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Composition and Cytotoxic Activity of Essential Oils from *Croton matourensis* and *Croton micans* from Venezuela

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Abstract: The chemical composition and cytotoxic activity of the essential oils extracted from leaves of *Croton matourensis* and flowers and leaves of *Croton micans* were investigated. GC-FID and GC-MS analysis revealed that forty six compounds comprised 95.5 % of *C. matourensis* essential oil, with the main components being fenchyl acetate (19.5%), methyleugenol (14.2%), isoelemicine (11.3%), elemicine (7.6%), spathulenol (6.9%) and valencene (5.8%). In the analysis of *C. micans* oils, 63 compounds that comprised 98.9% were identified from the flower oil, the principal being fenchyl acetate (41.6%), α-caryophyllene (12.6%), β-cubebene (5.0%), β-caryophyllene (5.5%), α-cubebene (5.3%), β-elemene (4.7%) and valencene (4.6%). The oil from leaves gave fenchyl acetate (25.3%) α-caryophyllene (20.7%), α-selinene (12.8%) and β-bourbene (9.3%) as major constituents. The cytoxicity of these oils was screened by the MTT method against three human tumour cell lines and primary culture of human dermis fibroblasts (normal control cells). The results indicate that the oils have a moderate cytotoxicity against LoVo (colon carcinoma), X-17 (colon carcinoma), HeLa (cervical cancer), and control cells.

Keywords: *Croton micans; Croton matourensis*; cytotoxicity; essential oil; fenchyl acetate; methyleugenol.

1. Introduction

Croton is a genus of the Euphorbiaceae, that comprises more than 1300 species, which grow in the tropical and subtropical regions of the world [1]. This genus is widespread in Venezuela and, up to now only several species have been studied. *Croton micans* Muell. Arg. and *Croton matourensis* Aubl., are two of the 80 endemic species reported in our country [2]. *C. micans* is a shrub that grows in the northern part of Venezuela. No common use of *C. micans* is known so far in our country,

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however, in Brazil, where is commonly called *alecrim-de-vaquero* it is considered a medicinal plant used in folk medicine against cardiac problems, influenza and as a calmative [3]. *C. micans*, was misidentified as *C. caracasana* in one of our previous publications, in which we reported the isolation of two 3,4-*seco*-entkaurenes [4]. *C. matourensis* is a medium-sized tree found mainly in the south of Venezuela where it is commonly known as *tabaquillo.* The isolation of a diterpene known as maravuic acid has been reported from a Brazilian specie of *C. matourensis* [5]. Many members of *Croton* genus are aromatic and produce essential oils on distillation, which are used for medicinal purposes such as inflammations, gastric ulcers, diabetes, diarrheas, rheumatism, wound healing, cancer and as anti-inflammatory and analgesic agents [6-11]. The composition of essential oils of a considerable number of species of *Croton* from all over the world has been reported in the literature, showing that the main components are phenylpropanoids, monoterpenes and sesquiterpenes [12-15].

In the context of a research project in which the chemistry and pharmacology of *Croton* species from Venezuela are investigated [16-18], the analysis of volatile constituents from *C. matourensis* and *C. micans* has been undertaken. In our pharmacological screening, the aqueous and dichloromethane extracts obtained from the leaves showed strong inhibition on three different leukemia cells lines. (unpublished results). Hence, in view of the results obtained with the aqueous extract, the essential oils obtained from leaves and flowers from these plants were screened for cytotoxic activity using the MTT assay against a panel of human tumour cell lines: LoVo (colon carcinoma), HeLa (cervical cancer), X-17 (colon carcinoma) and normal control cells, and their chemical composition determined by analysis of GC and GC-MS.

2. Materials and Methods

2.1. Plant Material

C. matourensis leaves were collected in February 2006 from trees growing wild near the airport of Santa Elena de Uairen, Bolivar State of Venezuela. The botanical identity of the plant was confirmed by Dr. Anibal Castillo and a voucher specimen with the number 22600 was deposited at the Herbarium of the Botanical Garden of Caracas (VEN). Fresh material was used for extraction of the essential oil. *C. micans* was collected during flowering in June 2006, from Ocumare de la Costa, Aragua State, Venezuela. The specimen was identified by Dr. Stephen Tillett and Dr. Ricarda Riina. A voucher specimen with the number MYF-26701 was deposited at the Herbarium Víctor Manuel Ovalles (MYF) of the Pharmacy Faculty, Universidad Central de Venezuela.

2.2 Extraction of the Essential Oils

Leaves of *C. matourensis* (0.3 Kg) and fresh flowers and leaves of *C. micans* were subjected to hydrodistillation for 3 h using a Clevenger–type apparatus. The oils were dried over anhydrous sodium sulphate, and stored in sealed vials under nitrogen at 5 ^{o}C until analyzed.

2.3 Gas Chromatography

GC analyses were accomplished using an Agilent 5890 gas chromatograph equipped with a FID and HP-5 capillary column (30 m x 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 7 min at 50^{o}C, and subsequently at 5 ^{o}C/min up to 200^{o}C; injector temperature 200^{o}C, detector temperature 250^{o}C. Helium was used as carrier gas, at a flow rate of 1mL/min. Split ratio, 1:10.

2.4. Gas Chromatography-Mass Spectrometry

GC/MS analyses were carried out using a Varian Saturn 2000 GC-MS system operating in the EI mode at 70 eV, using a DB-5 capillary column (30 m x 0.25 mm, 0.25 μm film thicknesses). The temperature program was 60°- 300°C at a rate of 5 °C/min. Injector and transfer line temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas, flow rate of 1mL/min, split ratio, 1: 10; injection of 0.2 μl (10% chloroform solution). Identification of the constituents was based on the comparison of their retention times in relation to those of the *n*-alkanes series and mass spectra, which were compared with those of NIST [19] and Wiley [20] libraries. The retention indices were compared with those of literature [22-23].

2.5. Human cell lines

Human colon adenocarcinoma cell line (LoVo, P53 wild type), colon adenocarcinoma, variant genetic cell of LoVo deficient in the protein P53 cell line (X-17) and human uterus cell line HeLa, were kindly provided by Dr. Marie France Poupon at The Institute Curie, Paris France, and were maintained in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum, 1% of L-glutamine and 1% streptomycin (all obtained from Sigma-Aldrich, USA). Primary culture of human dermis fibroblast were grown in DMEM medium. Cells were grown in a humidified incubator with 5% CO_2 and 95% air at 37 °C until they reached the exponential growth phase.

2.6. Cytotoxicity assay

A 96-well microtiter plate (tissue culture grade) containing 0.2 ml of growth medium/per well (RPMI) was seeded with sufficient cells to provide approximately 70% growth confluence after 24-48 h of culture. At this point, cells were exposed to DMSO solution of the essential oils for 72 h at concentrations ranging from 5 to 25 μg/mL, and then evaluated for cytotoxicity. In all cases although the oil was dissolved in DMSO, for which the final concentration in the culture medium was lower than 1%, a concentration that has neither cytotoxic effect nor causes any interference with the colorimetric detection method. Cytotoxicity was determined following the reduction of 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). Cells grown in the microtiter were first incubated with MTT at 37°C for 3h, then they were washed with phosphate buffered saline (PBS) and, finally the colorimetric detection was done by the addition of Formazan (dissolved in 1% DMSO). Absorbance was measured by a microplate reader (SpectraFluor®, Tecan) set at a wavelength of 570 nm. The IC_{50} value in the MTT assay was defined as the concentration of test oil resulting in a 50% reduction of absorbance compared with untreated cells. Adriamycin was used as control drug.

3. Results and Discussion

Fresh leaves and flowers of *C. micans,* extracted by hydrodistillation, afforded pleasant-smelling yellow oils, the yields being 0.13% (w/w) in leaves and 0.25% (w/w) in flowers, according to their dry weight. The yield of oil of *C. matourensis* leaves was 1.0 %. The oils were separately subjected to GC and GC/MS analysis. In the flower oil of *C. micans*, 63 compounds were identified accounting for 98.9% of the oil, and 56 components in the leaf oil which represent 97.3 % of its total composition. The components identified in each oil, their retention indices and their percentage composition are summarised in Table **1.** The main components identified from the flower oil were: fenchyl acetate (41.6%), α-caryophyllene (12.6%), β-caryophyllene (5.5%), β-cubebene (5.0%), α-cubebene (5.3%), β-elemene (4.7%), and valencene (4.6%). The leaf oil consisted mainly of: fenchyl acetate (25.3%), α-caryophyllene (20.7%), α-selinene (12.8%), β-bourbene (9.3%). Comparing the

chemical composition of the two oils, they share a high number of constituents although at different levels. Quantitative rather than qualitative differences in oil composition were observed. Many of the compounds identified in these oils have previously been identified in the oil from other *Croton* species, previously studied by us [16-17], and also in other species described in the literature [12-13]. However, the chemical composition of the oil from the leaves, found in the present study, seems to be quite different from that reported for the Brazilian material a few years ago [22], where the major components were the sesquiterpenes β-caryophyllene, β-elemene and germacrene B. Monoterpenes were minor elements and no phenylpropanoids were found. This result confirms that the chemical composition of the oil of species of *Croton* can vary depending on where the plants are grown, or from genetic differences in distinct populations.

In the oil obtained from *C. matourensis*, more than 95.5% of the volatile oil was identified. Results obtained in the qualitative and quantitative analyses are also shown in Table 1, where all the compounds are arranged in the order of elution on DB-5 column. A total of 46 components have been identified. The main components are fenchyl acetate (19.5%), methyleugenol (14.2%), isoelemicine (11.3%), elemicine (7.6%), spathulenol (6.9%) and valencene (5.8%). Other minor components with smaller amounts are limonene (3.4%). α-cubebene (2.3%), α-cadinol (2.2%) and terpinen-4-ol (2.0%).

The essential oils showed a very diverse composition. The most representative compounds in the oils from leaves of *C. micans,* were sesquiterpene (53.7%), followed by monoterpenes (38.8%) and small amount of phenylpropanoids (3.5%). However, the oil from leaves of *C. matourensis,* is predominantly composed by monoterpenes (35.1%) followed by phenylpropanoids (25.9%), and sesquiterpenes (24.2%) represent the minor group in this complex mixture. The component found in major concentration in the oils (fenchyl acetate), belongs to the type of monoterpene compounds. The only report of the chemical composition of the oil from *C. matourensis* from Brazilian origin shows that our results differ from those obtained by Brazilian investigators, which indicated that the oil also was rich in monoterpenes, but noteworthy was the high content of α-pinene with (86.7%) in the composition of the Brazilian oil [23]. In our case the oil presents a high diversity in components with remarkable richness in phenylpropanoids. The differences and similarities in composition herein discussed, show again that the variability in the composition of essential oils depends essentially upon the origin of the samples, the influence of geographic circumstances and climate. Our results, however, are in general accordance with those of many species in which the characteristic constituents of oil of *Croton* species were in some cases phenylpropanoids and in other monoterpenes hydrocarbons [14, 24].

The *in vitro* cytotoxicity bioassays of the essential oils against three human cancer cell lines LoVo (colon carcinoma), HeLa (cervical cancer), X-17 (colon carcinoma) and one normal control represented by dermis fibroblast, revealed a regular cytotoxiciy. The results are summarized in Table **2**. Cytotoxicity was expressed as the concentration of oil inhibiting cell growth by 50% (IC_{50}). All the cell lines were submitted to increasing concentrations of essential oils (5, 15, 25 μg/mL). The results, in which the IC_{50} values are between 36.6 μg/mL for LoVo and 87.9 μg/mL for HeLa cells, indicate a moderate activity on the cancer cells and the normal cell line (50.0 μg/ML) used in the current investigation. Interestingly the IC_{50} values obtained with the normal dermis fibroblasts indicates that the tumor cells are more sensitive to the oil components, since the IC_{50} obtained with the normal fibroblast is higher that the IC_{50} shown for the tumor cells. It is evident that the oil isolated from leaves of *C. micans* was more active against Hela and LoVo cells. The three oils were inactive against the X-17, which represents a cancer cell that has a mutation in the P-53 protein, making it more aggressive. Some of the components in high concentration such as terpinen-4-ol, D-limonene, spathulenol, α-cadinol, β-elemene, have been reported with activity against breast cancer, colon cancer, gastric cancer cells, and lung, ovarian and laryngeal cancer cell lines [25].

Table 1. Chemical composition of *Croton micans and Croton matourensis* essential oils.

Compound	RI	Peak area (%)			Identification
		C.micans Leaf oil	*C. micans* Flower oil	*C. mataourensis* Leaf	
α-thujene	925	0.1	0.2	nd	RI,MS
α-pinene	936	0.3	0.1	1.3	RI, MS
α-fenchene	945	0.6	1.5	nd	RI
Sabinene	962	0.1	0.3	nd	RI, MS
Verbenene	965	0.3	0.1	2.1	RI, MS
β-myrcene	972	0.2	1.3	nd	RI, MS
β-pinene	986	0.9	0.2	1.5	RI, MS
δ-3-carene	1010	0.2	0.1	nd	RI, MS
D-limonene	1018	2.9	1.3	3.4	RI
p-Cymene	1030	0.7	1.1	0.1	RI, MS
δ-terpinene	1063	nd	0.1	1.1	RI,MS
Fenchone	1068	0.3	0.4	0.5	RI, MS
Linalool	1091	2.2	1.5	1.2	RI, MS
α-fenchol	1104	2.0	0.1	nd	RI, MS
β-fenchol	1110	0.6	0.2	nd	RI, MS
Terpinen-4-ol	1158	1.1	1.2	2.0	RI, MS
Norborneol	1167	nd	tr	tr	RI, MS
α-Terpineol	1190	0.5	tr	1.2	RI, MS
Myrtenal	1198	0.3	0.1	tr	RI, MS
trans-carveol	1212	nd	0.2	1.0	RI,MS
Fenchyl acetate	1223	25.3	41.6	19.5	RI, MS
Geraniol	1242	nd	0.1	0.2	RI, MS
Pulegone	1234	0.2	0.2	nd	RI
Thymol	1291	nd	tr	0.1	RI, MS
Carvacrol	1293	0.1	nd	0.4	RI
Eugenol	1347	1.1	tr	0.5	RI, MS
α-cubebene	1380	0.8	5.3	2.3	RI, MS
β-bourbene	1382	9.3	nd	nd	MS
β-cubebene	1386	2.7	5.0	nd	RI, MS
β-elemene	1392	0.5	4.7	nd	RI, MS
Methyleugenol	1400	nd	0.1	14.2	RI,MS
β-caryophyllene	1422	0.7	5.5	0.5	RI, MS
α-humulene	1442	nd	1.1	0.4	RI, MS
α-caryophyllene	1448	20.7	12.6	nd	RI, MS
Unknown	1459	nd	nd	0.2	--
Germacrene-D	1467	nd	1.1	0.1	RI, MS
Alloaromadendrene	1474	01	nd	0.1	RI, MS
Veratral	1480	0.4	0.2	nd	RI, MS
Ar-curcumene	1482	nd	nd	0.1	MS
Valencene	1485	2.9	4.6	5.8	RI, MS
α-Selinene	1501	12.8	nd	1.0	RI, MS
Methylisoeugenol	1509	tr	tr	0.5	RI, MS
β-bisabolene	1509	0.2	tr	0.3	RI, MS
Bisabolene	1520	nd	0.3	nd	RI, MS
δ-Cadinene	1526	0.2	tr	1.0	RI, MS
Calamenene	1532	tr	tr	nd	RI, MS
Elemol	1540	0.2	0.2	nd	RI, MS
Ledol	1545	0.2	tr	nd	RI,MS

Table 1 Continued

Unknown	1548	1.0	0.1	0.1	---
Elemicin	1550	1.6	1.1	7.6	RI,MS
γ- Eudesmol	1560	tr	0.2	nd	RI,MS
Spathulenol	1565	0.9	0.1	6.9	RI,MS
Globulol	1580	0.5	tr	nd	RI,MS
Caryophillene oxide	1587	0.2	1.1	0.9	RI,MS
Humulene epoxide	1593	nd	t	0.3	RI,MS
3,4,5-trimethoxybenzaldehyde	1623	0.1	nd	1.3	RI,MS
Unknown	1595	0.1	0.1	nd	---
Cedrol	1638	0.1	t	t	RI,MS
Carotol	1649	0.1	nd	0.1	MS
Bisabolol	1653	0.1	0.3	0.2	RI,MS
Isoelimicine	1656	0.2	t	11.3	RI,MS
Agarospirol	1664	t	t	0.1	RI,MS
α-bisabolol	1667	nd	0.1	1.8	RI,MS
Cubenol	1669	0.1	nd	t	RI,MS
α-Cadinol	1672	t	2.1	2.2	RI,MS
τ-Cadinol	1673	nd	0.1	nd	RI,MS
δ- Cadinol	1684	0.1	t	nd	RI,MS
α-11-Eudesmene	1688	nd	0.2	nd	RI,MS
Unknown	1690	0.2	0.3	nd	--
4α-Seleniol	1692	nd	0.2	nd	RI,MS
Ledenol	1761	0.2	t	0.1	RI,MS
α-Cyperone	1783	0.1	0.3	nd	RI,MS
Total identified		97.3	98.9	95.5	
Monoterpenes Sesquiterpenes		38.8	51.9	35.1	
Phenylpropanoids		53.7	45.1	24.2	
Unknown		3.5	1.4	25.9	
		1.3	0.5	0.3	

nd: not detected
t :trace < 0.05 %

Table 2. Evaluation of cytotoxicity in cell lines

Cell	Oil-CmF (IC_{50})	Oil-CmL (IC_{50})	Oil-Cma (IC_{50})
LoVo	103.27	45.85	36.60
HeLa	87.91	54.95	83.90
X-17	*	*	*
Fibroblasts	50.00	81.28	132.73

Oil-CmiF : essential oil of flowers from C. micans
Oil-CmiL: essential oil of leaves C. micans
Oil-Cma: essential oil of from C. matourensis
* No effect

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