Comparison of the chemical composition of the hexanics extracts from species of *Croton.* Biological activity

Comparación de la composición química de los extractos hexánico de especies de *Croton*. Actividad biológica

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ABSTRACT

The species endemics *Croton micradenus*, *Croton myricifolius* and *Croton spiralis* among others the genus, integrate the main populations in this Oriental region of Cuba. A wide range of uses are attributed to the species of the genus, among which medicinal uses stand out. Among the secondary metabolites present in *Croton* species, nonpolar compounds constitute a group that has shown biological activity against pathogenic microorganisms. In this sense, our objective is to compare the chemical composition, determine the major components and the potential chemo-taxonomic markers; as well as, the antimicrobial activity of the hexanic extracts of the leaves and stems of the three species, by GC-MS and pharmacological assays. The samples tested showed activity against at least two of the four microorganisms studied. The presence of octacosanol and palmitic acid (majority components), as oleic acid, stigmasterol and sitosterol in the extracts of the three species confer responsibility for the biological activity expressed.

Keywords: euphorbiaceae; *croton*; hexanics extracts; antimicrobial activity and GC-MS.

RESUMEN

Las especies endémicas *Croton micradenus*, *Croton myricifolius* y *Croton spiralis* entre otras del género, integran las principales poblaciones en la región oriental de Cuba. A éstas se les atribuyen una amplia gama de usos, entre los que se destacan los medicinales. Entre los metabolitos secundarios presentes en las especies de *Croton*, los compuestos apolares constituyen un grupo que ha mostrado actividad biológica frente a microorganismos patógenos. En este sentido, el objetivo de este trabajo, es comparar la composición química, determinar los componentes mayoritarios y los potenciales marcadores quimio-taxonómicos; así como, la actividad antimicrobiana de los extractos hexánicos de las hojas y tallos de las tres especies, mediante CG-MS y ensayos farmacológicos. Las muestras analizadas mostraron actividad contra al menos dos de los cuatro microorganismos estudiados. La presencia de octacosanol y ácido palmítico (componentes mayoritarios), así como ácido oleico, estigmasterol y sitosterol en los extractos de las tres especies confieren responsabilidad de la actividad biológica expresada.

Palabras clave: euphorbiaceae; *crotón*; extractos hexánicos; actividad antimicrobiana y GC-MS.

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Introduction

The fundamental populations of the species from genus *Croton* in the Cuban archipelago they are characterized to grow in coastal and pre-coastal ecosystems, under conditions of extreme drought, solar high-intensity and strong winds (breezes) sustained most of the time.[(1)]

Croton micradenus Urb. (Euphorbiaceae) is one of the species that characterizes to the District Phytogeographical of the Coastal Area Maisí-Guantánamo for its representativeness in the most part of the xerophytic vegetation the south of the oriental counties mainly and that together to the species endemics *Croton myricifolius* Griseb. and

Croton spiralis Muell. Arg. among others the genus, integrate the main populations in this Oriental region of Cuba.[1,2]

In Cuba, *Croton* genus it is compound for 53 species, of them 37 endemics that represent the 69,8 %. [3] Inside her, occupying an important place the medicinal ones [4,5], to mention some examples, they can be related its effectiveness like diuretic, diaphoretic, detersive, spasmolytic, antimalarial and antimicrobial. Many are the secondary metabolites of interest for phytochemicals investigations of plants with potential biological activity; among them, the components of lipophilic extracts like the alcohols, saturated free fatty acids molecular weight, sterols and fatty acids, etc.[6,7]

The tools on which botanists and biologists in general have relied to establish the correct taxonomic classification of families, genera and species of biota at a global level have been dissimilar. The presence of groups of phytocompounds, such as those previously mentioned, in species of the same genus has enhanced the determination of chemo-taxonomic markers as a complement to these investigations.

Previously, we reported the chemical compositions of the hexanic extract from the leaves and steam bark of the endemic Cuban species potentially medicinal *Croton micradenus* Urb. [8] In this sense, our objective is to compare for the first time the chemical composition and determine potential chemo-taxonomic markers of the Cubans endemics species *C. micradenus*, *C. myricifolius* and *C. spiralis*; as well as, their antimicrobial activity against of the references strains *Escherichia coli* (Migula) Castellani & Chalmers (ATCC 10536), *Staphylococcus aureus* Rosenbach (ATCC 6538), *Candida albicans* (Robin) Berkhout (ATCC 10231) and *Pseudomonas aeruginosa* (Schoroeter) Migula (ATCC 9027).

Materials and methods

General: GC-MS, Gas Chromatograph 6890 N to a selective detector mass 5975 B inert (Agilent, USA) with a computation system and a capillary column HP-5 Ms (30 m x 0,25 mm d.i. and 0,25m thickness, Agilent, USA). The identification was carried out by comparison of the spectra obtained with the libraries NIST 2011 and Wiley-275, as well as with substances commercial references available and the literature. The retention times (Rt) were also compared with substances commercial of reference. The determination of the relative percentage was based on the internal normalization method (n=3).

Plant material: the leaves and steam bark of individual adults sterile of the species *C. micradenus*, *C. myricifolius* and *C. spiralis* were recollected in May 2012 in San Antonio del Sur, Guantánamo, Cuba, and identified by Dra. Ramona Oviedo Prieto. A voucher specimen (number HAC 41947, HAC 4148 and HAC 4149, respectively) is retained in the Herbarium of the Institute, CITMA, Havana, Cuba.

Extraction and obtaining of chromatograms: the dried (40°C) and ground leaves and steam bark of *C. micradenus C. myricifolius* and *C. spiralis* (100g) were extracted with n-hexane to reflux at 100°C (4h) of residue upon evaporation in vacuum. For GC-MS analysis, 4mg of the hexanic extract from the leaves and steam bark was dissolved in 0.2mL of chloroform and derivatized with 100µL of N-metil-N-trimetilsilil trifluoroacetamida (MSTFA) to 70ºC during 30min in a dry thermostat.

Biological assay: the antimicrobial activity of the hexanics extracts from the leaves and steam bark was determined for microdilution plating method [(9)] for determining the Minimum Inhibitory Concentration (MIC) according to the National Committee for Clinical Laboratory Standar (NCCLS). The microorganisms *S. aereus* (ATCC 6538), *P. aeruginosa* (ATCC 9027), *E. coli* (ATCC 10536) and *C. albicans* (ATCC 10231) were adjusted with the turbidity standard McFarland 0.5. It was used as control the antibiotic chloramphenicol to a concentration of 5mg/mL. The Minimum Inhibitory Concentration (MIC) is the lowest concentration capable of inhibiting bacterial growth.

Results and discussion

The identification and comparison of the compounds of the hexanics extracts of *C. micradenus*, *C. myricifolius* and *C. spiralis* is given in table 1.

Starting from the 65 compounds found in the extract of *C. micradenus*, 61 were identified what represents 93,8 %. The majority components were 1-octacosanol (C28OH) (12,5 %), triacontanal (C30Al) (9,11 %), octacosanal (C28Al) (7,67 %), heptacosanal (C27Al) (6,58 %), 1-hexacosanol (C26OH) (6,58 %), 5,5-dimetil-1-etil 1,3-cyclopentadiene (5,70 %), triacontanol (C30OH) (4,62 %), nonacosane (C29) (3,46 %), triacontanoic acid (C30:0) (3,44 %), palmitic acid (C16:0) (3,36 %), 1-tetracosanol (C24OH) (2,90 %) and 1-nonacosanol (C29OH) (2,24 %), which represent 68,2 % of the total.

From the 63 compounds found in the extract of *C myricifolius*, 56 were identified what represents 88,9 %. The majority components were 1-octacosanol (C28OH) (16,7 %), octacosanoic acid (C28:0) (8,64 %), 1-hexacosanol (C26OH) (7,82 %), triacontanoic acid (C30:0) (7,43 %), triacontanol (C30OH) (7,30 %), palmitic acid (C16:0) (3,69 %), phytol (3,46 %), hexacosanoic acid (C26:0) (3,05 %), 1-heptacosanol (C27OH) (2,82 %), β-sitosterol (2,42 %), which represent 63,3 % of the total.

Starting from the 64 compounds found in the extract of *C. spiralis*, 53 were identified what represents 82,9 %. The majority components were 1-octacosanol (C28OH) (14,54 %), octacosanoic acid (C28:0) (10,05 %), palmitic acid (C16:0) (9,95 %), triacontanoic acid (C30:0) (8,50 %), triacontanol (C30OH) (6,49 %), 1-hexacosanol (C26OH) (4,76 %), stearic acid (C18:0) + NI (3,97 %), hexacosanoic acid (C26:0) (3,96 %), dotriacontanoic acid (C32:0) (2,98 %) y dotriacontanol (C32OH) (2,81 %), which represent 68 % of the total.

Table 1. Comparison among the chemical composition of the hexanics extracts from the leaves and steam bark of the species *C. micradenus, C. myricifolius* and *C. spiralis*.

No.	Compound	Rt (min)	Content (%)		
			C. micradenus	*C. myricifolius*	*C. spiralis*
1	*cis*-verbenone	8,800	0,26	-	-
2	borneol	8,899	1,08	1,45	-
3	NI	8,961	-	1,62	-
4	benzoic acid	9,050	1,16	0,64	0,06
5	glycerine	9,264	0,10	-	0,10
6	bornyl acetate	9,384	0,29	-	-
7	NI	9,481	-	0,82	0,16
8	5,5-dimethyl-1-ethyl-1,3-ciclopentadiene	9,679	5,70	0,57	-
9	3-methyl hexanedioic acid	9,849	-	-	0,16
10	NI	10,407	0,03	1,13	-
11	NI	10,526	tr	-	1,05
12	*cis*-calamenene	11,062	0,55	1,25	-
13	phytol	11,232	1,32	3,46	0,35
14	NI	11,324	-	1,23	-
15	NI	11,508	0,88	1,65	-
16	lauric acid (C12:0)	11,754	1,29	0,92	0,63
17	NI	12,386	-	2,11	-
18	NI	12,529	-	-	0,52
19	azelaic acid	12,758	0,10	2,06	1,34
20	myristic acid (C14:0)	13,123	1,05	0,87	1,85
21	NI	13,462	0,68	-	0,52
22	pentadecanoic acid (C15:0)	13,883	0,05	0,12	0,38
23	NI	14,045	-	-	1,05
24	NI	14,360	-	-	0,63
25	palmitic acid (C16:0)	14,711	3,36	3,69	9,95
26	margaric acid (C17:0)	15,564	0,11	0,13	0,52
27	1-octadecanol (C18OH)	15,663	0,18	0,10	0,84
28	linoleic acid (C18:2)	16,189	0,34	0,17	0,13
29	oleic acid (C18:1)	16,232	1,48	0,49	0,27
30	NI	16,390	-	-	0,57
31	stearic acid (C18:0)+ NI	16,456	1,91	1,26	3,97
32	tricosane (C23)	16,937	0,18	0,24	0,18
33	NI	17,000	-	-	1,61
34	1-eicosanol (C20OH)	17,438	0,36	0,19	0,20
35	tetracosane (C24)	17,841	0,24	-	0,12
36	NI		-	-	1,23
37	eicosanoic acid (C20:0)	18,247	0,14	0,19	0,68
38	pentacosane (C25)	18,750	1,62	0,11	0,55
39	1-docosanol (C22OH)	19,216	0,32	0,23	0,60

No.	Compound	Rt (min)	Content (%)		
			C. micradenus	*C. myricifolius*	*C. spiralis*
39	hexacosane (C26)	19,637	0,69	0.02	-
40	NI	19,660	-	-	0,84
41	docosanoic acid (C22:0)	20,015	0,16	0,43	1,33
42	heptacosane (C27)	20,516	1.46	0,08	0,14
43	pentacosanal (C25Al)	20,846	0,22	-	-
44	tricosanoic acid (C23:0)	20,881	0,03	0,10	0,53
45	1-tetracosanol (C24OH)	20,948	2,90	0,70	0,46
46	octacosane (C28)	21,367	1,75	-	-
47	hexacosanal (C26Al)	21,711	1,43	-	-
48	tetracosanoic acid (C24:0)	21,726	0,30	1,89	1,81
49	1-pentacosanol (C25OH)	21,776	0,67	0,26	0,23
50	nonacosane (C29)	22,205	3,46	0,24	0,30
51	pentacosanoic acid (C25:0)	22,539	0,03	0,49	0,82
52	heptacosanal (C27Al)	22,559	1,59	tr	tr
53	1-hexacosanol (C26OH)	22,597	6,58	7,82	4,76
54	triacontano (C30)	23,011	0,57	0,10	0,23
55	hexacosanoic acid (C26:0)	23,336	0,57	3,05	3.96
56	octacosanal (C28Al)	23,379	7,67	tr	tr
57	1-heptacosanol (C27OH)	23,394	0,10	2,82	1,60
58	hentriacontane (C31)	23,811	1,84	0,19	0,19
59	heptacosanoic acid (C27:0)	24,116	0,20	0,63	0,46
60	1-octacosanol (C28OH)	24,171	12,50	16,70	14,54
61	dotriacontane (C32)	24,692	0,43	0,75	-
62	octacosanoic acid (C28:0)	24,884	1,78	8,64	10,05
63	1-nonacosanol (C29OH)	24,930	2,24	0,68	0,42
64	triacontanal (C30Al)	24,955	9,11	1,50	0,78
65	tritriacontane (C33)	25,321	0,24	0,40	0,53
66	stigmasterol	25,525	0,65	0,44	0,07
67	nonacosanoic acid (C29:0)	25,592	0,20	0,11	0,20
68	triacontanol (C30OH)	25,636	4,62	7,30	6,49
69	hentriacontanal (C31Al)	25,694	0,61	tr	tr
70	β-sitosterol	25,928	0,97	2.42	0,79
71	β-amirine	26,067	0,33	0.66	0,15
72	lup-20(2)-en-3-ona	26,232	-	0.60	-
73	triacontanoic acid (C30:0)	26,337	3,44	7.43	8,50
74	lupeol	26,406	0,88	1,35	0,50
75	dotriacontanal (C32Al)	26,446	1,23	0.22	0,33
76	NI	26,887	-	-	0,55
77	dotriacontanol (C32OH)	27,144	1,08	1,87	2,81
78	dotriacontanoic acid (C32:0)	28,017	0,05	0,92	2,98
79	tetratriacontanol (C34OH)	29,050	0,57	0,60	0,79
80	ester NI	32,153	-	1,14	-

NI- No Identified, tr- trace, Rt- Time retention

From the 80 compounds found among the three extracts of *C. micradenus*, *C myricifolius* and *C. spiralis*, 63 were identified, which 50 were common for the three samples, among them all the alcohols (12), saturated free fatty acids of high molecular weight (11) and 8 of the 9 fatty acids you present. It stands out that among the alcohols that share the three species they are majority for all 1-octacosanol (C28OH) (12,5%, 14,54% and 16,7), 1-hexacosanol (C26OH) (6,58 %, 4,76 % and 7,82 %) and triacontanol (C30OH) (4,62%, 6,49 % and 7.30%), among the saturated free fatty acids of high molecular weight the triacontanoic acid (C30:0) (3,44 %, 8,50 % and 7,43 %) and octacosanoic acid (C28:0) (1,78 %, 8,64 % and 10,05 %); as well as among the fatty acids the palmitic acid (C16:0) (3,36 %, 9,95 % and 3,69 %), which could be potential markers chemo-taxonomic of this genus. (**Supplementary Information)**

Some alkanes, aldehydes and other groups of compound it appears indistinctly in one or two of the samples in the three possible combinations: *C. micradenus/C. myricifolius, C. micradenus/ C. spiralis* and *C. myricifolius/C. spiralis*. As well as, five compounds are recognized that alone they appear in *C. micradenus*; a sterol (lup-20-(2)-in-3-ona) and four compounds NI for the hexanic extract of *C. spiralis*, while that the 17 compounds NI of the three species, eight are part of the constituents of the hexanic extract of *C. myricifolius*, the rest it shares it with the other two studied species, except a fatty acid (acid 3-metil hexane dioic) that alone it appears in this species (Table 2).

Table 2. Differ found in the composition of the obtained samples of the hexanics extracts from the leaves and steam bark of the three species

Sample	Compounds	Type of compounds
C. micradenus	octacosane (C28)	alkane
	pentacosanal (C25Al) hexacosanal (C26Al)	aldehyde
	cis-verbenone	terpene
	bornil acetate	ester
C. myricifolius	3-metil hexanedioic acid	Fatty acid
C. spiralis	lup-20-(2)-en-3-one	sterol

When analyzing the obtained results, of the chemical composition of the hexanics extracts of *C. micradenus, C. myricifolius* and *C. spiralis*, it is verified that they don't differ of that

reported in the literature for the different types of compounds that compose these non polars extracts in species of the *Croton* genus.[10-20] The presence of sterols like the β-sitosterol, compound as the phytol, saturated free fatty acids of high molecular weight, among other, they support this idea. However, it should be stood out that these results are novel when characterizing chemically for the first-time these extracts of three Cuban endemic species.

All the samples showed the inhibition halo at least in front of two the four studied microorganisms. They stand out the result of 3,1 mg/mL IMC shown by *C. micradenus* in front of *S. aureus* and *E. coli*, results to consider compared with the control. Not being this way in front of the bacteria *P. aeruginosa* and to the mushroom *C. albicans* that I turn out to be negative. The hexanics extracts of C. *myricifolius* and *C. spiralis* showed inhibition halo front *S. aereus* and *E. coli*, to inhibiting minimal concentrations (IMC) inferiors for the case of the first microorganism of 4,0 and 3,75 mg/mL and superiors for the second of 32,0 and 30,0 mg/mL, respectively. In front of the bacteria *P. aeruginosa* and to the mushroom *C. albicans* only was evaluated the extract of *C. myricifolius*, the one that showed a different answer in front of both pathogens with an IMC of 32,0 and 8,0 mg/mL, respectively. They stand out the results shown by the two extracts like antimicrobians agents in front of the bacteria positive Gram *S. aureus* and the one of *C. myricifolius* in front of the mushroom *C. albicans*, compared with the concentration assayed for the control (5 mg/mL) (Table 3).

Table 3. Antimicrobial activity of the hexanics extracts from the leaves and steam bark of *C. micradenus*, *C. myricifolius* and *C. spiralis*

	Microorganisms IMC (mg/mL)				
Sample	*S. aureus*	*E. coli*	*P. aeruginosa*	*C. albicans*	Control
C. micradenus	3,1	3,1	Negative	Negative	Cloranfenicol 5 mg/mL
C. myricifolius	4,0	32,0	32,0	8,0	
C. spiralis	3,75	30,0	No Valued	No Valued	

The presence in the extract of *C. micradenus*, *C. myricifolius* and *C. spiralis* of octacosanol (12,5 %; 14,5 % and 16,7 %) [5], palmitic acid (3,36 %; 9,95 % and 3,69 %), oleic acid (1,48 %; 0,27% and 0,49%), estigmasterol (0,65 %; 0,07 % and 0,44 %) and sitosterol

(0,97 %; 0,79 % and 2,42 %).[6] They confer him responsibility on the antimicrobial activity expressed by the samples [21,22,23,24], but the synergy action with other constituents of the samples it could potentiates the same one.[25]

Conclusions

The chemical composition of the hexanic extracts was determined and compared, as well as the major compounds in the three species and potential markers chemo-taxonomic of this genus. All the extracts showed the inhibition halo at least in front of two the four studied microorganisms. For the first time the chemical composition and antimicrobial activity of these three Cuban endemic species was determined.

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Authors declare no conflict of interests.

Authors' contribution

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Conceptualization - Ideas; formulation or evolution of general research goals and objectives.

Research: carrying out or conducting a research process, specifically conducting experiments or data collection/testing.

Project administration: Responsibility for management and coordination of the planning and execution of the research activity.

Visualization: preparation, creation and/or presentation of published work, specifically data visualization/presentation.

Writing - original draft: Preparation, creation and/or presentation of published work, specifically writing of the initial draft (including substantive translation).

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Formal analysis: application of statistical, mathematical, computational or other formal techniques to analyze or synthesize study data.

Resources: provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computer resources or other analysis tools.

Writing – review and editing: Preparation, creation and/or presentation of published work by those in the original research group, specifically critical review, commentary or review, including pre- or post-publication stages.

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Conceptualization - Ideas; formulation or evolution of general research goals and objectives.

Supervision: Supervisory and leadership responsibility for the planning and execution of the research activity, including external mentoring to the core team.

Writing – review and editing: Preparation, creation and/or presentation of published work by those in the original research group, specifically critical review, commentary or review, including pre- or post-publication stages.

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