteria represented by *Bacillus subtilis* (*B. subtilis* ATCC 6633), *Staphylococcus aureus* (*S. aureus* ATCC 25923), *Bacillus cereus* (*B. cereus* ATCC 10876), and Gram negative bacteria: *Escherichia coli* (*E. coli* ATCC 25922), *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 27853), and *Enterobacter cloacae* (*E. cloacae* ATCC 13047) and the yeast *Candida albicans (C. albicans* ATCC 10231*)*. The tested strains were obtained from the American Type Culture Collection (ATCC, Rockville MD, USA).

Antimicrobial activity

Disc diffusion

The agar disc diffusion method was used to determine the antibacterial activity of essential oils as follow: A 1 mL of suspension of an 18 hours culture bacteria containing about 10^6 UFC/mL were spread on Mueller Hinton agar medium using sterile swabs. Filter paper discs (6 mm in diameter) were soaked in 5 or 10 µL of pure essential oil and placed on the inoculated plates and allowed to dry for 30 min, then incubated at 37 °C for 24 hours. The diameters of the inhibition zones were measured in millimeters. Two controls were included in the test: the first was a control involving the presence of microorganisms but without the test oils sample and the last was a standard antibiotic penicillin G which was used in order to control the sensitivity of the tested micro-organisms. Studies were performed in triplicate, and the developing inhibition zones were compared with those of reference discs.

Minimum inhibitory and minimum bactericidal concentration

An aliquot (10 µL) of a 10^6 CFU/mL overnight culture was added to wells of a sterile 96-well micro-plate titer. Essential oil (EO) was diluted in Mueller Hinton broth (MHB) containing 0.1% (v/v) Tween 80 and added to wells to give final concentrations ranging from 0.03 to 10 µL/mL. The positive control wells contained MHB+ bacteria suspension without essential oil while negative control wells contained MHB only. Optical density (OD) was measured at 630 nm using a microplate reader (Titertek Twin-reader, Finland) and again after incubation for 24 hours at 37 °C. The minimum inhibitory concentration (MIC) was determined as the lowest EO concentration at which the OD after 24 h of incubation of the inoculum remained the same or reduced compared with the initial reading. MTT (30 µL) in aqueous solution (0.01%) was used to evaluate the micro-organism viability. For minimum bactericidal (MBC) determination, 10 µL was taken from each well after incubation and spot inoculated on to MHB and incubated for 24, 48 and 72 hours at 37 °C. The concentration at which no growth observed on subculture was determined as the MBC [19]. The mean MBC/MIC ratio was evaluated for each sample.

Statistical analysis

All statistical calculations were carried out with GraphPadPrism4. The results are expressed as the mean ± standard error of mean (S.E.M) of three independent experiments with individual values. Unpaired Student's t-test was used for statistical comparison; p values less than 0.01 were considered as significantly different against the control.

RESULTS

Ethno-botanical survey

During ethno-botanical survey, twenty five traditional healers were interviewed about medicinal plants used in folk medicine to treat wounds and bacterial infections. The most cited plant was *H. voyronii* with the use value and informant consensus factor of 0.59 and 0.35 respectively.

Extraction yields and Chemical composition

The yield of the root barks essential oils of *H. voyronii* (Jum.) Capuron (Hernandiaceae) obtained by hydro-distillation was 4.12%.

From GC-FID and GC/SM analyses, a total of 28 volatiles compounds were identified corresponding to 92.2% of the total essential oils composition. The essential oil from the root bark of the *H. voyronii* contained 13.4% of monoterpens hydrocarbons, 0.08% of sesquiterpens hydrocarbons, 36.6% of oxygenated monoterpens and 42.2% of oxygenated sesquiterpens. The major compounds were (-) spathunelol (42.3%), eucalyptol (22.0%), limonene (10.3%), borneol (10.2%), myrtenal (2.0%), perrylaldehyde (1.3%) and α-pinene (1.447%) (Table 1).

Antimicrobial activity

The antimicrobial activity of essential oils from *H. voyronii* against some pathogenic microorganisms was determined. The results are shown in Tables 2. Compared to the standard antibiotic, all microbial strains displayed sensitivity to the tested essential oils with the inhibition zones varying from 6.50±0.577 (or 7.20±1.302) to 29.33±3.32 (34.33±3.32) mm (Table 2). The most sensitive microbes were *B. subtilis* followed respectively by *B. cereus; S. aureus,* E. *cloacae, E. coli* and *P. aeruginosa.*

The minimum inhibitory concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values ranged from 0.312 to 2.5 µL/mL (Table 3).

Table 1: Composition of H. voyronii root bark essential oils

	%	RI	Identification
Monoterpens hydrocarbons			
α-thujene	0.338	919	a, c
α-Pinene	1.447	939	a, b, c
α-fenchene	0.147	951	a, c
Camphene	0.612	953	a, b, c
Bicyclo[3.1.1]heptane-6,6-dimethyle-2-mzthylene-(1S)	0.023	972	a, b
Sabinene	0.028	975	a, c
β-pinene	0.211	978	a, b, c
laevo β-pinene	0.015	981	a, b
Nopinene	0.021	983	a, b
β-Myrcene	0.046	992	a, b, c
Limonene	10. 360	1028	a, b, c
p-cymene	0.101	1026	a, b, c
D-limonene	0.013	1030	a, b
TOTAL		**13.4%**	
Oxygenated Monoterpens			
2-heptanol	0.021	928	a, b
Eucalyptol	22.031	1033	a, b, c
Linalool	0.146	1098	a, b, c
p-menth-1-en-4-ol	0.476	1177	a, b
Borneol	10.226	1182	a, b
p-menth-1-en-8-ol	0.411	1188	a, b
Myrtenal	2.011	1193	a
Perrylaldehyde	1.260	1272	a
TOTAL		**36.6%**	
Sesquiterpens hydrocarbons			
S-Silanene	0.042	1483	a, b
Caryophyllene	0.035	1494	a, b, c
TOTAL		**0.08%**	
Oxygenated Sesquiterpens			
1H-Cycloprop[e]azulen-7-ol decahydro-1,1,7-trimethyl-4-methylene	0.036	1328	a, b
6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a octahydronaphtalen-2ol	0.665	1562	a, b
2(4,8a-dimethyl-1,2,3,5,6,7,8,8a octahydronaphtalen-2yl)-prop-2en'	0.111	1558	a, b
(-)Spathunelol	41.28	1578	a, b
Caryophyllene oxide	0.135	1584	a, b, c
TOTAL		**42.2%**	

Total identified	**92.2%**

Table 2: Inhibitory effect of H. voyroniii essential oils against microorganisms (expressed as the inhibition zones of microbial growth, n=6)

Microbial strains	Diameter of inhibition zones (mm)		
	5 µL/disc	10 µL/disc	Penicillin G (10 units)
B. subtilis ATCC 6633	29.33±3.32	34.33±3.32	16±0.7
S. aureus ATCC 25923	21.66 ±2.80	25.16±3.314	14±0.5
B. cereus ATCC 10876	25.00±3.577	29.00 ±3.517	13±0.2
E. coli ATCC 25922	14.66±4.41	17.66±4.132	16±0.3
E. cloacae ATCC 13047	15.50±3.72	20.50±1.87	13±0.1
P. aeruginosa ATCC 27853	8.50±0.77	12.63±1.032	9±0.2

Table 3: Inhibitory effect of H. voyroniii essential oils against bacteria (expressed as the minimum inhibitory concentration MIC and the minimum bactericidal concentration MBC).

Bacterial strains	MIC (µL/mL) (24 hours)	MBC (µL/mL) (72 hours)
B. subtilis	0.312	0.312
B. cereus	0.312	0.312
S. aureus	0.625	0.625
E. cloacae	1.25	1.25
E. coli	1.25	1.25
P. aeruginosa	2.5	2.5

DISCUSSION

The results of the present study revealed a variation in chemical composition of essential oils (EO). The major components of *H. voyronii* EO were (-) spathunelol (41.3%), eucalyptol (22.0%), limonene (10.3%), borneol (10.2%), myrtenal (2.0%), perrylaldehyde (1.3%) and α-pinene (1.4%). The presence of these secondary metabolites could be correlated to the displayed activity.

The essential oil from *H. voyronii* was more active than the positive control (Penicillin G). Results of the antimicrobial assay showed also that all MIC values were less than 15 µg/mL. Cunha and coworkers [20] reported that EO with MIC values below 100 µg/mL is considered promising as potential antimicrobial agents. The values of MIC and MBC were identical for all tested bacterial strains indicating that EO from *H. voyroniii* has bactericidal activity (MBC/MIC ≤ 4).

H. voyronii essential oil act by killing bacteria. The present study indicates that *H. voyronii* can serve as source of antimicrobials. Historically, plants always have been confronted with microorganisms. They have evolved numerous chemical strategies for deterring pathogen attack, including the production of bactericidal and anti-infective compounds, leading to their use as medicines [21]. However, despite the fact that plant pathogenic microorganisms have played a key role in the early evolution of the secondary metabolites diversity, there is little chance for a microbe to gain resistance from a plant as it is known for antibiotic-producing microbes which possess genes protecting them from the toxic effects of these compounds. Plants, on the other hand, are genetically dissimilar from the microorganisms they are trying to eliminate. Like microbial antibiotics, plant antimicrobial secondary metabolites could kill pathogenic agent via a non-species specific mechanism such as disrupting microbial cell membranes [22].

CONCLUSION

The present research work evaluated the chemical composition and the *in vitro* antimicrobial activity of the essential oil from *H. voyronii*. This essential oil displayed promising antimicrobial activity *in vitro*. The ability of the oil to kill bacteria may represent a rational explanation for the use of this plant species by the traditional healers to treat incurable wounds and bacterial infections in Madagascar. Further studies involving the evaluation of both cytotoxicity of this oil toward human cells and the role of the lead molecules in the antimicrobial activity of the essential oil and combinational studies with conventional drugs are in progress.

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