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Original Article

Chemical composition and antibacterial activity of essential oil from leaves of *Croton heliotropiifolius* in different seasons of the year

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ABSTRACT

This paper reports the first study of the variation of the chemical composition and abundance of the essential oil of *Croton heliotropiifolius*, in four seasons, and the evaluation of its antibacterial activity. Essential oil obtained from leaves of *C. heliotropiifolius* were analyzed by GC/MS and evaluated against eight bacteria strains by broth microdilution method. The chemical constituents identified were the same in all samples, but with different proportions. The total percentages identified were 96.58% in summer, 92.08% in autumn, 98.44% in winter and 90.78% in spring. The majors constituents are β-caryophyllene, bicyclogermacrene, germacrene-D, limonene and 1,8-cineole. β-Caryophyllene was the major compound in all samples. The results of the antibacterial evaluations showed weak to moderate activity against the analyzed strains. In all analyzes was observed that essential oil sample collected in summer stands out from the others, displaying stronger activity against Gram-positive as Gram-negative bacteria.

Introduction

Croton is the second largest genus of the Euphorbiaceae family and comprises around 1200 species. Predominantly in America, *Croton* has a pantropical distribution and about 350 occur in Brazil (Berry et al., 2005). Several biological assays were carried out with essential oil from species of this genus in diverse models of activity to demonstrate their popular use, including anti-inflammatory (Ramos et al., 2013), antinociceptive (Bighetti et al., 1999), gastroprotective (Coelho-de-Souza et al., 2013), wound healing (Cavalcanti et al., 2012), anticancer (Sylvestre et al., 2006), synergistic effect with antibiotics (Rodrigues et al., 2009) and cardiovascular activity (Siqueira et al., 2006).

Popularly known as "velame" or "marmeleiro", *Croton heliotropiifolius* Kunth is traditionally used as remedy in folk medicine and is commonly found in the Northeast, Midwest and Southeast regions of Brazil. The leaves are used as infusion particularly for the treatment of gastrointestinal disturbances and weight loss (Govaerts et al., 2000). Doria and co-workers (2010) demonstrated the larvicidal activity against *Aedes aegypti* of the essential oil of *C. heliotropiifolius*. Neves and Camara (2012) demonstrated that the major constituent of de essential oil of the leaves of this specie was β-caryophyllene (20.82 ± 0.48), unlike the major found in the stem, β-elemene (17.28 ± 0.06). The ethanolic extract from leaves of *C. heliotropiifolius* presented insecticidal activity against *Sitophilus zeamais* (Silva et al., 2012).

Essential oils are volatile mixture and have complex composition, characterized by a strong odor. Formed by aromatic plants as secondary metabolites, it is known for their antiseptic, bactericidal, virucidal, fungicidal and other medicinal properties. These volatile oils may be used as food preservatives and as analgesic, sedative, anti-inflammatory, spasmolytic and as local anesthetic (Bakkali et al., 2008).

Several studies shown the influence of the year's seasons not only on the content of chemical constituents of essential oils, but also on their biological activities (Angioni et al., 2006; Hussain et al., 2008; Al-Hamwi et al., 2011; Almeida et al., 2014). In a continuation to our investigations on of the medicinal plants from Brazilian Caatinga Biome, the aim of this study was to investigate the possible variation of the chemical constituents of essential oils from leaves of *C. heliotropiifolius* and to evaluate its antibacterial activity. Covering a region of approximately one million square kilometers,

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the Caatinga Biome is present in much of the Brazilian Northeast, especially the semiarid region (Menezes et al., 2012).

Material and methods

Plant material

Wild populations of *Croton heliotropiifolius* Kunth, Euphorbiaceae, growing in Caboclo locality, Afrânio, Pernambuco State (08°28′37,50″ S, 040°56′13,30″ W, 565 m), Brazilian northeast were collected. The samples were identified by the botanist José Alves de Siqueira Filho from Centro de Recuperação de Áreas Degradadas da Caatinga – CRAD. A voucher specimen was deposited in the HVASF Federal University of San Francisco Valley Herbarium (voucher number 13963). Leaves have been collected in August (winter), November (spring) 2010 and February (summer) and May (autumn) 2011 (Southern Hemisphere seasons), always every three months.

Essential oil extraction

Fresh leaves were submitted to hydro-distillation in a Clevenger apparatus. A total of 250 g of fresh plant material and 2500 ml of water were used, and the distillation was carried out for 2 h. Traces of water were removed by freezing the sample below 0 °C followed by transferring unfrozen essential oil to a new vial to yield yellowish volatile oils. Samples were dried with anhydrous sodium sulfate.

Analytical conditions and GC–MS and GC–FID analysis

To analyze the chemical composition of the essential oils, they were previously diluted in 1 ml of ethyl acetate. The analysis by GC/FID chromatograph used a Shimadzu GC-2010® equipped with autosampler AOC-20i. Conditions were: Rtx-5 capillary column (30 m × 0.25 mm), positioned 00:25 film thickness microns injector temperature of 220 °C and detector 240 °C; helium carrier gas (1.2 ml min^{-1}) with the oven temperature program 60–240 °C at 3 °C min^{-1} maintained at 240 °C for 20 min; split 1:20, injection volume 1 μl. Analyses by GC/MS was performed on Shimadzu chromatograph® CG-2010 Mass Spectrometer coupled to GC/MS; Shimadzu QP 2010® with AOC-20i autosampler; capillary column DB-5 ms (30 m × 0.25 mm) thickness 00:25 film microns injector temperature 220 °C; helium carrier gas (1 ml min^{-1});temperature of the interface and the ionization source 240 °C; ionization energy 70 eV; ionization current 0.7 kV, and program temperature and split similar to that described above. Co-injection of the sample with a homologous series of *n*-alkanes (C8–C24) was held at the GC/MS for the standardization of retention time.

Qualitative and quantitative analysis

The data were acquired and processed with a PC with Shimadzu GC-MS – Solution software. The identification of the constituents was assigned on basis of comparison of their relative retention indices to a *n*-alkane homologous series (C8–C24) obtained by co-injecting the oil sample with a linear hydrocarbon mixture, as well as, by computerized matching of the acquired mass spectra with those stored in Wiley 8 and NIST05 mass spectral libraries of the GC–MS data system and other published mass spectra (Adams, 1995; Leffingwell and Alford, 2005; Özel et al., 2006). The results are expressed in relative percentage of each compound, calculated by normalization of the chromatographic peak areas.

Microorganisms and determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The reference bacterial strains used in this study were purchased from National Institute of Quality Control in Health (INCQS/FIOCRUZ–Brazil). Microorganisms used were *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 10708), *Serratia marcescens* (ATCC 13880), *Shigella flexneri* (ATCC 12022) and *Staphylococcus aureus* (ATCC 25923).

Antibacterial activity was evaluated by the method of broth microdilution (Santos et al., 2012) as recommended by The National Committee for Clinical Laboratory Standards (CLSI, 2003). Initially a stock solution of 1 mg/ml of essential oils was prepared using an aqueous solution of 20% DMSO (v/v). A hundred μl of this dilution have been transferred to the microplate containing 100 μl of Müller-Hinton broth. Then, serial dilutions were performed resulting in concentrations of 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 μg/ml. Inoculum containing 5×10^5 CFU ml^{-1} (0.5 in McFarland scale) was added to each well. Wells in microplate have been dedicated to sterility control of the broth, bacterial growth and action of antimicrobial reference (Gentamicin). A solution of gentamicin was used prepared at an initial concentration of 1.6 mg/ml, which was diluted to concentrations of 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 mg/ml. Microplates were incubated under conditions of aerobically for 24 h at 37 °C. 10 μl of 2,3,5-triphenyl-tetrazolium (CTT) 2% were added to each well to detect the color change of the CTT (colorless) to red, reflecting the bacterial metabolism as active. The MIC was defined as the lowest concentration of the samples that visibly inhibited bacterial growth. To determine the MBC, before adding the CTT to determine de MIC, aliquots of 10 μl were withdrawn from each well containing extracts and transferred to Petri dishes containing Müller-Hinton agar. The plates were incubated for 24 h at 37 °C. The appearance of bacterial colony for a given concentration indicates that the samples was not able to kill 99.9% of bacterial inoculum used, so that, the MBC was defined as the lowest concentration of the samples that visibly inhibited bacterial growth. Assays were performed in triplicate. The density of the oils was employed to convert μl/ml to mg/ml.

Results and discussion

Harvest of leaves (250 g) was carried out at 7 am, ranging from three months according to the seasons of year summer, autumn, winter and spring. Chemical constituents identified were the same in all samples (Table 1). β-Caryophyllene is the major constituent in all samples and their percentage were 46.99% in winter, 43.85% in spring, 41.04% in summer and 28.61% in autumn, followed by bicyclogermacrene, germacrene-D, limonene and 1,8-cineole. Sesquiterpenes were in highest proportions in all samples.

Co-occurrence of α/β-pinene might be a characteristic of the genus *Croton* (Bracho and Crowley, 1966); indeed, along with β-caryophyllene, these terpenoids occur in essential oil of several species as *C. cordiifolius* (Nogueira et al., 2015), *C. conduplicatus* (Almeida et al., 2014), *C. campestris* (Almeida et al., 2013), *C. antanosiensis*, *C. decaryi* and *C. sakamaliensis* (Radulovic et al., 2006).

Table 2 shows the minimum and maximum temperature and relative humidity of harvest's day, monthly rainfall and the yield of each extraction. Within the collection period, the highest rainfall regimens were registered in summer (66.4 mm), the same month of higher maximum temperature (33.6 °C), rainy season in San Francisco Valley Brazilian's region. Lowest rainfall and relative humidity were observed in winter (0.0 mm and 47.5%, respectively), local dry season (BDMEP, 2016). The best essential oil

Table 1
Essential oil chemical composition from leaves of *Croton heliotropiifolius* in different seasons of the year.

Compound	Leaves (%)[a]				LRIcalc[b]	LRIlit[c]
	Summer (February)	Autumn (May)	Winter (August)	Spring (November)		
α-Pinene	0.31	3.95	0.61	0.19	933	932
Sabinene	0.29	2.36	0.82	0.17	973	969
β-Pinene	0.30	0.73	Trace	Trace	977	974
β-Myrcene	0.47	1.51	0.58	0.18	990	988
p-Cymene	2.83	2.61	0.58	0.31	1024	1020
Limonene	5.24	9.56	4.54	1.76	1028	1024
1,8-Cineole	8.66	16.31	4.60	1.12	1031	1026
γ-Terpinene	1.32	2.21	1.98	1.05	1058	1054
Terpinolene	Trace	Trace	0.17	Trace	1089	1086
Linalool	1.40	1.47	0.39	0.25	1095	1100
Borneol	4.58	2.09	0.38	0.39	1166	1165
Terpinen-4-ol	2.31	1.18	Trace	0.21	1178	1174
α-Terpineol	1.31	0.51	0.26	Trace	1191	1186
Bornyl acetate	2.70	0.58	1.14	0.41	1287	1287
β-Caryophyllene	41.04	28.61	46.99	43.85	1421	1417
α-Humulene	4.57	3.62	4.73	4.81	1456	1452
Germacrene-D	4.03	3.06	7.80	6.11	1484	1484
Bicyclogermacrene	9.24	8.47	21.83	22.60	1502	1500
Spathulenol	2.92	3.03	0.40	2.78	1581	1577
Caryophyllene oxide	2.49	3.38	0.64	3.56	1586	1582
α-Cadinol	0.57	0.79	Trace	1.03	1658	1652
Monoterpenes	10.76	18.98	9.28	3.66		
Monoterpenoids	20.96	22.14	6.77	2.38		
Sesquiterpenes	58.88	43.76	81.35	77.37		
Sesquiterpenoids	5.98	7.20	1.04	7.37		
Total identified	96.58	92.08	98.44	90.78		

[a] Compound percentage by CG/FID analysis.
[b] LRIcalc = linear retention index calculated on a DB5 column (comparison with *n*-alkanes C8–C24).
[c] LRIlit = linear retention index from literature.

Table 2
Minimum and maximum temperature, relative humidity of harvest day, monthly rainfall and the yield of extractions.

	Minimum (°C)	Maximum (°C)	Relative humidity (%)	Rainfall (mm)	Yield (%)
Winter	17.7	32.4	47.5	0.0	0.60
Spring	20.2	32.4	52.2	1.1	0.24
Summer	23.4	33.6	58.5	66.4	0.36
Autumn	21.7	27.9	70.0	9.4	0.16

yield (0.6%), as well as the highest proportion of β-caryophyllene (46.99%), happened in winter, the same month of lower rainfall (0.0 mm), lower relative humidity (47.5%) and maximum temperature (32.4 °C) very close to the highest observed (33.6 °C). The worst oil yield (0.16%) and proportion of β-caryophyllene (28.61%) happened in the autumn, the same month with the highest relative humidity (70.0%) and lower maximum temperatures (27.9 °C) among the months observed. The most probable explanation to find β-caryophyllene in larger proportions in the essential oil, when temperatures are higher, is that this compound is a sesquiterpene, and their loss to the environment in a natural way occurs more slowly than the monoterpenes, which are compounds with lower molecular weight that evaporate faster. Following a similar reasoning, the monoterpene 1,8-cineol was found in higher proportion during fall (16.31%), exactly the season where β-caryophyllene was in smaller proportion. Thus, we can observe for samples obtained in rainy season that, as the sesquiterpenes like β-caryophyllene, germacrene-D and bicyclogermacrene occurred in reduced proportion in the essential oil of *C. heliotropiifolius*, some monoterpenes increase, such as *p*-cymene, 1,8-cineol, limonene, borneol and linalool.

Genetic factors determine chemical composition of essential oils, but other factors may result in significant changes in the production of secondary metabolites. These metabolites represent a chemical interface between plants and environment, and stimuli arising from environment in which the plant is located may redirect the metabolic pathway, leading to the biosynthesis of different compounds, or the same compounds but in different proportions (Morais, 2009).

Among these factors, one can stress the plant interactions with microorganisms, insect or other plants, age and stage of development, and abiotic factors such as light, temperature, rainfall, nutrition, season, time of collection and harvesting techniques. It is worth noting that these factors may have correlations among them, not acting alone, and exert joint influence in secondary metabolism. Temperature and luminosity have a relevant role in photosynthesis, because the interaction of these factors ensures optimum environment for physiological process. Although the species can to adapt to their natural habitat, plants are able to withstand temperature variations, and these variations are responsible for changes in the production of secondary metabolites (Souza et al., 2008; Morais, 2009).

Antibacterial activity was evaluated against eight reference bacteria, three Gram-positive and five Gram-negative (Table 3). Essential oils obtained in all seasons showed weak to moderate activity against *Enterococcus faecalis* and *Serratia marcescens* strains. In all analyzes were observed that essential oil sample obtained in summer stands out from the others, showing capacity to inhibit the growing of six strains. All samples showed no effect against bacteria *Staphylococcus aureus* and *Klebsiella pneumonia*. The capacity to inhibit the growth of *Shigella flexneri* and *Escherichia coli* strains, even at the highest concentrations tested (500 μg/ml), may justify

Table 3
Antibacterial activity of essential oil from leaves of *Croton heliotropiifolius* in different seasons of the year.

Microorganisms	EOCh-Summer		EOCh-Autumn		EOCh-Winter		EOCh-Spring		GEN	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Bacillus cereus (ATCC 11778)	500	[a]	[a]	[a]	[a]	[a]	[a]	[a]	0.4	0.4
Enterococcus faecalis (ATCC 19433)	62.5	[a]	125	[a]	500	[a]	500	[a]	0.4	0.4
Staphylococcus aureus (ATCC 25923)	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	0.025	0.025
Escherichia coli (ATCC 25922)	500	[a]	[a]	[a]	500	[a]	500	[a]	[b]	0.4
Klebsiella pneumonia (ATCC13882)	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	0.05	0.05
Salmonella enterica (ATCC 10708)	500	[a]	[a]	[a]	[a]	[a]	500	[a]	0.05	0.05
Serratia marcescens (ATCC 13880)	500	[a]	500	[a]	500	500	500	[a]	[b]	0.025
Shigella flexneri (ATCC 12022)	500	500	500	[a]	[a]	[a]	[a]	[a]	[b]	0.025

MIC, minimal inhibitory concentration (μg/ml). MBC, minimum bactericidal concentration (μg/ml).
[a] Bacterial growth at all concentrations.
[b] Absence of bacterial increase at all concentrations tested ($n = 3$).
EOCh, essential oil of *Croton heliotropiifolius*; GEN, gentamicin.

the popular use of the species to treat gastrointestinal tract disorders. Thus, a way to use a sample, which could probably have an effect against these two microorganisms, would performing the harvest plant in summer.

The increasing incidence of multiple resistance of pathogenic microorganisms to drugs that are currently in clinical use makes the discovery of new antibiotics one intense research field (Oliveira-Júnior et al., 2012). Cytotoxic characteristics of essential oils have been studied to understand effects on bacteria. This biological activity appears to be related to its ability to cause damage to cell wall. The lipophilic constituents of essential oils can pass through cell wall and cytoplasmic membrane and affect the structure of different layers of polysaccharides, fatty acids and phospholipids. In bacteria, permeabilization is associated with loss of ions, reduction of membrane potential, collapse proton pump and depletion of ATP (Di Pasqua et al., 2006). The damage to cell wall and cell membrane cause escape of macromolecules, progressing to cell lysis (Gustafson, 1998).

This study showed that the period of harvest influences the percentage of chemical constituents of *C. heliotropiifolius* essential oil, and the antibacterial activity observed may be related to the chemical composition variation. To the best of our knowledge, this is the first study that shows chemical composition and evaluation of antibacterial activity for essential oil of *C. heliotropiifolius* in different seasons of the year.

Authors' contributions

JMTAF and LCA contributed in collecting plant samples and obtaining the essential oils. ECCA and JMTAF drafted the paper and contributed to critical reading of the manuscript. LSC and JRGSA contributed to critical reading of the manuscript. AML and ECCA contributed to chromatographic analysis. FSS designed the study and supervised the laboratory work. APO, AGMP and ALG contributed to microbiological study. All the authors had read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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