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Chemical composition and antioxidant activity of essential oils isolated from Colombian plants

Jesús Olivero-Verbel,[,1,2] Tulia González-Cervera,[1] Jorge Güette-Fernandez,[1] Beatriz Jaramillo-Colorado,[1,2] Elena Stashenko[2]*

[1]Environmental and Computational Chemistry Group. Faculty of Pharmaceutical Sciences. Campus of Zaragocilla. University of Cartagena. Cartagena, Colombia,
[2]Chromatography Laboratory, Research Centre of Excellence, Cenivam, Industrial University of Santander, Building 45, Bucaramanga, Colombia.

RESUMO: "Composição química e atividade antioxidante de óleos essenciais isolados de plantas da Colombia." Treze óleos essenciais de plantas da Colômbia, obtido por hidrodestilação ou hidrodestilação assistida por microondas, da planta inteira, caule, folhas e flores, foram analisados por técnicas de espectrometria de massa acoplada a cromatografia gasosa. Foi avaliada a citotoxicidade de óleos essenciais utilizando o ensaio com *Artemia salina* e suas atividades antioxidantes medindo seus efeitos sobre os níveis de substâncias reativas ao ácido tiobarbitúrico em microssomas de fígado de rato induzida por Fe^{2+}/H_2O_2. Cinco óleos apresentaram alta citotoxicidade (LC50<10 µg/mL) sobre o ensaio de *Artemia salina*. A atividade antioxidante do óleo essencial de *Ocotea* sp., *Tagetes lucida* e *Lippia alba* (quimiotipo geranial) apresentaram os menores valores de Concentração Eficaz (CE50), na ordem de 31,1, 37,9 e 94,9 µg/mL, respectivamente. Os principais componentes desses óleos foram α-pineno (42%), estragol (95,7%) e geranial (30,4%), respectivamente. *Elettaria cardamomum* e *Lippia alba* (quimiotipo carvona, Tolima) apresentaram moderada atividade antioxidante com valores de CE50 de 130,5 e 174,4 µg/mL, respectivamente. Os óleos essenciais de mollis *Minthostachys mollis, Lippia alba* (quimiotipo carvona, Cundinamarca), e *Piper sanctifelisis* não mostraram atividade antioxidante (CE50>1000 µg/mL).

Unitermos: Óleo essencial, hidrodestilação, atividade antioxidante, TBARS, citotoxicidade, Colombia.

ABSTRACT: Thirteen essential oils from Colombian plants, obtained by hydrodistillation or microwave-assisted hydrodistillation of total plant, stem, leaves, and flowers were analyzed by gas-chromatography-mass spectrometry techniques. Cytotoxicity of essential oils was assessed using the brine shrimp assay, and their antioxidant activities measuring their effects on the levels of thiobarbituric acid reactive substances on rat liver microsomes induced by Fe^{2+}/H_2O_2. Five oils showed high cytotoxicity (LC50<10 µg/mL) on the brine shrimp assay. The antioxidant activity of the essential oil from *Ocotea* sp., *Tagetes lucida* and *Lippia alba* (geranial chemotype) showed the lowest mean effective concentrations (EC50), with values of 31.1, 37.9 and 94.9 µg/mL, respectively. The main components for these oils were α-pinene (42%), estragole (95.7%) and geranial (30.4%), respectively. *Elettaria cardamomum* and *Lippia alba* (carvone chemotype, Tolima) presented moderate antioxidant activities with EC50 values of 130.5 and 174.4 µg/mL, respectively. Essential oils from *Minthostachys mollis, Lippia alba* (carvone chemotype, Cundinamarca), and *Piper sanctifelisis* did not show antioxidant activity (CE50>1000 µg/mL).

Keywords: Essential oil, hydrodistillation, antioxidant activity, TBARS, Cytotoxicity, Colombia.

INTRODUCTION

Essential oils are natural products with many applications and demand in the food, perfumery, cosmetics, pharmaceutical and winery industries, among others (Arbujarai & Natsheh, 2003; Ajose, 2007; Natsch & Wasescha, 2007; Fisher, 2008). These complex mixtures have different pharmacological properties, including anti-inflammatory (Tabanca et al., 2007; Tekeoglu, 2007), antibacterial (Ravishankar et al., 2008; Sharififar et al., 2007), antifungal (Farzaneh et al., 2006), protective of the gastrointestinal tract (Monteiro et al., 2007), anticonvulsant (Viana et al., 2000) and antioxidant properties (Safaei-Ghomi et al., 2009; Li et al., 2007; Magwa et al., 2006). This last characteristic makes these chemicals suitable as food preservatives, as the free radical oxidation of the lipid

*E-mail: joliverov@unicartagena.edu.co

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components in these products is a major strategic problem for manufacturers (Pokorny et al., 2001). A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and the related compound butylated hydroxytoluene (BHT) are currently used in food processing (Soarez et al., 2003; Henderson & Slickman, 1999). However, these compounds are known by their capability of promoting tumor formation (Malkinson et al., 1997). Thus, the search for new, natural, and low toxicity antioxidants is extensive, and numerous compounds have been tested using a diversity of methodologies (Ruberto et al., 2000; Kulisic et al., 2004). However, much remains to be clarified about how they exert their antioxidant properties, although it has been suggested that some have the ability to trap radicals (Foti & Ingold, 2003).

Despite the fact that Colombia has an immense biodiversity, few studies have been reported on the biological properties of essential oils from plants that grow in this country. The aim of this work was to determine the composition of essential oils from plants collected in different regions of this country, and to evaluate their cytotoxicity and antioxidant activity in a lipid peroxidation model using rat liver homogenates.

MATERIAL AND METHODS

Plant material collection and treatment

Thirteen plants of six different families (Gramineae, Labiateae, Magnoliaceae, Piperaceae, Verbenaceae, and Zingiberaceae) were collected at several regions of Colombia (Antioquia, Arauca, Bolívar, Boyacá, Chocó, Cundinamarca, Nariño, and Tolima. Undamaged plants (stems, leaves, flowers) were transported to the Cenivam Laboratory at the Industrial University of Santander, Bucaramanga (Colombia). Species were identified by botanists from the Institute of Natural Sciences at the National University of Colombia (Bogotá); specimen voucher numbers were assigned to each one (Table 1), and deposited at the National Herbarium. Permission to collect these samples was given by the Colombian Housing and Environment Ministry.

Essential oil extraction and analysis

Oils from collected plants were obtained from different parts (total plant, stem, and combination of leaves and flowers) by hydrodistillation (HD) and microwave-assisted hydrodistillation (MWHD) (Table 1). HD was performed in a 5 L round flash with 500 g of plant material and 4 L of water, using an electric heater (boiling water) for 2 h. The oil was decanted from the condensate, previously saturated with NaCl, and dried with anhydrous sodium sulfate, as described elsewhere (Stashenko et al., 2004a,b). On the other hand, for the MWHD, the hydrodistillation apparatus was placed inside a domestic microwave oven (2.45 GHz, 800 W) with a side orifice through which an external glass condenser joined the round flask with the plant material (500 g) and water (0.2 L), inside the oven. The oven was operated for 30 min at full power, which caused water to boil vigorously and reflux. Essential oil was decanted from the condensate and dried with anhydrous sodium sulfate.

Essential oil analyses were performed using an Agilent Technologies 6890 Plus equipped with a HP-5 MS capillary column (30 m x 0.25 mm i.d. x 0.25 μm, d_f) and an Agilent Technologies MSD 5973 mass-selective detector. For gas chromatography-mass spectrometry detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas at a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. Column temperature was initially at 50 °C, and then gradually increased to 150 °C at a 3 °C/min rate, held for 10 min, and finally raised to 250 °C at 10 °C/min. Diluted samples (1:100 v/v, in CH_2Cl_2) of 1.0 μL were injected manually and in the *Splitless* mode. The components were identified on the basis of comparison of their relative retention time and mass spectra with those of some standard compounds, NBS75K library data of the GC-MS system, and literature data (Adams, 1995; Davies, 1990).

Cytotoxicity assays

The essential oil acute toxicity (mean lethal concentration) (LC50) was evaluated using the brine shrimp (*Artemia franciscana*) assay as described elsewhere (D'Souza et al., 2002; Massele, 1995). Briefly, 10 mg of dried brine shrimp eggs (Great Lake, Salt Lake City, UT, USA) were deposited in fresh filtered seawater and allowed to hatch for 24 h. The assay was performed dissolving the essential oil in DMSO at a maximum concentration of 1.3 % v/v. Ten larvae were put in 10 mL seawater containing the essential oil at concentrations between 0.01 and 1000 μg/mL. The LC50 assay was done using three replicates for each concentration, and counting of dead organisms was carried out 24 and 48 h after exposure to the different essential oil concentrations. All the experiments were conducted by quadruplicates and LC50 values as well as 95% confidence intervals were calculated using the Probit method (Finney, 1971).

Antioxidant assay using the thiobarbituric acid reactive species (TBARS) method

The essential oil antioxidant effects were tested by measuring their ability to inhibit the formation of TBARS, including malondialdehyde (MDA), induced by Fe (II) and H_2O_2 in rat liver tissues (Kade et al., 2008; Oliveira et al., 2006). This assay quantifies the peroxidation of polyunsaturated fatty acids (PUFA) present in liver microsomal fraction. Male Wistar rats (298±6.5 g) were

decapitated under mild ether anesthesia and hepatic (liver) tissues were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in ice cold saline solution (0.15 M KCl, pH 7.4), maintaining a relationship of 100 mg of liver per each 0.9 mL of KCl, with 10 up-and-down strokes at approximately 1200 rev/min in a Teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1600 g at 4 °C to yield a pellet and a low-speed supernatant, followed by centrifugation of the supernatant at 20000 g for another 10 min at 4 °C. The resulted supernatant extract was used as liver homogenate (Yildirim et al., 2000). Peroxidation of liver homogenate was induced by addition of $FeCl_2$ (0.1 mM) and H_2O_2 (3 mM) (Oliveira et al., 2006; Zhang et al., 2001).

Each essential oil was dissolved in the homogenate to obtain final concentrations of 0.1, 1, 10 and 100 µg/mL, in a final volume of 500 µL (fraction S10 with $FeCl_2$ and H_2O_2), incubated at 37±1 °C during 1 h, and then centrifuged at 5000 g for 5 min at 25 °C. After incubation, lipid peroxidation in the supernatant was determined by rapid addition of 2.5 mL TCA-TBA reagent (15% w/v trichloroacetic acid, TCA, and 0.375% thiobarbituric acid, TBA) to the reaction mixture, incubated at 95 ºC for 60 min in a boiling water bath, and after cooling, the precipitated flocculent was removed by centrifugation at 20000 g for 5 min at 25 °C. Absorbance of TBARS in the supernatant was recorded at 540 nm against a blank containing all the reagents (Haraguchi et al., 1997). TBARS concentration was normalized to protein content. Butylated hydroxytoluene (BHT) (2.5 µg/mL) was used as standard antioxidant and dimethyl sulfoxide (DMSO) was utilized as vehicle control (Kulisic, 2004). The results were expressed as nmoles of MDA/mg protein. Each essential oil was assayed by triplicate.

Statistical analysis

The results were expressed as mean±S.D. Significant differences between means for different oil concentrations were analyzed by ANOVA using Dunnet test as a post-test. In all cases, normal distribution and equality between standard deviations were evaluated using Kolmogorov-Smirnov and Barlett test, respectively. In absence of normality comparison between concentrations were carried out by Kruskal Wallys. Differences between groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Chemical composition of essential oils

The major compounds of the essential oils analyzed by GC-MS are presented in Table 2. These compounds are mainly hydrocarbons, oxygenated monoterpenes and oxygenated sesquiterpenes. It is clear that the composition of the essential oil of *Lippia alba* depends on the collection site and the corresponding chemotype. The main constituents of *Lippia alba* (VEmtT01E, VEmtT02E and VEmcT02E) were carvone, limonene and bicyclosesquiphellandrene. However, in *Lippia alba* VEboW02E, the main components were geranial and neral, wheras for Lippia alba VEsrW01E were

Table 1. Taxonomical classification, geographical collection and extraction type for analyzed essential oils.

Plant	CENIVAM Code	Family	Common name	Sampling site	Department	Voucher	Extraction method
Ocotea sp.	**cb*01T	Lauraceae	Cascarillo	Cubará	Boyacá	CENIVAM-457	MWHD[1]
Tagetes lucida	MAbrW01E	Magnoliaceae	Estragón	Barrancabermeja	Santander	512074	MWHD
Lippia alba	VEboW02E	Verbenaceae	Pronto alivio	Colorado	Bolívar	512272	MWHD
Elettaria cardamomum	ZlanT01*	Zingiberaceae	Cardamomo	Rionegro	Antioquia	CENIVAM-450	HD[2]
Lippia alba	VEmtT02E	Verbenaceae	Pronto alivio	Flandes	Tolima	484350	HD
Cymbopogon nardus	GRnaT02E	Gramineae	Citronela	Mercaderes	Cauca	CENIVAM-454	HD
Lippia alba	VEmtT01E	Verbenaceae	Pronto alivio	Flandes	Tolima	484350	HD
Lippia origanoides	VEchW03B	Verbenaceae	Orégano	Jordán Sube	Santander	512270	MWHD
Cymbopogon nardus	GRnaT01E	Gramineae	Citronela	Mercaderes	Cauca	CENIVAM-454	HD
Minthostachys mollis	LTmcT02E	Labiateae	Muña	Anolaima	Cundinamarca	521089	HD
Lippia alba	VEmcT02E	Verbenaceae	Pronto alivio	Anolaima	Cundinamarca	484350	HD
Lippia alba	VEsrW01E	Verbenaceae	Pronto alivio	Saravena	Arauca	512084	MWHD
Piper sanctifelisis	PPccH01E	Piperaceae	Cordoncillo	Quibdó	Chocó	519979	MWHD

1. MWHD, Microwave-assisted hydrodistillation; 2. HD, Hydrodistillation
Samples with vouchers named CENIVAM are stored in our herbarium in Bucaramanga

Table 2. Major constituents identified and quantified in the studied essential oils.

Essential Oil	Major components
Minthostachys mollis (LTmcT02E)	Pulegone (19%), *trans*-β-caryophyllene (18%), menthone (11.9%), bicyclogermacrene (11.3%), germacrene D (11%)
Ocotea sp. (**cb*01T)	α-Pinene (42%), *p*-cymene (14.6%), β-pinene (12.7%)
Elettaria cardamomum (ZIanT01*)	1,8-Cineol (29.7%), α-terpineol acetate (26.1%)
Cymbopogon nardus (GRnaT02E)	Citronellal (42.1%), geraniol (14.9%)
Cymbopogon nardus (GRnaT01E)	Citronellal (30.7%), geraniol (16.9%), citronellol (12.1%)
Lippia alba (VEmtT01E)	Bicyclosesquiphellandrene (35.9%), carvone (24.5%), limonene (14.7%)
Lippia alba (VEmtT02E)	Bicyclosesquiphelandrene (27.4%), carvone (26.9%), limonene (18.6%)
Lippia alba (VEmcT02E	Carvone (25.5%), bicyclosesquifelandrene (27.4%), limonene (20.6%)
Lippia alba (VEboW02E)	Geranial (30.4%), neral (23.9%), *trans*-β-caryophyllene (8%)
Tagetes lucida (MAbrW01E)	Estragole (95.7%)
Lippia origanoides (VEchW03B)	*p*-Cymene (15.7%), *trans*-β-caryophyllene (9.4%), α-terpinene (6.9%)
Piper sanctifelisis (PPccH01E)	α-terpinene (35.3%), bicyclogermacrene (7.1%), *trans*-β-caryophyllene (5%)
Lippia alba (VEsrW01E)	Carvone (25.3%), *cis*-β-ocimene (22.4%), neral (10.4%), geranial (10.4%)

carvone, *cis*-β-ocimene and neral. Although quantitative differences are verified, the composition of the essential oils of *Lippia alba* shows agreement with the existence of different chemotypes as previously described (Bounatirou et al., 2007; Oliveira et al., 2006; Zoghbi et al., 1998).

The principal components of *Ocotea* sp. were α-pinene (42%), *p*-cymene (14.6%), and β-pinene (12.7%). This composition is different from that found for *Ocotea quixos* (Ishpingo) from Ecuador, oil that has *trans*-cinnamaldehyde (27.9%) and methyl cinnamate (21.7) as major constituents (Bruni et al., 2004). Ishpingo essential oil differs from commercially exploited *O. pretiosa* and *O. cymbarum* oils () by the absence of both methyleugenol and safrole. In particular, the absence of safrole allows only minor constraints on the use of Ecuadorian Ishpingo essential oil (due to the recognized toxicity of this compound) (Ioannides et al., 1981). More than 350 species belong to the *Ocotea* genus, mainly distributed in the Americas and in Southern Africa, and those are widely known as commercial sources of spicy essential oils.

Estragole (95.7%) was the major constituent in the essential oil of *Tagetes lucida* from Colombia. This plant is an aromatic herb distributed naturally in Central and South America, where it is used as a spice, for medicinal purposes, as insecticide, and as ornamental plant. The essential oil obtained from this species cultivated in Costa Rica has methyl chavicol (95-97%) as a major component (Cicció, 2004).

The main components found in the volatile oil from *Colombian Elettaria cardamomum* were 1,8-cineol (29.7%) and α-terpineol acetate (26.1%). In contrast, Marongiu et al. (2004) found as majority compounds in *E. cardamomun* α-terpinyl acetate (42.3%), 1,8-cineole (21.4%), linalyl acetate (8.2%), limonene (5.6%), and linalool (5.4%). Regarding the other studied species, major constituents for *Minthostachys mollis* were pulegone, mentone and *trans*-β-caryophyllene; for *Cymbopogon nardus* were citronellal and geraniol; for *Lippia origanoides*: p-cymene, *trans*-β-caryophyllene, α-terpinene; and for *Piper sanctifelisis*: α-terpinene (35.3%), bicyclogermacrene (7.1%), *trans*-β-caryophyllene (Table 2).

Cytotoxicity of essential oils against *Artemia franciscana*

The LC50 of the essential oils based on the brine shrimp assay after 24 and 48 h exposure are presented in Table 3. Values varied between 4.36-64.3 µg/mL and 1.2-20.8 µg/mL, for 24 and 48 h exposure, respectively. These results, although indicate that most tested essential oils can be considered cytotoxic (LC50<10 µg/mL), also pointed out that that bioactivity is highly dependent on chemotype, type of extraction and sampling location (Medina-Holguín et al., 2008).

The ratio of the estimated LC50s (24 h-LC50/48 h-LC50) for *Ocotea* sp., *Piper sanctifelisis, Elettaria cardamomum, Lippia alba* (VEboW02E) and *Cymbopogon nardus* ranged between 1.03 and 1.69. This indicates that an increase in the exposure time from 24 to 48 h does not lead to a marked augment in toxicity, suggesting that naupliar susceptibility to these essential oils do not change throughout development. However, time-dependent larvae development was highly susceptible to essential oils obtained from *Minthostachys mollis* and *Lippia alba* (chemotype carvone, Tolima), with LC50 ratios of 9.43 and 5.91 µg/mL.

In general, it has been suggested that the cytotoxic activity of essential oils is mostly due to the presence of phenols, aldehydes and alcohols (Bruni et al., 2004; Sacchetti et al., 2005, Oliveira et al., 2006).

Table 3. Cytotoxicity against *Artemia franciscana* and antioxidant capacity of essential oils.

Essential oils (Code)	LC50-24 h (µg/mL)	LC50-48 h (µg/mL)	LC50-24 h/LC50-48 h	Antioxidant activity (CE_{50}) (µg/mL)
Piper sanctifelisis (PPccH01E)	4.36 (4.84-3.92)	3.27 (3.64-2.93)	1.33	> 000
Lippia alba (VEmtT01E)	4.53 (5.41-3.79)	1.17 (1.47-0.94)	3.86	331.6 (291.5-377.2)
Lippia alba (VEsrW01E)	6.95 (8.03-6.03)	2.42 (2.83-2.06)	2.88	>1000
Ocotea sp. Cascarillo (**cb*01T)	7.84 (8.32-7.39)	7.59 (8.88-6.48)	1.03	31.1(27.9-34.7)
Lippia alba (VEmcT02E)	8.14 (9.39-7.05)	2.21(2.83-1.72)	3.68	>1000
Cymbopogon nardus (GRnaT02E)	12.41 (13.70-11.24)	4.61 (5.60-3.86)	2.67	309.8 (264.8-362.5)
Lippia origanoides (VEchW03B)	14.41 (15.61-13.30)	5.08 (5.82-4.44)	2.84	463.5 (408.1-526.4)
Cymbopogon nardus (GRnaT01E)	19.73 (20.77-18.75)	11.66 (12.72-10.68)	1.69	860.5 (730.0-1014.4)
Lippia alba (VEboW02E)	21.05 (22.58-19.63)	15.21 (16.63-13.90)	1.38	94.9 (82.6-109.0)
Minthostachys mollis (LTmcT02E)	21.29 (25.04 - 18.11)	2.26 (2.71-1.88)	9.43	>1000
Tagetes lucida (MAbrW01E)	22.14 (23.54-20.82)	17.68 (19.03-16.42)	1.25	37.9 (34.5-41.6)
Elettaria cardamomum (ZIanT01*)	27.79 (29.42-26.26)	20.83 (22.54-19.26)	1.33	130.5 (115.8-147.2)
Lippia alba (VEmtT02E)	64.33 (68.05-60.82)	10.88 (14.12-8.38)	5.91	174.4 (152.0-200.0)
Butylated hydroxytoluene, BHT (5 µM)				55.22% (52.49-57.95)[a]

[a]Antioxidant activity exhibited by 5 µM BHT, calculated as the percentage inhibition of MDA production when compared to control.

Antioxidant activity of essential oils

The ability of the Colombian essential oils to act as antioxidant in the TBARS assay is presented in Table 3. CE50 values varied between 31.1 µg/mL and >1000 µg/mL. Based on these data, the essential oil of *Tagetes lucida* showed the highest antioxidant activity (CE50=37.9 µg/mL), followed by *Ocotea* sp., and *Lippia alba* (citral chemotype) (CE50 values lower than 100 µg/mL). Essential oils from *Elettaria cardamomum* and *Lippia alba*-carvone (from Tolima) displayed moderate activity, whereas the essential oils isolated from *Minthostachys mollis, Lippia alba* (carvone chemotype, from Cundinamarca) and *Piper sanctifelisis* did not show antioxidant capacity, with CE50 values greater than 1000 µg/mL.

Medicinal plants and foods from vegetal origin usually contain natural antioxidants that can scavenge free radicals (Miller et al., 2000; Sawai & Moon, 2000; Bandoniene & Murkovic, 2002). Antioxidants prevent the radical chain oxidation which destroys membrane integrity, resulting most often in cell lyses. Phenolic compounds such as estragole and eugenol, both found in essential oils presented here are well-known antioxidants (Gil et al., 2000; Soarez et al., 2003; Goupy et al., 2003), whereas some monoterpenes such as α-terpinene, γ-terpinene, and terpinolene only have modest protective action in some models. However, it is important to mention that different components in the same essential oil can generate synergistic interactions (Teissedre & Waterhouse, 2000).

Finally, although Colombian flora is rich in plants producing essential oils with potential pharmacological properties (Stashenko et al., 2004a), there have been few reports on them. Results provided here showed that there are promissory plant species, some with several chemotypes in Colombian flora, with interesting antioxidant properties, for which more extensive pharmacological activities should be evaluated.

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