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Alírica I Suárez
Departamento de Química, Universidad Técnica Particular de Loja / San Cayetano alto, calle Marcelino Champagnat, s/n. AP: 11 01 608, Loja, Ecuador.

Alírica I Suárez
Facultad de Farmacia, Universidad Central de Venezuela, Caracas, Venezuela.

Chabaco Armijos
Departamento de Química, Universidad Técnica Particular de Loja / San Cayetano alto, calle Marcelino Champagnat, s/n. AP: 11 01 608, Loja, Ecuador.

José Miguel Andrade
Departamento de Química, Universidad Técnica Particular de Loja / San Cayetano alto, calle Marcelino Champagnat, s/n. AP: 11 01 608, Loja, Ecuador.

Enrique V Quisatagsi
Departamento de Química, Universidad Técnica Particular de Loja / San Cayetano alto, calle Marcelino Champagnat, s/n. AP: 11 01 608, Loja, Ecuador.

Marco Cuenca
Departamento de Química, Universidad Técnica Particular de Loja / San Cayetano alto, calle Marcelino Champagnat, s/n. AP: 11 01 608, Loja, Ecuador.

Sandra Cuenca-Camacho
Sección de Genética Humana, Microbiología y Bioquímica Clínica, Departamento de Ciencias de la Salud, Universidad Técnica Particular de Loja.

Natalia Bailon-Moscoso
Sección de Genética Humana, Microbiología y Bioquímica Clínica, Departamento de Ciencias de la Salud, Universidad Técnica Particular de Loja.

Correspondence
Alírica I Suárez
Departamento de Química, Universidad Técnica Particular de Loja / San Cayetano alto, calle Marcelino Champagnat, s/n. AP: 11 01 608, Loja, Ecuador.

The cytotoxic principle of *Bejaria resinosa* from Ecuador

Alírica I Suárez, Chabaco Armijos, José Miguel Andrade, Enrique V Quisatagsi, Marco Cuenca, Sandra Cuenca-Camacho, Natalia Bailon-Moscoso

Abstract
Bejaria resinosa Mutis ex LF is also known as "pena de cerro" and is a plant species used traditionally by the Saraguro ethnic group in Ecuador to treat nervous system problems, swollen wounds and inflammations of the genital organs, as well liver diseases and cancer. The aim of this study was to screen *Bejaria resinosa* aqueous extract and isolated compounds from organic extracts, for activity against the following cancer cell lines: MCF-7 (breast carcinoma), PC-3 (prostate carcinoma), RKO (colon carcinoma), and D-384 (astrocytoma). This was followed by identification of the compounds responsible for this effect. The results indicated that ursolic acid is the principle active responsible for the cytotoxicity on tumor cells.

Keywords: *Bejaria resinosa*, Ericaceae, triterpene acids, flavonoids, cytotoxicity, Ecuador

1. Introduction
Ecuador is one of the most biodiverse countries in the world and is also well-known for its old tradition in medicine; the population uses many species of flora in the management of diseases and ailments. The Ericacea comprises 100 genera and 3000 species and is widespread around the world from the temperate and cold areas to the mountains and neotropics [1]. The genus *Bejaria* has 15 species that belong to the family Ericaceae. It is distributed in the southeastern United States, Cuba and from Mexico to Bolivia. In Ecuador, they are represented by five species in the south Andes; four species are typical of areas of higher vegetation, especially the subpáramo: *B. aestuans* Mutis ex L., *B. mathewsii* Fielding & Gardner, *B. resinosa* Mutis ex LF, and *B. subsessilis* Benth, the fifth species is 2000 m [2-3].

In the literature some species are cited as *Befaria*, this is due that, originally, Mutis described the genus as *Bejaria* dedicated to Don Bejar of Cadiz, in a letter to Linnaeus, who subsequently edited and published describing the genus as *Befaria* producing the typographical mistake [4].

Plants of this genus are widely used in traditional medicine of some countries, *B. aestuans* is used in Peru to treat infections and gastrointestinal diseases, [5] and *B. cinnamomea* have been used as a vaginal antiseptic and for menstrual irregularities [6]. Regarding to the phytochemical information, the Ericaceae produce grayanoids diterpenes, well known by their toxic effects in genera such as *Pieris*, *Rhododendron*, *Leucothoe*, *Lyenis* and *Kalmia,* [7] however phytochemical and pharmacological studies of *Bejaria* species are scarce, the previous phytochemical studies on *B. resinosa* from Colombia, where is widely used to treat wounds, as purgative, and to prevent heart attacks, indicated that the main secondary metabolites are triterpenes as taraxerol, α and β amyrin, lupeol, ursolic acid, and flavonoids as quercetin and quercetin-rutinoside [8-10].

B. resinosa, a flowering evergreen tree is broadly distributed in the Loja Province of South Ecuador, and it is locally called as *pena de cerro* and *payama de cerro*. Traditionally the plant, is used by the Saraguro ethnic group, to treat problems of the nervous system [11]; with leaves, stem, bark, and particularly the flowers infusions for swollen wounds and inflammations in the genital organs are made, as well to treat liver diseases [2, 12].

To the best of our knowledge so far, the searched literature data indicated that neither the phytochemical profile nor pharmacological study of this species from Ecuador was previously reported. Due to the medicinal significance of *B. resinosa* on the Saraguro population, and as part of one ongoing project on medicinal plants of Ecuador in which the phytochemical and pharmacology are investigated, this plant was chosen to evaluate, the antiproliferative activity

on four cancer cells, MCF-7 (breast adenocarcinoma), PC3 (prostate carcinoma), RKO (colon RKO (colon carcinoma), and D-384 (astrocytoma), of the main secondary metabolites, and an aqueous extract obtained from the aerial parts of the plant, trying to find results that explain the wide use of it under the Saraguro ethnic group.

2. Materials and Methods

2.1. General experimental procedures

Melting points were determined using a Fisher Johns melting point apparatus and were uncorrected. NMR spectra were recorded in the indicated solvent on a Varian 400 MHz spectrometer and ^{1}H and ^{13}C chemical shifts (δ, ppm) were relative to the solvent signals used as references. Mass spectra analysis was performed on an Agilent Technologies, G890N. Silica gel 60 (E. Merck, Darmstardt, Germany; 60-120 mesh) was used for column chromatography and pre-coated silica gel 60 GF-254 (Merck) was used for thin layer chromatography (TLC) analysis. Spots were visualized by spraying with sulfuric acid (10% in methanol) and heating at on a hot plate.

2.2. Plant material

The leaves and flowers of *B. resinosa* were collected near Saraguro town, Parroquia San Lucas, Provincia de Loja, Ecuador, in March 2015 located 335226 S, 79155822 W at 2870 m.a.s.l. The plant material was identified by Bolivar Merino, curator of the Universidad Nacional de Loja Herbarium and a voucher sample (PPN-er-007) was deposited in the Universidad Técnica Particular de Loja Herbarium.

2.3. Extraction and isolation

The dried aerial parts, flowers and leaves of *B. resinosa* (627 g) were extracted under maceration with MeOH by 48h, after filtration the solvent was evaporated at reduced pressure to give a crude methanol extract (97.271 g). Half of this extract (48.63 g) was suspended in water/methanol (3:7) solution and was in turn sequentially partitioned with n-hexane (3 x 100 mL), dichloromethane (3 x 100 mL), ethyl acetate (3 x 100 mL). The solvents fractions were dried with anhydrous sodium sulfate, filtered and evaporated under vacuum to afford n-hexane (6.74 g), dichloromethane (16.77 g), ethyl acetate (3.43 g), and (18.67 g) of hydro-methanolic fraction. The compounds were isolated from n-hexane, dichloromethane and MeOH fractions by repeated column chromatography.

The dichloromethane fraction (4 g) was subjected to open column chromatography (CC), using silica gel and eluting with hexane, and increasing gradient with ethyl acetate until 100% ethyl acetate, and finally ethyl acetate/methanol to give 40 fractions; these were combined according to similarity in TLC to obtain finally eleven fractions (FI-FXI). From the fraction FIII yellowish solid (486 mg) crystallized, it was identified as ursolic acid. The separation of the fraction FV by silica gel column chromatography eluted with dichloromethane-ethyl acetate (3:1) yielded mixtures of α and β amyrin (183 mg). Fraction FVI gave pure β amyrin (67 mg). Fraction FX yielded after crystallization from MeOH, taraxerol (30.5 mg).

The n-hexane extract (3 g) was loaded on silica gel column. The column was packed and eluted with 100% n-hexane, gradually increasing the concentration of ethyl acetate. Each fraction of 250 mL was collected. Fractions (FH-A) eluted with 10% ethyl acetate were pooled together based on TLC profile. Likewise, fractions (FH-B) eluted with 20% ethyl acetate gave ursolic acid (892 mg) and taraxerol (101.7 mg).

From the methanolic extract, one yellow solid crystallized, it was washed with dichloromethane to give 1.22 g of quercetin-3-O-rahmnoside.

2.4. Cell lines

Human astrocytoma D384 cells, colon cell carcinoma RKO cells, breast cancer MCF-7 cells, prostatic carcinoma PC-3 cells were cultured in RPMI-supplemented medium (100 units/mL penicillin G, 100µg/mL streptomycin, 0.25µg/mL amphotericin B), 2mM L-glutamine with 10% fetal bovine serum (FBS, v/v, Invitrogen) in a humidified incubator (37 °C, 5% CO2).

2.5. Cell Viability Assay

Cell viability was analyzed by the MTS assay (Invitrogen) used to assess the viability and/or the metabolic state of the cancer cells based on mitochondrial respiratory activity. A total of 5×103 cells were seeded into each well of 96-well plates. After 24 h the cells were treated with seven different concentrations (1-100 µg/mL) of *B. resinosa* extract. Doxorubicin (Sigma) was used at five different concentrations (0.01–5 µM) as a positive control. Negative control cells were treated with vehicle DMSO to get the final concentration of 0.1% v/v. Each concentration/assay was performed 3 times in triplicate. The cells were then incubated with treatments for 48h. After 46h, MTS (5mg/mL) was added and cells were further incubated for 2h at 37 °C. The absorbance was measured at 570 nm against the reference wavelength of 650 nm was recorded using a Tecan microplate reader (Model Sunrise; Tecan Austria GmbH, Grödig/Salzburg, Austria). The percentage of viability was calculated based on the formula: Viability (%) = (absorbance of treated cells/absorbance of control cells) x 100%.

3. Results and Discussion

3.1. Structure elucidation

The phytochemical study from the aerial parts of. *B. resinosa* led to the isolation of the flavonoid quercetin-3-O-rahmmnoside (5) [13] along with the known triterpenes, α and β amyrin (2, 3), [14] taraxerol (4), [15] and ursolic acid (1), [16] being the last one the main secondary metabolite present in this species. These compounds are under typical profile of the phytochemical studies found in the *Bejaria* genus. The structures of the isolated metabolites (Fig. 1) were identified on the basis of spectroscopic data (^{1}H, ^{13}C NMR, MS) and by comparison of these data with literature values.

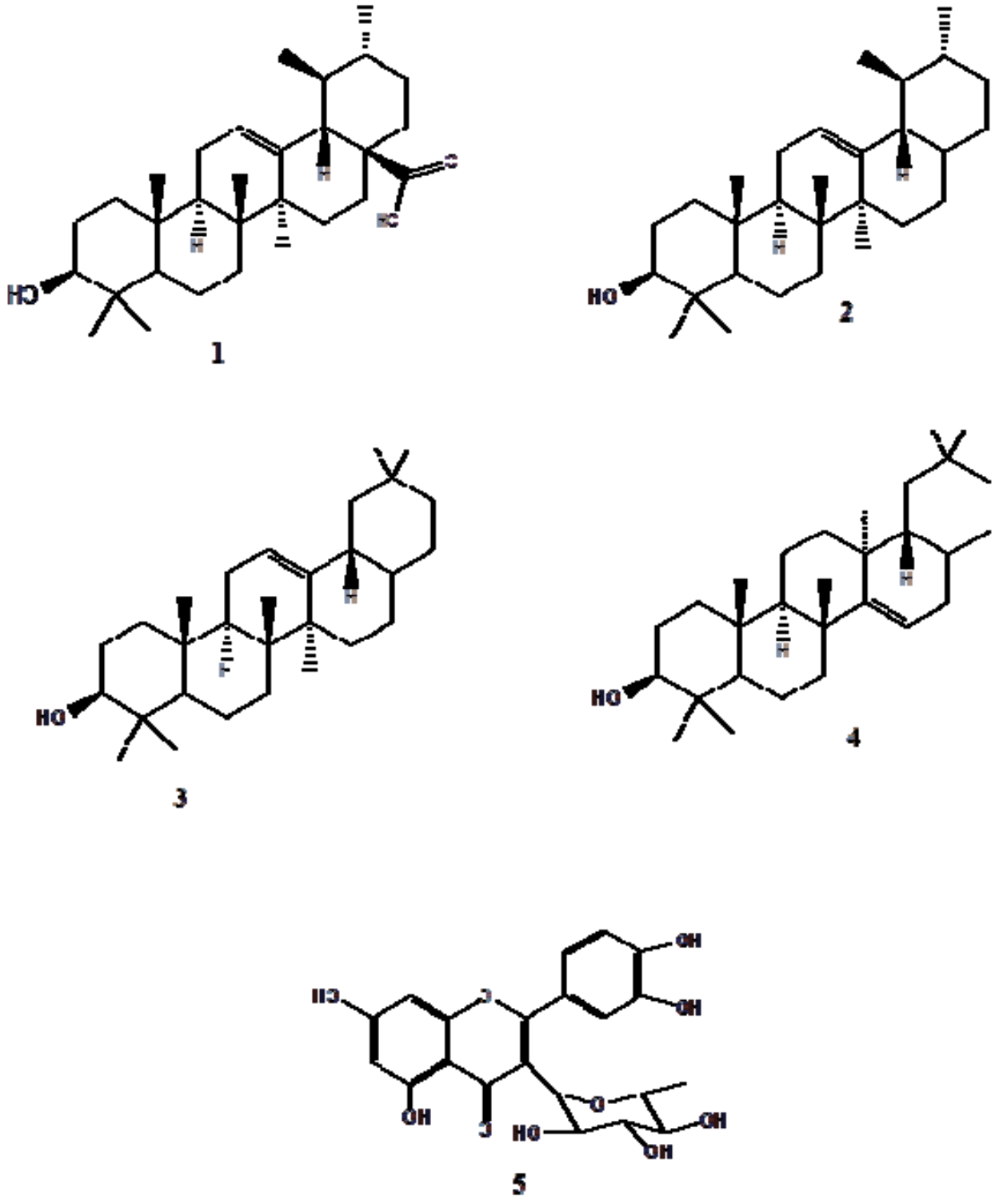

Figure 1 Structures of isolated compounds

Cytotoxicity effects of the aqueous extract and isolated compounds were tested against the four human tumor cell lines, D384, RKO, MCF-7 and PC-3 using the MTS method. Doxorubicin was used as positive control. The results clearly indicated that from the isolated, ursolic acid UA (1), was the compound with the best cytotoxic effect against the above mentioned cell lines. The Table **1** present only the results obtained with the aqueous extract and UA (1) on the cell lines.

This evaluation showed that the aqueous extract possessed moderate activity against D384 (IC_{50} 12.19 ± 0.24) and PC-3 (IC_{50} 11.22 ± 0.3), it was observed that from the all cell lines tested, MCF-7 was the only that not showed activity with the aqueous extract, whilst with UA (1), the main isolated metabolite gave the best cytotoxic effect (IC_{50} 7.43 ± 0.64), maybe other compounds present in the extract inhibit the effect of UA or perhaps because in the extract, it is in less concentration.

Table 1: Effect of leaves extract of *B. resinosa* and Ursolic acid on the growth of human cancer cell lines

	IC_{50} ± SEM			
	Human cancer cell lines			
Treatment	D384 (Astrocytoma)	RKO (Colon cancer)	MCF-7 (Breast cancer)	PC-3 (Prostate cancer)
B. resinous (µg/mL)	12.19 ± 0.24	71.49 ± 23.27	>100	11.22 ± 0.3
Ursolic Acid (uM)	10.39 ± 1.46	17.16 ± 8.99	7.43 ± 0.64	12.11 ± 0.52
Doxorubicin (µM)	2.45 ± 0.68	1.93 ± 0.09	5. 28 ± 0.11	3.83 ± 0.15

Each data is given as the mean and its standard error (SEM) of at least three independent experiments

Ursolic acid is pervasive triterpene reported with a wide spectra of biological activities, it has been mentioned to possess properties such as anti-inflammatory, antioxidative, analgesic, diuretic, antitumoral, hepatoprotective, hypolipidemic, antibacterial, anti-HIV, anti-angiogenic and immunomodulator between others [18-26]. In relation to cytotoxic activity, UA has been shown to have effect against several cancer cells, also showed be apoptotic and had activity on the angiogenesis [27].

The triterpenoids are one of the more profuse and diverse compounds found on plants. In the phytochemical screening of many plants used in traditional medicine, this group of compounds have shown to be responsible for the therapeutic activity attributed to those species. Triterpene acids like ursolic, oleanolic, and betulinic, have been isolated from plant extracts, demonstrating have multiple biological activities, including antitumor. Moreover the results herein shown, indicate that UA, the main compound found in the aerial parts of *B. resinosa*, is the cytotoxic principle of this plant, and these results validate the use of this plant under the traditional medicine of the Saraguro ethnia in Ecuador.

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