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GC-FID and GC/MS analyses and Antimicrobial activity of *Croton greveanus, C. borarium* and *C. geayi* (Euphorbiaceae) essential oils from Madagascar

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Abstract
The aim of the present study was to evaluate the chemical composition and antimicrobial activity of essential oils from *Croton greveanus*, *Croton borarium* and *Croton geayi*. An ethno-botanical survey was conducted in the south of Madagascar from January to February 2010. Essential oils were extracted from the leaves of selected plants species by hydro-distillation. Antimicrobial activities were assessed using agar disc diffusion and micro-dilution broth methods with Gram-positive and Gram-negative bacteria as model. The chemical compositions of the essential oils were determined by Gas chromatography-Flame Ionization Detector (GC-FID) and Gas chromatography-Mass spectrometry (GC/MS). The essential oil from *Croton greveanus* exhibited great number of components (90) followed by *Croton geayi* (64) and *Croton borarium* (63). The essential oil of *Croton greveana* was rich in terpenic hydrocarbon compared to that of *Croton geayi*. The major compounds were β-pinene (28.73%) and limonene (25.65%). While the essential oil of *Croton borarium* was found to be rich in β-phillandrein (33.79%), α-terpineol (25.12%) and camphene (13.74%). The antimicrobial activity of each essential oil against seven bacteria strains was evaluated using disc and micro-dilution methods respectively. The essential oils from the leaves of Croton genus originated from Madagascar showed bactericidal activity (MBC/MIC ≤ 4) against most of the tested bacteria.
The ability of essential oils in this study to display antibacterial activity may represent a rational explanation for the use of these aromatic and medicinal plants species against pathogenic microorganisms by Malagasy traditional healers. Further studies are, therefore, necessary to evaluate better the selectivity index before developing these essential oils as novel antimicrobial agents.

Keywords: Pathogenic bacteria, medicinal plants, *Croton* genus, essential oils, antimicrobial activity, Madagascar.

1. Introduction
Infectious diseases remain the major public health problem, and are currently the world's leading cause of death [1]. The control of these diseases constitutes new challenges because of the emergence of multidrug resistance among several pathogens to some of the drugs commonly used in the treatment of infectious diseases [2]. This explains the need to intensify the search for more efficient drugs to combat these diseases. In addition, some conventional drugs are sometimes associated with adverse effects on the human cell host including hypersensitivity, immune-suppression, allergic reactions and even loss of hearing [3]. Despite the efforts of research bio-scientists, few new antimicrobial drugs have emerged. For efficient drug discovery, it's important to identify new biologically active chemical complex that will lead to effective antimicrobial drugs. The plant kingdom constitutes a good target for this purpose because of its enormous chemical and structural diversity [4].
The genus *Croton* consists of about 1200 species located in tropical and subtropical zones. There are about 200 species in Madagascar which are mainly located in the South and Southwest parts of Madagascar [5]. Many species of Croton genus are used in folk medicine for their various pharmacological properties [6-8].
Recent findings revealed that several essential oils possess strong antimicrobial activity suggesting the possibility of using them as alternative of synthetic antimicrobials to overcome the increasing resistance of some pathogens to the conventional antibiotics [9]. During an

ethno-botanical survey conducted in the South-west part of Madagascar, it was reported that *Croton greveanus, Croton borarium and Croton geayi* known under the vernacular name of Andriambolafotsy, Somorombohitse and Pisopisovavy respectively [Malagasy name] are used by the local communities to treat malaria, asthma, hypertension, cough and stomach disease and for their odor (vapor inhalation). They are also widely used as tea. The three plants belonging to the Euphorbiaceae family are endemic to Madagascar [5]. The chemical composition and biological activity of essential oils from the selected plant species has not been fully studied yet. They could be promising sources of secondary metabolites with pharmacological properties, including antimicrobial activity.

The aim of this study was to determine the chemical composition of essential oils extracted from the leaves of *Croton greveana, Croton borarium and Croton geayi* and to investigate the *in vitro* antimicrobial activity of these essential oils against Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Bacillus cereus*) strains and Gram-negative bacteria (*Escherichia coli*, *Salmonella typhii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*) strains respectively, in order to validate scientifically the traditional use of these aromatic plant species. The search for natural antibacterial products is the new challenges for the control and prevention of bacterial diseases in human health. This is the first report involving the complete chemical composition and antimicrobial activity of essential oils of the selected plants originated from the south of Madagascar.

2. Materials and methods

2.1. Selection and collection of plant material

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. For each plant species, a sample of leaves (approximately 400 g) of *Croton greveanus, Croton borarium and Croton geayi*, was collected during rainy season, on January and February 2010. They were deducted in the morning between 7 O'clok to 10 O'clock from plants in empty flowering. The plants samples were identified by comparison with reference specimens available at the Department of Botany, Tsimbazaza Zoological and Botanical Park, Antananarivo. Voucher specimens with assigned sample number AR-01, AR-02 and AR-03 were deposited at the Herbarium of the Laboratory of Applied Chemistry, Layflaylle Street, University of Toliara.

2.2. Essential oil extraction

Fresh leaves of each plant species were separately cut and about 400 grams of each sample were extracted for four hours by hydro-distillation using a Clevenger-type apparatus. Briefly, the plant leaves were completely immersed in water 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 in a dark glass bottle, and kept at 0 °C until the time of further analysis. The percent yield was calculated relative to the dried mass of the initial sample.

2.3. Determination of physico-chemical indices of extracted essential oils

Physico-chemical characterization of essential oils was carried out according to the International Organization of Normalization and the French Association of Normalization standard [10] in order to determine relative density, refractive index, optical rotation, acidity index and ester index. Specific gravity was measured with an Aton Paar densimeter (DMA 23_N model). Optical rotation was measured with a CETI Polaris polarimeter instrument. Essential oil sample were previously dissolved in analytical grade chloroform (Merk). And then, the readings were made with 1 dm tubes at 20 °C. Refractive index was measured at 20 °C with a CETI Quartz refractometer. The acid value (mg/KOH/g) of essential oils was evaluated by neutralizing the free acids of those by KOH. The saponification value (mg/KOH/g) was calculated as the quantity of KOH necessary to saponify esters of acids and to neutralize the free acids in one gram of essential oil. The ester index is the difference between the saponification value and the acid value.

2.4. Chemical analysis of essential oils and identification of the constituents

The quantification and analysis of the three essential oils samples of the leaves of *Croton greveanus*, *Croton geayi* and *Croton borarium* were carried out using Gas chromatography (GC) and gas chromatography coupled to a mass spectroscopy (GC/MS).

The quantification of oils was performed in a Varian model 3400 gas chromatograph/flame ionization detector (GC-FID), under the following conditions: one-column injector, a DB-Wax (Université Blaise Pascal de Clermont, France) 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 oils were performed under the same conditions with GC using an AGILENT 5973 gas chromatography equipped with an AGILENT 6890 series mass selective detector. Analytic conditions were: injector and transfer line temperature, 220 °C and 240 °C, respectively; oven temperature programmed from 50 °C to 200 °C at 5 °C/min; carried gas used was helium at a flow rate of 1 mL/min; and the injection volume of 0.1µL (10% hexane solution); and the split ratio was 1:50. The electron impact energy was set at 70 eV. The identifications of the constituents were based on the comparison of their mass spectra with those of Wiley and NIST (National Institute of Standards and Technology) libraries and literature data [11, 12].

2.5. Biological screening

2.5.1. 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), *Salmonella typhii* (S. *typhii* ATCC 13311), *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 27853), and *Enterobacter cloacae* (*E. cloacae* ATCC 13047). The tested strains were obtained from the American Type Culture Collection (ATCC, Rockville MD, USA).

2.5.2. Antimicrobial activity

2.5.2.1. 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 any tested bacteria containing about 10^6 UFC/mL were spreaded on Mueller Hinton agar medium using sterile swabs. Filter paper discs (6 mm in diameter) were soaked in 20 µL of pure essential oil and placed on the inoculated plates and allowed to dry for 15 min, then incubated at 37 °C for 24 hours. The diameters of the inhibition zones were measured in millimeters. Chloraphenicol or cycloheximid (10 µg/mL) was included as control.

2.5.2.2. 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-titre plate. Each 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 oils 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 microorganism 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 hours at 37 °C. The concentration at which no growth observed on subculture was determined as the MBC [13]. The mean MBC/MIC ratio was evaluated for each sample.

2.6. 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 n 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.

3. Results

3.1. Extraction yields

Extraction yields of essential oils from the leaves of *C. greveanus, C. geayi* and *C. borarium* are 0.96%, 0.72% and 0.68% respectively. This result indicates that among three *Croton* species samples, *C. borarium* leaves exhibeted a low content of essential oil.

3.2. Physico-chemical indices

Physico-chemical indices of the extracted essential oils are given in the Table 1. From this table, it can be noticed that the relative density values of three essential oils are less than 1. However, the relative density value of *C. borarium* (0.921) essential oil is greater than that of *C. geay*i (0.914) and *C. greveanus* (0.904). All extracted essential oils were found to be dextrogyre but the optical rotation of *C. greveanus* oil is three times greater than that of the others studied essential oils. The refractive index and the acidity index of *C. borarium* essential oil were also found to be greater than that of *C. geay*i and *C. greveanus* respectively. According to the ester index value, the Table 1 revealed that *C. greveanus* possesses high value followed respectively by *C. geay*i and *C. borarium.*

Table 1: Physico-chemical indices of essential oils from Malagasy Croton species

Physico-chemical indices	*C. greveanus*	*C. geayi*	*C. borarium*
Relative density	0.901± 0.117	0.904± 0.155	0.921±0.160
Optical rotation	159.7°±0.925	50.64° ± 0.194	50.84° ± 0.745
Refractive index	1.4785 ± 0.311	1.4831 ± 0.115	1.5428 ±0.234
Acidity index	0.73 ± 0.00012	16.191 ± 0.00420	19.830 ± 0.00123
Ester index	372.33 ± 13.193	74.930 ± 4.311	53. 150 ± 1.866

3.3. Chemical compositions

3.3.1. Croton greveanus essential oil

The analysis of the *Croton greveanus* essential oil chromatogram have shown that it contains 90 compounds of which 9 hydrocarbons monoterpens, 25 oxygenated monoterpens, 15 oxygenated sesquiterpens, 28 hydrocarbons sesquiterpens and 8 others non terpenic compounds corresponding to 96.1% of the essential oil. The major compounds are 1, 8 cineol (40.40%), linalol (23.81%) and α-terpineol (8.2%), sabinen transhydrate (10.17%), sabinen (6.87%) and finally terpinen-4-ol (1.52%). The other compounds are less than 1% or in one spoor (Table 2).

Table 2: Composition of the essential oil of Croton greveanus leaves

N°	Component name	Percentage	R.I	Identification method
1	n-octane	Traces	789	b
2	Hex-2-enal	Traces	849	b

3	n-butanol	Traces	867	b
4	n-nonane	0.05	896	b
5	5-éthylcyclopent-1-ene carbaldehyde	Traces	899	b
6	α-thujene	0.33	922	b
7	α-pinene	0.99	929	b
8	Sabinene	6.87	971	b
9	Octen-3-ol	0.3	982	b
10	Myrcene	0.84	988	b
11	n-octanal	Traces	1002	b
12	α-terpinene	Traces	1014	b
13	p-cymene	Traces	1024	b
14	Limonene	Traces	1030	b
15	1,8-cineole	40.40	1038	b
16	γ-terpinene	0.47	1058	b
17	Cis hydrate sabinene	Traces	1071	b
18	Terpinolene	Traces	1083	b
19	Cis-oxyde de linalool	Traces	1086	b
20	Linalol	23.81	1114	b
21	Trans hydrate de sabinene	10.17	1116	b
22	Cis-p-menth-2-en-1-ol	Traces	1129	b
23	Trans-p-menth-2-en-1-ol	Traces	1145	b
24	Sabinacetone	Traces	1159	b
25	Cis-chrystanthenol	Traces	1167	b
26	δ-terpineol	0.32	1173	b
27	Terpinen-4-ol	1.52	1183	a ; b
28	α-terpineol	8.2	1203	a ; b
29	n-decanal	0.2	1207	a ; b
30	Trans-pipertiol	Traces	1212	a ; b
31	Nerol	Traces	1226	a ; b
32	Methyl-ether-thymol	Traces	1229	a ; b
33	Methyl-ether-carvacol	Traces	1238	a ; b
34	Cuminaldehyde	Traces	1244	a ; b
35	Acetate de linalal	Traces	1249	b
36	Geraniol	Traces	1251	b
37	E-farnesol	Traces	1255	b
38	Geranial	Traces	1267	b
39	1-nonen-3-ol	Traces	1282	b
40	α-terpinen-7-al	Traces	1286	b
41	2-n-octyl furane	Traces	1290	b
42	Thymol	Traces	1293	b
43	Carvacrol	Traces	1302	b
44	n-undercanal	Traces	1307	a ; b
45	Bicycloelemene	Traces	1330	a ; b
46	δ-elemene	Traces	1345	a ; b
47	Eugenol	Traces	1352	b
48	α-amorphene	Traces	1369	a ; b
49	α-compaene	Traces	1374	a ; b
50	β-bourbounene	Traces	1383	a ; b
51	β-elemene	Traces	1388	b
52	Cyperene	Traces	1402	b
53	α-gurjunene	Traces	1406	b
54	n-dodecanal	Traces	1408	b
55	β-caryophyllen	0.94	1420	b
56	β-copaene	Traces	1430	b
57	Trans- α-bergamotene	Traces	1431	b
58	Selina-4(15), 5-diene	Traces	1433	b
59	Isogermacrene-D	Traces	1443	b
60	SesquisabineneB	Traces	1451	b
61	α-humulene	Traces	1455	a ; b
62	Allo-aromadendrene	Traces	1459	a ; b
63	Cis-muurola-4(14),5-diene	Traces	1463	a ; b
64	γ-muurolene	Traces	1474	a ; b
65	7-epi-helifolene	Traces	1476	a ; c

66	Germacrene-D	0.76	1481	b
67	Bicyclosesquiphellandrene	Traces	1492	b
68	Bicyclogermacrene	Traces	1494	b
69	γ-cadinene	Traces	1512	b
70	δ-cadinene	Traces	1516	b
71	Cis-calamenene	Traces	1519	a ; b
72	Cadina-1,4-diene	Traces	1531	a ; b
73	α-cadinene	Traces	1536	a ; b
74	α-calacorene	Traces	1540	a ; b
75	Elemol	0.87	1551	b
76	Ledol	Traces	1571	b
77	Spathulenol	0.33	1578	b
78	Epoxycaryophyllene	0.39	1583	b
79	Cis-guai-6-en-10-ol	Traces	1595	b
80	Epi-gobulol	0.14	1605	b
81	Humulen-1, 2, epoxyde	Traces	1610	b
82	10-epi-γ-eudesmol	Traces	1623	a ; b
83	1-epi-cubenol	Traces	1627	a ; b
83	γ-eudesmol	Traces	1633	b
85	Epi- α-cadinol	Traces	1642	b
86	Epi- α-muurolol	Traces	1644	b
87	Muurolol	Traces	1647	b
88	α-elemol	0.20	1657	b
89	Bulnesol	Traces	1665	b
90	Daucalene	Traces	1671	b

a: Retention Index; b: GC-SM (NIST & Wiley library); c: Co-injection.

3.3.2. Croton borarium essential oil

The essential oil of *Croton borarium* leaves is constituted of 63 compounds of which 61.25% of terpenic hydrocarbons, 25.50% of oxygenated compounds and 13.05% of non terpenic derived (Table 3), corresponding to 99.80% of the essential oil. That is characterized by a strong proportion of β-phellandren (39.72%), α-terpineol (25.121%), and camphene (13.74%), α- pinene (10.70%). The minor compounds where terpinen-4-ol (1.71%), germacren-D (6.68%), α-copaen (4.71%), sabinen (3.63%), β-pinen (2.46%), limonene (2.31%), β- caryophylen (2.18%), α-hulemen (1.76%), p-cymen (1.051%), γ-terpinen (1.29%), β-myrcen (1.22%) and epoxy-caryophyllen (1.092%). The other compounds are in trace state ie less than 1% (Table 3).

Table 3: Composition of the essential oil of *Croton borarium* leaves

N°	Component name	Percentage	RI	Identification method
1	α-thujene	0.82	516	a
2	α-pinene	10.70	527	a
3	Camphene	13.74	572	a
4	β-pinene	2.46	613	a
5	Sabinene	3.63	626	a
6	β-Myrcene	1.22	668	a ; b
7	α-terpinene	0.64	686	b
8	Limonene	2.31	707	b
9	β-phellandrene	39.72	717	b
10	Cis- β-ocimene	0.04	739	b
11	γ-terpinene	1.29	750	b
12	Trans- β-ocimene	0.26	756	b
13	p-cimene	1.051	776	b
14	Terpinolene	0.27	788	b ; c
15	Hex-2-enal	Trace	849	a ; c
16	5-ethyl pent-1-ene carbaldehyde	Trace	899	b
17	α-cubebene	0.25	957	a; b
18	δ-elemene	0.20	968	b ; c
19	α-copaene	4.71	992	b
20	Linalol	0.47	1049	b
21	β-elemene	0.51	1086	b
22	β-Caryophyllene	2.18	1090	b
23	Terpinene-4-ol	1.71	1102	a ; b
24	Trans-p-menth-2-en-1ol	Trace	1145	b
25	Sabinacetone	Trace	1159	b

26	α-humulene	1.76	1162	b
27	Cis-chrystanthenol	Trace	1167	b
28	Germacrene	6.68	1196	b
29	α-terpineol	25.121	1198	b ; c
30	1,8 Cineole	0.253	1207	a ; b
31	β-silenene	0.85	1209	a ; b
32	Methyl ether thymol	Trace	1229	a ; b
33	Methyl ether Carvacrol	Trace	1238	a ; b
34	Cuminaldehyde	Trace	1244	a ; b
35	Acetate linalyl	Trace	1249	a ; b
36	6-Methyl-5-hepten-2-one	Trace	1279	a ; b
37	2-hexen-1-ol	Trace	1288	a ; b
38	2-n-octyl furane	Trace	1290	a ; b
39	Thymol	Trace	1293	a ; b
40	Perillene	Trace	1294	a ; b
41	Geraniol	0.11	1343	a ; b
42	α-amorphene	0.326	1369	a ; b
43	α-gurjunene	Trace	1406	a ; b
45	Selina-4(15), 5-diene	Trace	1433	a ; b
46	Caryophyllene oxide	0.21	1469	a ; b
47	γ-muurolene	Trace	1474	a ; b
48	γ-cadinene	Trace	1516	a ; b
49	β-cubebene	Trace	1535	b
50	α-calacorene	Trace	1540	b
51	Camphor	Trace	1549	b
52	Epoxycaryophyllene	1.092	1583	b
53	Cis-guai-6-en-10-ol	Trace	1595	a ; b
54	1-epi-cubenol	Trace	1627	a ; b
55	Epi-α-cadinol	Trace	1642	a ; b
56	Epi-α-muurolol	Trace	1644	a ; b
57	Bulnesol	Trace	1665	a ; b
58	Epi-Bicyclosesquiphellandrene	0.652	1667	a ; b
59	(E)- 2,6 dimethyl-3,7-octadien-2,6 diol	Trace	1669	a ; b
60	Cadina-1,4-diene	Trace	1783	b
61	Cis calamenene	0.218	1853	b
62	Torreyol	Trace	2147	b
63	Levomenol	Trace	2197	b

a: Retention Index; b: GC-SM (NIST & Wiley library); c: Co-injection.

3.3.3. *Croton geayi* essential oil

The *Croton geayi* essential oil analysis has revealed the presence of multiple secondary metabolites of which 60.19% constituted of terpenic hydrocarbons, 23.46% of oxygenated compounds and 16.15% of non terpenic derived, corresponding to 96.71% of the essential oil. This essential oil is principally constituted of β-pinene (28.74%), limonene (22.92%) and secondarily by eucalyptol (10.42%), α-terpineol (8.2%), transhydrate of sabinen (5.67%), β-Phellandren (7.47%), β- caryophylen (4.80%), α-pinene (4.32%), trans-nerolidol (3.88%), β-myrcen (3.06%), germacren-D (2.56%), cis-nerolidol (2.50), aromadren (2.35%), fenchol (2.04%), sabinen and terpinen-4-ol (1.05%), caryophyllen oxide (1.09%) respectively (Table 4).

Table 4: Composition of the essential oil of *Croton greayi* leaves

N°	Component name	Percentage	RI	Identification method
1	α-pinene	4.32	527	a
2	Camphene	0.039	573	a ; b
3	β-pinene	28.74	617	a
4	Sabinene	1.057	627	a
5	β-Myrcene	3.067	670	a
6	α-terpinen	0.25	688	a
7	Limonene	22.92	710	a ; c
8	β-phellandrene	7.472	717	a
9	Trans-β-ocimene	0.183	741	a
10	γ-terpinene	1.038	752	a
11	Cis-β-ocimene	0.152	758	a
12	p-cimene	0.853	778	a
13	α-thujene	0.33	922	a
14	β-cubebene	0.053	959	a ; b ; c

15	δ-elemene	0.158	971	a ; b
16	Linalol	0.931	1051	a ; b
17	β-elemene	0.191	1088	a ; b
18	β-Caryophyllene	4.804	1093	a ; b
19	Terpinene-4-ol	1.058	1105	a ; b
20	Trans hydrate sabinene	5.67	1116	a ; b
21	δ-3-carene	0.52	1142	a ; b
22	α-humulene	0.98	1163	a
23	Germacrene-D	2.561	1202	a ; b
24	β-silenene	0.09	1211	a ; b
25	Trans-pipentol	Trace	1212	a ; b
26	Cis-p-menth-2-en-1-ol	0.43	1279	a ; b
27	Allo-ocimene	Trace	1284	a ; b
28	2-hexa-1-ol	0.19	1288	b
29	Carvacrol	Trace	1302	b
30	Cyprene	Trace	1402	b
31	Trans-α-bergamotene	Trace	1431	b
32	Cis oxyde linalol	0.76	1439	b
33	Isogermacrene	Trace	1443	a
34	SesquisabineneB	Trace	1451	b
35	α-cubebene	0.18	1455	b
36	Allo-aromadendrene	Trace	1459	a ; b
37	Caryophyllene oxyde	1.09	1471	a
38	7-epi-helifolene	Trace	1479	b
39	α-cadinene	Trace	1536	b
40	Fenchol	2.04	1574	b
41	Aromadrene	2.35	1607	b
42	Humulen-1,2-epoxyde	Trace	1610	b
43	Menthol	0.027	1633	b
44	Eugenol	0.734	1651	b
45	Myrtenal	Trace	1686	b
46	α-terpinyl acetate	0.13	1688	b
47	Geranial	Trace	1735	b
48	Neral	Trace	1736	b
49	Citronellol	Trace	1751	a ; c
50	δ-cadinene	0.61	1760	b
51	Myrtenol	0.09	1777	a ; b
52	Nerol	Trace	1786	a ; b
53	Isogeranial	0.11	1811	a ; b
54	Carveol	Trace	1818	a ; b
55	Geraniol	0.80	1845	a ; b ; c
56	Cis-myrtanol	Trace	1858	a ; b ; c
57	Cis-nerolidol	2.50	1974	a ; b
58	Trans-nerolidol	3.88	2024	a ; b
59	Elemol	Trace	2083	a ; b
60	γ-eudesmol	0.13	2166	a ; b
61	α-eudesmol	Trace	2205	a ; b
62	β-eudesmol	0.12	2213	a ; b
63	Driminol	Trace	2287	a ; b
64	Farnesol	0.34	2304	a ; c

a: Retention Index; b: GC-SM (NIST & Wiley library); c: Co-injection.

3.4. Antimicrobial activity

The antimicrobial activity of essential oils from Croton species against microorganisms was determined. The results are shown in Tables 5 and 6. All bacteria demonstrated some degree of sensitivity to the essential oils within the concentrations tested.

The essential oil of *C. greveanus* leaves displayed antimicrobial activity against all bacterial strains tested with the inhibition zones varying from 7 to 16 mm (Table 5). *B. cereus, B. subtilis* and *S. aureus* are the most sensitive strains while *E. coli, P. aeruginosa, E. cloacae* and *S. typhii* are the least sensitive. The essential oil from *C. borarium* leaves displayed interesting bioactivity on all tested germs showing the inhibition zones from 10 to 23 mm. All the germs were very sensitive. The *C. geayi* leaves essential oil was inactive on *E. cloacae*, *P. aeruginosa* and *S. typhii*; but it was bioactive on *B. subtilis*, *B. cereus* and *E. coli*. The inhibition zones varied from 3 to 10 mm. The minimum inhibitory concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values ranged from 0.312 to 10 µg/mL (Table 6).

Table 5: Inhibitory effect of essential oils against bacteria (expressed as the inhibition zones of bacterial growth)

Bacterial strains	Diameter of inhibition zones (mm)		
	C. greveanus	***C. geayi***	***C. borarium***
B. cereus	15.5	08.5	17
S. aureus	11.5	10	22.5
B. subtilis	13.2	09	15
E. coli	09	07.5	16.2
E. cloacae	07.5	04.3	10.5
P. aeruginosa	08.01	03.17	18.3
S. typhii	07.2	03.5	14.12

Table 6: Inhibitory effect of essential oils against bacteria (expressed as the Minimum Inhibitory Concentration MIC and the Minimum Bactericidal Concentration MBC).

Bacterial strains	MIC (µL/mL)			MBC (µL/mL)		
	C. greveanus	***C. geayi***	***C. borarium***	***C.greveanus***	***C. geayi***	***C. borarium***
B. cereus	1.25	2.5	0.625	1.25	2.5	0.625
S. aureus	1.25	2.5	1.25	1.25	2.5	1.25
B. subtilis	1.25	5	0.312	1.25	5	0.312
E. coli	5	-	1.25	5	-	1.25
E. cloacae	10	-	2.5	10	-	2.5
P. aeruginosa	5	-	1.25	5	-	1.25
S. typhii	2.5	5	0.312	2.5	5	0.312

4. Discussion

During the extraction, it was noticed that the essential oils of the leaves of *Croton* species were white color. This character was demonstrated by the relative density measured at 20 °C. The refractive index and optical rotation (dextrogyre) values of each essential oil were almost similar to the values found by others authors [14, 15]. For the other physico-chemical indices such as the ester index, we found different values. The ester index of *Croton greveanus* oil is 372.33 ± 13.193. This value was very high because it's richness in oxygenated hydrocarbon components.

The results of the present study revealed a variation in chemical composition of essential oils. The *Croton greveana* is rich in sesquiterpens with a prevalence of 1,8 cineol (40.40%) and linalol (23.81%). The two others *Croton* species (*C. borarium* and *C. geayi*) were rich in hydrocarbon terpenic with a high proportion of β-phellandren (33.72%), α- terpineol (25.12%) for the *C. borarium*, and an occurrence of β-pinen (28.74%) and limonene (25.62%) in the *Croton geayi* essential oil. The study revealed also the presence of camphene (13.74%) in *Croton borarium* essential oil which was absent in the essential oils of the two other *Croton* species. Some compounds were common to the three plant essential oil samples. This is the case of α-terpineol, transhydrate of sabinen and terpinen-4-ol. The difference in the chemical composition of essential oils could explain the difference in antimicrobial activities of tested oils. This chemical composition is controlled by the environmental factors such as the climate, the geological nature of the site of harvest and the period of the harvest of the aromatic plants samples [16, 17].

Recent findings have indicated that essential oil extracts with MIC values below 100 µg/mL are considered promising as potential antimicrobial agents. In this study, the essential oils of the leaves of Croton species exhibited strong antibacterial effect against tested microorganisms showing MIC values lower than 15 µg/mL and lower than other studies in the literature [18]. Results of antimicrobial assay showed that the essential oils of *Croton geayi* and *Croton greveanus* are less active than that of *Croton borarium*. The essential oil from *Croton borarium* was more active than the positive control (Chloramphenicol/ cycloheximid). The values of MIC and MBC were identical for all tested bacterial strains. So, the Malagasy *Croton* essential oils could act both by inhibiting bacterial growth and/or by killing them. This activity was found to be bacterial strains dependent. The present study indicates that medicinal plants species should be a powerful source of antimicrobial compounds due to their environment. Indeed, the plant kingdom offers a way of hope because of the enormous structural and chemical diversity of its secondary metabolites [4]. 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 [19]. 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 eradicate. Like microbial antibiotics, plant antimicrobial compounds could kill pathogen via a non-species specific mechanism such as disrupting microbial cell membranes [20]. However, it was recently reported that, plant secondary metabolites could also act against microbes by targeting cell's communication system (quorum sensing). The breakdown of this system causes an attenuation of microbial pathogenicity [21].

The effect of essential oils on bacterial growth and quorum sensing is well known in the literature [22-24]. It was reported that essential oils act by destabilizing bacterial communities in

the host. The anti-quorum sensing effect of essential oils may reduce pathogenicity, antibiotic resistance and biofilm formation. It has been suggested that targeting pathogenesis instead of killing the microbial organism may provide less selective pressure and therefore decreased emergence of resistant strains [25].

5. Conclusion

This study focused on the evaluation of chemical composition and the antimicrobial activity of the essential oils from *Croton greveanus*, *Croton borarium* and *Croton geayi* which have been selected through ethno-botanical survey conducted in the South of Madagascar. At the end of this study, we have demonstrated that the essential oils from *Croton* species possess promising antimicrobial activity *in vitro* against tested bacteria. Interactions between the major and minor constituents within each of oil could be responsible for the displayed inhibitory effects. The ability of the oils to display antibacterial activity may represent a rational explanation for the use of these aromatic plant species by the traditional healers to treat infections caused by pathogenic bacteria in Madagascar. This study indicated, so this makes them particularly interesting for further studies including cytotoxicity bioassay in order to evaluate the selectivity/therapeutic index of these essential oils before developing them as novel antimicrobial agents.

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