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Chemical composition and biological activity of essential oil of *Achyrocline ramosissima* Britton ex Rusby (Asteraceae)

[Composición química y actividad biológica del aceite esencial de *Achyrocline ramosissima* Britton ex Rusby (Asteraceae)]

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Abstract: Essential oil from fresh aerial parts of *Achyrocline ramosissima* Britton ex Rusby (Asteraceae) collected in the Venezuelan Andes was obtained by hydrodistillation and analyzed by GC/MS. A yield of 0.10% w/v was afforded, and thirty seven components were identified by comparison of their retention indices (RI) and mass spectra with the Wiley GC-MS Library Data. The major components identified were rosifoliol (31.69%), β-caryophyllene (8.61%), guaiol (3.95%), β-eudesmol (3.33%) and selina-3,7 (11)-diene (2.99%). Antimicrobial activity of *A. ramosissima* essential oil was also evaluated against Gram positive, Gram negative bacterial strains and *Candida albicans* yeast. The results showed that, this oil is effective against Gram positive bacteria *Staphylococcus aureus* ATCC 6538 with MIC values ranging from 50 to 100 µg/mL and MBC values > 200 µg/mL as well as *Bacillus subtilis* CECT 39 with MIC values of 50 µg/mL and MBC of 100 µg/mL, however a low activity was observed against Gram negative bacterial strains, *Pseudomonas aeruginosa* AK 958, *Escherichia coli* CECT 99 and *C. albicans* yeast performing MIC and MBC values > 200 µg/mL. Cytotoxic activity was also determined against HeLa (cervix carcinoma), A-459 (lung carcinoma), MCF-7 (breast adenocarcinoma) human cancer cell lines and against normal Vero cells (African green monkey kidney), exhibiting antiproliferative effects with IC_{50} values of 28.2 µg/mL (HeLa cells). This is the first report regarding the chemical composition, antibacterial and cytotoxic activities of the essential oil from this species.

Keywords: *Achyrocline ramosissima*, essential oil, antibacterial activity, cytotoxic activity

Resumen: El aceite esencial de las partes aéreas frescas de *Achyrocline ramosissima* Britton ex Rusby (Asteraceae) recolectada en los Andes venezolanos fue extraído por hidrodestilación y analizado por CG/EM. Se obtuvo un rendimiento de 0,10% m/v, treinta y siete componentes fueron identificados por comparación de los índices de retención (IR) y sus espectros de masas con los datos de la biblioteca Wiley GC-MS. Los principales componentes identificados fueron rosifoliol (31,69%), β-cariofileno (8,61%), guaiol (3,95%), β-eudesmol (3,33%) y selina-3,7 (11)-dieno (2,99%). La actividad antimicrobiana del aceite esencial de *A. ramosissima* fue evaluada contra cepas bacterianas Gram positivas, Gram negativas y la levadura *Candida albicans*. Los resultados mostraron que el aceite esencial fue activo contra las bacterias Gram positivas *Staphylococcus aureus* ATCC 6538 con una CIM entre 50-100 µg/mL y CBM de valores > 200 µg/mL y *Bacillus subtilis* CECT 39 con CIM de 50 µg/mL y CBM de 100 µg/mL, sin embargo se observó una baja actividad contra las cepas bacterianas Gram negativas *Pseudomonas aeruginosa* AK 958, *Escherichia coli* CECT 99 y la levadura *C. albicans* cuyos valores de CIM y CBM fueron > 200 µg/mL. La actividad citotóxica fue determinada frente a las líneas celulares cancerígenas HeLa (carcinoma de cuello uterino), A-459 (carcinoma de pulmón), MCF-7 (adenocarcinoma de mama) y frente a las células normales Vero (células renales de mono verde), mostrando efectos antiproliferativos con valores de CI_{50} 28.2 µg/mL frente a las células HeLa. Este es el primer reporte sobre la composición química, actividad antibacteriana y citotóxica del aceite esencial de esta especie.

Palabras clave: *Achyrocline ramosissima*, aceite esencial, actividad antibacteriana, actividad citotóxica

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INTRODUCTION

A*chyrocline* (Less.) DC genus belongs to Gnaphalieae tribe, Asteraceae family and comprises about 20 species. It is widely distributed in South and Central America extended to tropical Africa and mountainous areas of Madagascar´s island (Nesom, 1990). In Venezuela, 10 species have been reported for the Andean region higher than 800 m.a.s.l. (metres above sea level) (Badillo, 2001; Hokche *et al.*, 2008). Somes species are known as "vira vira" in Venezuela, Colombia, Ecuador and Perú, and in other countries such as Argentina and Brazil as "marcela" (Badillo & González, 1999). For many years species of *Achyrocline* genus, mainly, *A. satureioides* has been used in natural medicine in Argentina, Uruguay, Brazil and Paraguay, for the treatment of a variety of human ailments, particularly those of the gastrointestinal tract (Simões *et al.*, 1988; Da Silva & Langeloh, 1994). Previous investigations conducted on different species have revealed antiviral (Bettega *et al.*, 2004; Sabini *et al.*, 2012), antimicrobial (Calvo *et al.*, 2006; Cezarotto *et al.*, 2011; Joray *et al.*, 2013), antioxidant, hepatoprotective (Kadarian *et al.*, 2002; Polydoro *et al.*, 2004), anti-inflammatory (Simões *et al.*, 1988) and anticancerous activities (Rivera *et al.*, 2004; Poglia *et al.*, 2014). On the other hand, extracts from species of *Achyrocline* genus are important sources of active substances with therapeutic potential and these have been used in the last years due to the secondary metabolites such as flavonoids (Mesquita *et al.*, 1986; Martino *et al.*, 1988; Broussalis *et al.*, 1993; Díaz & Heinzein, 2006;) and caffeoyl derivatives (Martino *et al.*, 1988; Broussalis *et al.*, 1993; López *et al.*, 2006). Essential oil´s composition of different *Achyrocline* species have also been investigated and revealed a variety of compounds such as monoterpenes and sesquiterpenes, of wich β-caryophyllene is present in almost all samples analyzed (Labuckas *et al.*, 1999; Rodrígues *et al.*, 2002; Retta *et al.*, 2010; Cezarotto *et al.*, 2011). Other compounds identified are α-pinene, β-pinene, limonene, *p*-cymene for *A. satureioides* (Ricciardi *et al.*, 1964); α-pinene, limonene,1,8-cineole and α-copaene in the essential oils of *A. satureioides* and *A. flaccida* (Retta *et al.*, 2010); germacrene D, caryophyllene oxide for *A. satureioides* (Schmeda-Hirschmann, 1984) and carvacrol, thymol identified for *A. alata* (Bueno-Sánchez *et al.*, 2009).

In this investigation, chemical composition, antibacterial and cytotoxic activities of the oil obtained from fresh aerial parts of *A. ramosissima* collected from Mérida, Venezuela were conducted. This species is used in the Venezuelan Andes for the treatment of diarrhea (Badillo & González, 1999), while in Perú is known in the popular indigenous medicine as "Huira-huira" and is used against bronchial affections (Mantilla & Otazábal, 2011) so as it is in Bolivia (Vidurre de la Riva, 2006). To the best of our knowledge there are no reports for the essential oil composition, antibacterial and cytotoxic activities of the essential oil from fresh aerial parts of *A. ramosissima*.

MATERIALS AND METHODS

Plant material

A. ramosissima, was collected in the Páramo Piedra Pirela, San José de Acequia, Mérida state at 3122 m.a.s.l. A voucher specimen (DB101) has been deposited in the Luis Ruiz Terán Herbarium of the Faculty of Pharmacy and Bioanalysis, Universidad de Los Andes, Mérida, Venezuela.

Isolation of essential oil

Fresh aerial parts (1900 g) were cut into small pieces and subjected to hydrodistillation for 3 h, using a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulfate and stored at 4 ºC.

Gas chromatography (GC) and Gas chromatography-Mass spectrometry (GC-MS) Analyses

GC analyses were performed on a Perkin- Elmer Auto System gas chromatograph equipped with flame ionization detectors (FID). A 5% phenylmethylpolysiloxane fused-silica capillary column (AT-5, Alltech Associates Inc., Deerfield, IL), 60 m x 0.25 mm, film thickness 0.25 μm, was used for the GC analysis. The initial oven temperature was 60 °C; this was then raised to 260 °C at 4 °C/min, and the final temperature maintained for 20 min. The injector and detector temperatures were 200 °C and 250 °C, respectively. The carrier gas was helium at 1.0 mL/min. The sample was injected using a split ratio of 1:100. Retention indices were calculated relative to C_8-C_{24} *n*-alkanes, and compared with values reported in the literature (Adams, 2007). The quantification was carried out according to percentages of relative areas.

The GC-MS analyses were carried out on a Hewlett Packard GC-MS system, Model 5973, fitted with a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d., film thickness 0.25 μm, Hewlett-Packard). The initial oven temperature was 60 °C; it was then heated to 280 °C at 4 °C/min, and the final temperature was maintained for 20 min. The injector and detector temperaturas were 200 °C and 230 °C, respectively. The carrier gas was helium, ajusted to a linear velocity of 34 m/s, the ionization energy 70 eV, and the scan range 40-500 amu at 3.9 scans/s. A Hewlett-Packard ALS injector was used with split ratio 1:100. The injected volume was 1.0 μL of a 2% dilution of oil in *n*-heptane. Identification of the oil components was based on the Wiley MS Data Library (6^{th} Ed).

Antimicrobial assay

Antimicrobial activity was determined against Gram positive (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* CECT39) and Gram negative (*Escherichia coli* CECT99, *Pseudomonas aeruginosa* AK 958) bacteria, and *Candida albicans* CECT 1039 (yeast). The bacterial strains were developed in either nutrient broth (NB), and the yeast was cultured in Sabouraud liquid medium at 37 °C. All media were purchased from Oxoid. The minimal inhibitory concentration (MIC) was determined for each sample by triplicate, using the broth microdilution method (De León *et al.*, 2005). All samples were dissolved in DMSO, several wells were also filled with the same proportions of DMSO as the controls and never exceeded 1%v/v. The starting microorganism concentration was approximately (1-5)×10^5 CFU/mL, growth was monitored by measuring the optical density increase at 550 nm (OD_{550}) using a microplate reader (Multiskan Plus II). The MIC was defined as the lowest concentration of the essential oil where growth inhibition was observed after 24 h of incubation in a rotatory shaker at 37 °C. All wells with no visible growth were sub-cultured by transferring 100 μL to nutrient brain-heart infusion or Sabouraud agar plates. After overnight incubation, colony counts were performed and the minimal bactericidal concentration (MBC) was defined as the lowest concentration of the essential oil that produced ≥ 99.9 % killing of the initial inoculum.

Cytotoxic assays

HeLa (human carcinoma of the cervix), A-549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma), and Vero (African green monkey kidney) cell lines were grown as a monolayer in Dulbecco's modified Eagle's medium, DMEM (Sigma), supplemented with 5% fetal calf serum (Gibco) and 1% of penicillin-streptomycin mixture. Cells were maintained at 37 °C in 5% CO_2 and 98% humidity. Cytotoxicity was assessed using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay (Mosmann, 1983), that detects the reduction of MTT by mitocondrial dehydrogenase to blue formazan product, which reflects the normal functioning of mitocondrial and cell viability (Lau *et al.*, 2004). Cell suspensions (2x10^4 cells/well) in growth phase were incubated in a microtiter well plate (96-well) along with the essential oil, pre-dissolved in DMSO at different concentrations. After 48 h, the optical density was measured using a micro ELISA reader (Multiskan Plus II) at 550 nm after dissolving the MTT formazan with DMSO (150 μL). The viability percentage was plotted against the sample concentration, and 50% cell viability (IC_{50}) was calculated from the curve. Cytotoxic assays were carried out by triplicate. Data are given in arithmetic means ± SD.

RESULTS AND DISCUSSION

Essential oil analysis

The essential oil from fresh aerial parts of *A. ramosissima* yielded (0.10% w/v). GC/MS analyses showed the presence of 37 components (representing 81.25% of the total oil). These were characterized by comparison of each MS with the Wiley GC/MS library data and also from its retention index. A list of identified compounds, along with their percentages of the total oil, is given in Table 1. The major components identified were rosifoliol (31.69%), β-caryophyllene (8.61%), guaiol (3.95%), α-eudesmol (3.33%), selina-3,7(11)-diene (2.99%). These compunds may be divided into four different groups: hydocarbons monoterpenes (1.47%), oxygenated monoterpenes (1.7%), hydrocarbons sesquiterpenes (26.79%) and oxygenated sesquiterpenes (51.29%). According to the references consulted, there are not studies on the composition of the essential oil of *A. ramosissima*. However, these results were compared to previous investigations of *A. satureioides* (Schmeda-Hirschmann, 1984; Labuckas *et al.*, 1999; Retta *et al.*, 2010; Cezarotto *et al.*, 2011), *A. flaccida* (Retta *et al.*, 2008; Retta *et al.*, 2010), *A. alata* (Labuckas *et al.*, 1999; Rodrígues *et al.*, 2002) and *A.*

tomentosa (Labuckas *et al.*, 1999), where β-caryophyllene is present within the main components similarly to *A. ramosissima*, but in different proportions. α-pinene, *p*-cimene, limonene, 1,8-cineole are also found in *A. satureioides*, *A. flaccida* and *A. alata* as well as in *A. ramosissima* (Ricciardi *et al.*, 1964; Rodrígues *et al.*, 2002; Retta *et al.*, 2010).

Table 1
Composition of the essential oil of aerial parts of *Achyrocline ramosissima*

COMPOUNDS[a]	%	RI cal[b]	RI tab
α-pinene	0.23	942	939
p-cymene	0.11	1032	1024
Limonene	1.05	1037	1029
1,8-cineole	1.20	1040	1031
γ-terpinene	0.08	1068	1059
Linalool	0.17	1108	1098
Terpinen-4-ol	0.14	1185	1177
α-terpineol	0.19	1197	1188
α-copaene	1.95	1384	1376
α-gurjunene	0.09	1417	1409
β-caryophyllene	8.61	1430	1419
α-guaiene	0.71	1449	1439
Myltayl-4(12)-ene	0.23	1452	1447
α-humulene	0.72	1465	1454
Allo-aromadendrene	0.46	1473	1460
Cadina-1(6),4-diene-trans	0.18	1486	1476
γ-muurolene	1.33	1489	1479
β-Selinene	2.04	1499	1490
α-Selinene	2.13	1508	1498
α-Muurolene	0.56	1513	1500
α-bulnesene	0.61	1518	1509
γ-cadinene	0.70	1526	1513
δ-Cadinene	2.47	1535	1523
Cadina-1,4-diene-trans	0.12	1543	1534
Selina-3,7 (11) diene	2.99	1552	1546
Germacrene B	0.89	1566	1561
Caryolan-8-ol	0.35	1577	1572
Spathulenol	0.20	1584	1578
Caryophyllene oxide	2.84	1589	1583
Guaiol	3.95	1605	1600
Rosifoliol	31.69	1620	1600
Agarospirol	2.04	1638	1648
β-eudesmol	2.71	1664	1650
α-eudesmol	3.33	1668	1653
Valerianol	1.98	1671	1658
Bulnesol	1.46	1682	1671
Eudesm-7-(11)-en-4-ol	0.74	1714	1700

[a] Compounds are listed in sequence from a HP-5 MS column elution.
[b] Kovats retention indices (RI) were calculated against C_8 to C_{24} *n*-alkanes series

Antimicrobial activity

Antimicrobial activty of *A. ramosissima* essential oil was evaluated against Gram positive, Gram negative bacterial strains and *C. albicans* yeast. The essential oil showed activity against *S. aureus* with MIC values ranging between 50 to 100 μg/mL, *B. subtilis* with MIC values of 50 μg/mL and MBC values > 200 μg/mL to *S. aureus* and *B. subtilis* with values of

100 µg/mL. For *E. coli*, *P. aeruginosa* and *C. albicans* higher concentration > 200 µg/mL was necessary to cause MBC. Table 2 summarizes these results.

Table 2
Antibacterial activity of the essential oil of aerial parts of *Achyrocline ramosissima*

Microorganism	Essential oil (µg/mL)		Antibiotic (µg/mL)	
	Achyrocline ramosissima		Cefotaxime[a]	
	MIC	MBC	MIC	MBC
S. aureus ATCC 6538	100-50	> 200	2.5-1.25	NT
B. subtilis CBCT39	50	100	8	NT
E. coli CECT99	> 200	> 200	NT	NT
P. aeruginosa AK 958	> 200	> 200	NT	NT
C. albicans CECT 1039	> 200	> 200	NT	NT

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration, a: Cefotaxime (positive control). ATCC: American type culture collection; CECT: Colección española de cultivos tipo (spanish type culture collection). NT: Untreated

In this work, antibacterial activity was observed only against Gram positive bacteria *S. aureus* and *B. subtilis*. Antibacterial activity against *S. aureus* has been reported previously for *A. satureioides* using different extracts from aerial parts of the same species (Asolini *et al.*, 2006; Mota *et al.*, 2011). *S. aureus* is well known for causing several human infections and showing resistance to antibacterial treatment using comercial medicines (Hsueh *et al.*, 2004; Guzmán & Lozada, 2007).

Oxygenated and hydrocarbons sesquiterpenes observed at important concentrations in the essential oil of the analyzed sample could be considered responsible for the antibacterial activity. Although they usually occur as complex mixtures, their activity may generally account for the major components. Previous investigations have reported activity of β-caryophyllene against *S. aureus* (Kim *et al.*, 2008), *P. aeruginosa* and *B. subtilis* (Oztürk *et al.*, 2009). Thus, the antibacterial results observed in this investigation might be related to the presence of this compound.

Cytotoxic activity

The essential oil was subjected to screening for possible cytotoxic activity using a representative panel of cancer cell lines, HeLa (cervix carcinoma), A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma), alone with Vero cells (African green monkey kidney), using 6-mercaptopurine as a positive control. As shown in Table 3, *A. ramosissima* essential oil turned to be active against the tested tumor cell lines especially on HeLa cells (IC_{50} 28.2 µg/mL), after 48 h of exposure. In addition, when comparing the activities against cancer cells with none tumorigenic (Vero) cells, some degree of selective cytotoxicity were observed. It is important to emphasize that *A. ramosissima* essential oil showed a selectivity index of 3.41 for HeLa cells. Low selectivity was found against A-549 with 1.69 as was demonstrated by a higher IC_{50} values against the non-tumor mammalian Vero cells.

Table 3
Cytotoxic activity (IC_{50} µg/mL) of the essential oil of *Achyrocline ramosissima*

Compounds	HeLa	A-549	MCF-7	Vero
Essential Oil	28.2 ± 0.45	56.9 ± 0.8	113 ± 2.24	96.29 ± 1.38
Control [a]	0.5 ± 0.01	8.0 ± 0.32	0.24 ± 0.01	11.5 ± 0.88

a: 6-mercaptopurine (positive control). All assays were repeated at least three times

Rosifoliol (31.69%) and α-eudesmol (3.33%) present in the essential oil are eudesmane type sesquiterpenes. This series of compounds have been characterized by cytotoxic activity (Bomfim *et al.*, 2013). β-caryophyllene (8.61%) has also been found to exhibit cytotoxic activity (Kubo *et al.,* 1996).

CONCLUSSION

Analysis of the chemical composition of the essential oil of *A. ramosissima* afforded rosifoliol, β-caryophyllene, guaiol, α-eudesmol and selina-3,7(11)-diene in major proportions; the main compounds belong to the sesquiterpene group (78.08%). The oil showed antibacterial activity against important human pathogenic Gram positive bacteria at concentrations (MIC 50-100 μg/mL). On the other hand, essential oil from *A. ramosissima* revealed an interesting cytotoxic activity against HeLa tumoral cell lines (IC_{50} 28.2 μg/mL), while it is not cytotoxic to Vero cell (no-tumoral cells). To the best of our knowledge, this is the first report on the chemical composition, antimicrobial and cytotoxic activities of *A. ramosissima* essential oil.

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