Article

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Chemical Analysis of Essential Oils from *Ocotea gomezii* W.C. Burger and *Ocotea morae* Gómez-Laur. (Lauraceae) Collected at "Reserva Biológica Alberto M. Brenes" in Costa Rica and their Cytotoxic Activity on Tumor Cell Lines

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A composição química dos óleos essenciais obtidos de folhas, cascas e troncos de *Ocotea gomezii* e *O. morae* de populações silvestres da Costa Rica, está sendo descrita pela primeira vez. Os óleos de *O. gomezii* são constituídos principalmente por sesquiterpenóides enquanto os de *O. morae* apresentaram mono- e sesquiterpenóides na mesma proporção. A análise da composição química por CG/EM e CG/DIC resultou na identificação de 166 componentes, correspondente a 89,4-98,1% dos óleos totais. Quando comparada a atividade de todos os óleos obtidos sobre linhagens de células CCF-STTG1, Hep3B, HepG2, H-460, AGS, N-87, SW-620, MCF-7 e VERO, observou-se que as células de astrocitoma foram as mais resistentes aos mesmos. Concluiu-se que os óleos essenciais de folhas, cascas e tronco de *Ocotea gomezii* e *Ocotea morae* podem conter alguns compostos tóxicos, mas o uso potencial dos mesmos contra as células tumorais foi muito baixo, pois são tóxicos na mesma extensão, para as linhagens de células tumorais e não-tumorais.

The chemical composition of the essential oils of the leaves, bark and wood of *Ocotea gomezii* and *O. morae* from Costa Rica, were analyzed by capillary GC-FID and GC-MS. The oils of *O. gomezii* were predominantly composed by sesquiterpenoids whereas the oils of *O. morae* had both monoterpenoids and sesquiterpenoids. Analysis by GC/MS and GC/FID resulted in the identification of 166 compounds, representing about 89.4–98.1% of the total oils. When we compared the effect of the oils on cell lines (CCF-STTG1, Hep3B, HepG2, H-460, AGS, N-87, SW-620 and MCF-7 and VERO), we found that astrocytoma cells were the most resistant ones. We conclude that the essential oils of *Ocotea gomezii* and *Ocotea morae* could have some toxic compounds, but the potential use of them against the tumor cells would be very low, since they could be toxic to tumor and non-tumor cells in the same extent.

Keywords: *Ocotea gomezii, O. morae*, Lauraceae, essential oils, cytotoxicity

Introduction

The genus *Ocotea* (Lauraceae) is widely represented in the American Tropics with 300-400 species, being the largest genus of this family in Mesoamerica, with 102 species.[1] Lauraceae is a family with about 2500-3000 species of mostly tropical trees.[2] This family is an important component of cloud forests in Costa Rica where the individuals occur in high abundance and diversity.[3,4] It can be recognized by the simple, alternate, stiff and aromatic elliptic to obovate leaves and fruits often borne in a cup. Worldwide, this family has a considerable economic importance because it is used as a source of timber for construction and furniture (*Nectandra*, *Ocotea*, *Persea* spp.), as a crop (*Persea americana* Mill., avocado), and to obtain flavors for food industry, perfumery and medicines (*Cinnamomum zeylanicum* Bl., *C. cassia* Pressl.).

Ocotea gomezii W.C. Burger is an unusual species distinguished by its ferruginous puberulence and broadly rounded leaves. It is a tree of about 6-10 m tall and endemic of Costa Rica. The geographic distribution of the species includes the Central Volcanic Mountain and extends from near Volcán Rincón de la Vieja in the West, to Moravia de Chirripó in the East of Costa Rica.[3] *O. morae* Gómez-Laur. is a tree of 18-22 m tall, with large fruits, readily recognized by their large 65 mm broad cupules, and 58 mm long and

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60 mm wide fruits.[5] This tree is also endemic of Costa Rica and its geographic distribution is restricted to the humid Cordillera de Tilarán slopes at *ca*. 850 m of elevation at the "Reserva Biológica Alberto M. Brenes", managed and administered by the Universidad de Costa Rica.[6]

Several phytochemical investigations have been performed on plants of the genus *Ocotea*. These plants are well known as a source of aporphine alkaloids,[7,8] lignans and neolignans[9,10] and phenylpropanoids.[11] The chemical composition of the volatile oils of *Ocotea* species has been the subject of several studies.[12-37]

These two endemic plants, *Ocotea gomezii* and *O. morae*, are barely studied from the chemical point of view. One report indicates that the aporphine alkaloid (+)-preocoteine is present in the bark of *O. gomezii*.[38]

Several *Ocotea* essential oils have been studied for their biological activities. For instance, oils from flower calyces and leaves of *O. quixos* ("flor de canela", American cinnamon) presented *in vitro* antioxidant, antibacterial and antifungal activities[24,39] and also anti-inflammatory[40] and antiplatelet properties.[41,42] Oil from the calyces of *O. bofo* also presented antimicrobial and antioxidant activities.[28] Essential oil from the stem bark of *O. bracteosa* presented molluscicidal activity,[33] and oils of *O. duckei* showed significant cardiovascular effects.[37] Setzer and co-workers[29] also determined the activity of leaf essential oils of ten *Ocotea* species from Monteverde, Costa Rica, against cruzain (Chagas disease).

Regarding the cytotoxic effect against human cells, there are several studies with *Ocotea* species that include: *O. endresiana*,[36] *O. floribunda*,[35] *O. meziana*,[43] *O. praetermissa*,[36] *O. tonduzii*,[34] *O. veraguensis*,[44] *O. whitei*[44] and some unidentified ones (*Ocotea* new species "los llanos" and *Ocotea* new species "small leaf").[43] Basically, it has been demonstrated that some of them showed some toxicity against breast cancer cells.[34,36,43,44] There is also one study with essential oil from *O. floribunda* that showed cytotoxicity against hepatoma cells HepG2.[35]

In this paper, we report the chemical composition and cytotoxic properties of six essential oils obtained from *O. gomezii* and *O. morae* from three different parts of the plants (leaves, bark and wood) and we show their complex composition and inespecific toxicities.

Experimental

Plant collection and oil isolation

Plant materials were collected in May, 2000 at the "Reserva Biológica Alberto M. Brenes" near the San Lorencito River, in the humid Caribbean slope of the Tilarán mountain range, province of Alajuela. Voucher specimens were deposited at the Herbarium of the Universidad de Costa Rica (herbarium numbers USJ-30631, USJ-77417). The samples were dried in the shade at room temperature (4 days). Then, the plant material was chopped and submitted to hydrodistillation (3 h) by using a modified Clevenger-type apparatus. The distilled oils were collected and dried over anhydrous sodium sulfate (Merck) and stored in a refrigerator. The yields (v/m) of the oils were: *O. gomezii* (leaves 0.4%, bark 0.1%, and wood 0.1%); *O. morae* (leaves 0.5%, bark 0.3%, and wood 0.2%). The oils were labeled as, *Og*L: *Ocotea gomezii* (leaves), *Og*B: *O. gomezii* (bark), *Og*W: *O. gomezii* (wood), *Om*L: *Ocotea morae* (leaves), *Om*B: *O. morae* (bark) and *Om*W: *O. morae* (wood).

Chemical analysis

The oils of *O. gomezii* and *O. morae* were analyzed by GC-FID using a Shimadzu GC-17 gas chromatograph with a Shimadzu Class-VP, version 4.3 software. The GC column was a Heliflex AT-5 (Alltech), 5% phenyl-95% methylpolysiloxane fused silica capillary (30 m × 0.25 mm; film thickness 0.20 μm). Operating conditions were: carrier gas N_2, flow 1.0 mL min^{-1}; oven temperature program: 60-220 °C at 3 °C min^{-1}, 220 °C (10 min); injection size: 0.1 μL (pure oil); sample injection port temperature 250 °C; detector temperature 275 °C; split 1:50.

The analysis by GC-MS was performed using a Shimadzu GC-17A gas chromatograph coupled with GCMS-QP5050 apparatus and CLASS 5000 software with Wiley138 computer database. The GC column was a Heliflex AT-5 (Alltech), 5% phenyl- 95% methylpolysiloxane fused silica capillary (30 m × 0.25 mm; film thickness 0.20 μm). Operating conditions were: carrier gas He, flow 1.0 mL min^{-1}; oven temperature program: 60-240 °C at 3 °C min^{-1}; injection size: 0.1 μL (pure oil); sample injection port temperature 250 °C; detector temperature 260 °C; ionization voltage: 70 eV; ionization current 60 μA; scanning speed 0.5 s over 38-400 amu range; split 1:70.

Identification of the oil components was performed using the retention indices on a DB-5 type column,[45] and by comparison of their mass spectra with either those published in the literature[46] or those from our own database. Integration of the total chromatogram, expressed as area percent, has been used to obtain quantitative compositional data.

Cell culture

Astrocytoma (CCF-STTG1), hepatocellular carcinoma (Hep3B, HepG2), lung large cell carcinoma (H-460),

gastric carcinoma (AGS, N-87), colon adenocarcinoma (SW-620), breast carcinoma (MCF-7) and kidney epithelial (Vero) cell lines were obtained from the American Type Culture Collection (ATCC) or National Cancer Institute (NCI), USA. Cells were maintained in Dulbecco essential medium supplemented with 10% fetal bovine serum, 2 mmol L^{-1} of glutamine, 100 IU mL^{-1} of penicillin and amphotericin B in a 37 °C humidified incubator under an atmosphere of 7% CO_2 on air. For the experiments, cells were cultured in 96-well plates (15,000 cells/well) and allowed to adhere overnight.

Cytotoxicity assay

Various concentrations of essential oils, previously dissolved in 95% ethanol, were added to the plates in 100 μL of fresh medium and incubated for 48 h. After incubation, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) was added to each well to a final concentration 0.5 mg mL^{-1} and after 2 h at 37 °C medium was carefully removed from the plates and 95% ethanol was added to the wells with the purpose of dissolving formazan crystals.[47] Absorbances were read at 570 nm and viability percentages were calculated, using samples incubated with 95% ethanol dissolved in culture medium as 100% viability values. (*R*)-(+) Limonene (Sigma Aldrich) was used as a standard to assure its values were always constant and the cells remained equally resistant to its effects in every performed experiment. LD_{50} values were calculated from concentration *versus* viability plots using SlideWrite® Plus 6.1 (Advanced Graphics Software, Inc., Carlsbad, CA), to obtain the concentrations able to induce 50% of cytotoxicity.

Statistical analysis

Cytotoxicity values were analyzed by ANOVA followed by Tukey's test and $p < 0.05$ were considered statistically significant.

Results and Discussion

From the hydrodistilled oils, a total of 166 compounds were identified, accounting for 89.4-98.1% of the total composition of the essential oils. The chemical composition of the volatiles is listed in Table S1 (see Supplementary Information, SI).

Essential oils from *O. gomezii* were rich in sesquiterpenoids (67.3-94.9%) with a minor quantity of monoterpenoids (0.7-12.6%). Main constituents of the leaf oil were pentan-2-ol (12.5%), *epi*-α-cadinol (9.8%), δ-cadinene (7.7%) and 1,8-cineole (6.0%) along with small amounts of γ-cadinene, *cis*-muurola-4(14),5-diene, α-muurolene and oxygenated sesquiterpenes viridiflorol, 1,10-di-*epi*-cubenol and globulol. *O. gomezii* bark essential oil was composed primarily of sesquiterpenoids (94.9%) and contained δ-cadinene (14.5%), 1,10-di-*epi*-cubenol (7.7%), and α-muurolene (6.9%) along with small amounts of γ-cadinene, *allo*-aromadendrene, α-cubebene, α-cadinol, *epi*-α-cadinol, globulol and viridiflorol. Main compounds of wood oil of *O. gomezii* were *epi*-α-muurolol (15.0%), *epi*-α-cadinol (10.0%), and δ-cadinene (7.7%), along with small amounts of 1,10-di-*epi*-cubenol, khusinol, epizonarene, viridiflorol and globulol. Moreover, essential oils from *O. gomezii* were rich in sesquiterpenes of the cadinene type (*ca*. 47-65%) mainly based on the cadinane and the muurolane skeletons.

Essential oils from the other analyzed species, *O. morae*, were all rich in sesquiterpenoids (54.0-71.0%) and monoterpenoids (24.3-42.5%). Leaf oil was composed by monoterpenoids β-pinene (17.5%), α-pinene (10.4%) and 1,8-cineole (7.3%), and sesquiterpenes bicyclogermacrene (8.8%), germacrene D (7.5%), β-caryophyllene (7.1%) and β-selinene. Major constituents of bark oil were 1,8-cineole (12.8%) and β-caryophyllene (6.1%), along with small amounts of δ-cadinene, caryophyllene oxide, β-selinene, α-cadinol, 1-*epi*-cubenol and spathulenol. Sesquiterpenoids (71.0%) were the main constituents of wood essential oil of *O. morae*, containing (*E*)-nerolidol as the main constituent (11.4%) accompanied by other sesquiterpenoids such as *epi*-α-muurolol (6.3%), δ-cadinene (6.2%), α-cadinol (6.0%), β-caryophyllene, β-cubebene and α-copaene. Also, there were present the monoterpenoids 1,8-cineole (7.1%), camphene and α-pinene.

The oils were predominantly terpenoid in nature like other studied *Ocotea* oils from Costa Rica.[26,30,32] Of the six oils analyzed in this work only the leaf oil from *O. morae* contained a very small quantity of the benzenoid compounds benzaldehyde (0.1%) and the esters benzyl benzoate (0.4%) and benzyl salicylate (0.1%). Oils from Costa Rica *Ocotea* spp. are lacking of phenylpropanoid constituents (like safrole, cinnamaldehyde, methylcinnamate, *O*-methyleugenol, asaricin, elemicin, and others, all volatiles with distinctive aromas) that are typical of some *Ocotea* essential oils mainly from South America origin.[12-25]

We determined the cytotoxicity of essential oils on eight different tumor cell lines, and non-tumoral cells (Vero). Cell lines were derived from tumors from lung, liver, colon, breast, stomach (primary tumor and liver metastasis) and an astrocytoma (Table 1). Except for bark

Table 1. LD_{50} of six essential oils obtained from different parts of the plants *O. gomezii* and *O. morae*

Cell line	*Og*L, (μg mL^{-1})	*Og*B (μg mL^{-1})	*Og*W (μg mL^{-1})	*Om*L (μg mL^{-1})	*Om*B (μg mL^{-1})	*Om*W (μg mL^{-1})	Limonene (μg mL^{-1})
Vero (non-tumoral)	175 ± 21	150 ± 28	456 ± 83[a]	344 ± 44	293 ± 47	234 ± 52	896 ± 152
H460 (lung)	160 ± 30	119 ± 9	414 ± 41[b]	353 ± 105	139 ± 31	218 ± 52[a]	616 ± 74
HepG2 (liver)	137 ± 48	79 ± 20	94 ± 4[c]	187 ± 55[a]	178 ± 50	166 ± 41[b]	1032 ± 45
Hep3B (liver)	137 ± 21	124 ± 33	293 ± 16[d]	282 ± 110[b]	201 ± 22	278 ± 23	466 ± 85
SW620 (colon)	122 ± 15	94 ± 10	187 ± 20[e]	201 ± 27[c]	132 ± 50	190 ± 30	924 ± 76
MCF7 (breast)	167 ± 22	160 ± 1	181 ± 79[f]	274 ± 19[d]	186 ± 18	260 ± 11	629 ± 211
AGS (stomach)	109 ± 27[a]	95 ± 7	260 ± 11[g]	183 ± 55[e]	185 ± 10	209 ± 36	774 ± 53
N87 (stomach, metastasis)	239 ± 52	132 ± 11	418 ± 117[h]	403 ± 46	234 ± 19	256 ± 15	796 ± 205
CCF-STTG1 (astrocytoma)	297 ± 12[a]	184 ± 38	862 ± 144[a,b,c,d,e,f,g,h]	744±2[a,b,c,d,e]	262 ± 67	587 ± 221[a,b]	833 ± 24

*Superscript letters represent statistically significant differences in the cytotoxicity observed between some of the oils on the different cell lines (comparison is made in each column). $p < 0.05$ is considered statistically significant. Limonene is used as an internal standard. *Og*L: *Ocotea gomezii* (leaves);*Og*B: *O. gomezii* (bark); *Og*W: *O. gomezii* (wood); *Om*L: *O. morae* (leaves); *Om*B*: O. morae* (bark); *Om*W: *O. morae* (wood).

oils, all the other volatiles showed statistically significant differences in toxicity between astrocytoma cells and the other ones, but the effect was not observed among the other cell lines, or between tumor cell lines and non-tumor Vero cells. When we compared the effect of the oils taking all the cell lines together, we only observed statistically significant differences between samples *Og*B and *Og*W; *Og*B and *Om*L and *Og*W and *Og*L. The effect of limonene was very low compared to the volatiles tested, but worked well as an internal standard for the experiments, to assure the cells were kept under the same degree of sensitivity along the time they were in culture.

One of the few *Ocotea* essential oils reported in the literature for its biological activities is *O. quixos*, which shows antifungical and antibacterial activities.[39] This leaf oil presents as main identified compounds: caryophyllene, humulene and eremophyllene. The first compound is present in significant amounts in *O. morae* oils too, whereas humulene is present in small amounts in the oils of both plants analyzed here.

There are just a few reports in the literature regarding the toxic effect of *Ocotea* essential oils on animal cells, and most of the studies have been carried out in breast cancer cells only.[34-36,43,44] We showed here that these species have some toxicity, but due to their complex chemical composition, no assumptions can be made about the compounds responsible for these activities. Compounds such as germacrene D, β-caryophyllene, α-cadinene and α- and β-pinene have been shown to be toxic on cell lines such as MCF-7, a breast carcinoma cell line.[43] All these compounds are present in the *Ocotea* species tested in this article, so they could be responsible for the relative toxicity observed here. Another compound found in these volatiles, 1,8-cineole, has been shown to induce apoptosis on KB cells (human oral epidermoid carcinoma), indicating that could play a role in the cytotoxicity observed here.[48] Some antagonistic effects between some of the compounds present in these oils have been also reported in the literature.[43]

Supplemantary Information

Supplementary data (Table S1) are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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