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EXTRACTION AND IDENTIFICATION OF SECONDARY METABOLITES FROM SIBERIAN GROUNDSEL

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ABSTRACT. Siberian groundsel (*Ligularia sibirica* (L.) Cass) contains many volatile compounds, pyrrolizidine alkaloids and eremophilane sesquiterpenes. This study focuses on the identification of some semi volatile and nonvolatile secondary metabolite components of from this plant, by using liquid and gas chromatography with mass spectrometry detection (LC-MS and GC-MS). From the root extracts has been identified one preudoguianolide compound by LC-MS method, in addition one guianolide and one germacrene sesquiterpene by GC-MS method. Leaf extracts analyses have been not resulted identification of any compounds.

Keywords: *Ligularia sibirica, mass spectrometry, fragmentation mechanism, eremophilane sesquiterpenes, pyrrolizidine alkaloids*

INTRODUCTION

Many plants produce valuable bioactive secondary metabolites for pharmaceutical industry, however studying the phytochemical composition and isolation of these compounds are important part of developing new medicines and therapies.

Genus *Ligularia* is widely spread in Europe and Asia, 20 to 40 species from the existing 211 species have been used in traditional folk medicine [1] being antipyretic, diuretic and choleretic agents for clearing heat and removing toxins from human body [2]. They have relieving phlegm and cough effect, invigorating circulation of blood and reducing pain [3, 4].

The most known chemical constituents from *Ligularia* species are eremophilane-type sesquiterpenes, with some cytotoxic and anti-tumor activities [3, 5-7].

The relict species of Siberian groundsel (*Ligularia sibirica* (*L.*) *Cass*) contain eremophilane sesquiterpenes and pyrrolizidine alkaloids, such as volatile compounds [8]. Isolated eremophilanes from this plant (fig. 1) [9,10] are not tested

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from biological activity viewpoint, but tussilagine and iso-tussilagine pyrrolizidine alkaloids are possessing antimicrobial and immune system stimulator effect and are used in anti HIV-1 [11], HSV-1 and HSV-2 treatments [3, 12, 13].

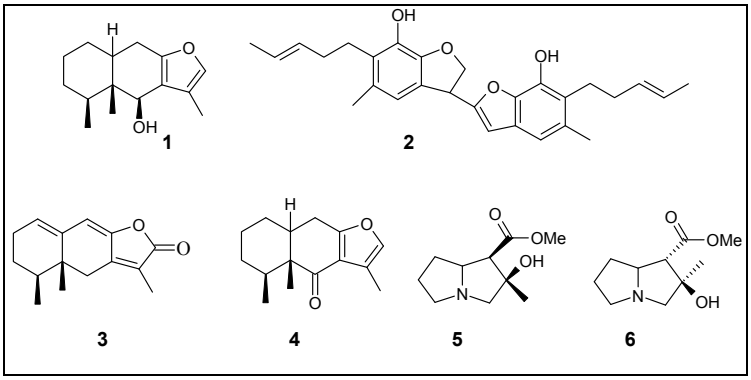

Figure 1. Identified compounds from *L. sibirica*; ligularol (1), ligularine A (2), ligularenolide (3), ligularone (4), tussilagine (5) and iso-tussilagine (6)

Chromatography - mainly the gas chromatography and high performance liquid chromatography - is mostly employed separation technique in analytical chemistry, particularly for the isolation of relatively small concentration of compounds and mainly quantitative estimation of compounds from different matrices, for example vegetal and mammalian tissue extracts [14].

Mass spectrometry (MS) coupled with GC and HPLC are powerful techniques for identifying molecular structures and quantitative measurement. Essentially, the mass spectrometry is an analytical method based on the separation of ionized molecules in inhomogeneous electric and magnetic fields and the separation is given by the mass/charge ratio differences of the ionized molecular fragments. After the separation of molecules on columns, the molecules are ionized and fragmented by different methods (fast atom bombardment (FAB), atmospheric pressure ionization and electrospray ionization (APCI and ESI), matrix assisted laser desorption/ionization (MALDI)), ions are formed in the source region and they are accelerated by an electric field in the mass analyzer [15, 16]. The quadrupole analyzer instrument executes the separation of the ions. It is constructed from four electrode rods, arranged opposite from each for other and opposite pair electrodes are connected electrically and a voltage, consisting of both radiofrequency and direct-current components that produce an oscillating electric field which functions as a band pass filter to transmit the specific mass/ charge ratio [15, 16]. In the mass spectrometer, molecular ions are energetically unstable, and some of them break up in smaller parts, named fragments. This process is named fragmentation mechanism that shows hard dependence from volatilization degree of molecules and from the applied ionization method [16].

In the case of GC-MS, the probe is almost totally volatilized and is more powerful ionized during electric impact ionization and that way the fragmentation process is more specific and more complete than in the case of LC-MS coupled by soft ionization modes, for examples ESI and APCI [15-17]. Consequently, identification of compounds is easier in case of GC-MS, having large a database. For or LC-MS (caused by incomplete fragmentation and large number of setting parameters) the identification is more difficult, because of lack of an extended database.

The aims of this study is developing an analytical method, like gas chromatography and liquid chromatography with mass spectrometry detection, for identification of compounds obtained from extracts of the studied plant species.

RESULTS AND DISCUSSION

Result of liquid chromatographic analysis of root and rhizome extract

The mass spectra of the identified compounds were obtained with LC-MS method (without using standard) by determination of their fragmentation mechanisms.

LC-MS analysis of extract resulted presumably identification of one pair of pseudoguaianolide lactone, named erigerolide or britanin, which are showing similar m/z 366 $[M]^{+\cdot}$ 306 $[M\text{-}CH3COOH]^{+}$ and 246 $[M\text{-}2\times CH_3COOH]^{+}$ characteristic ions for GC-MS detection [18]. Two from the three identical fragments, m/z 367 $[M+H]^{+}$ (100) and m/z 307 have been observed in the peak at 18.4 min retention time (fig. 2). The fragmentation mechanisms of pseudoguianlolides, including erigerolide and britanin has been described by Tsai and his coworkers [19], when m/z 307 ion brake into m/z 109, 123 and 95 representative fragment ions (fig. 3 and 4).

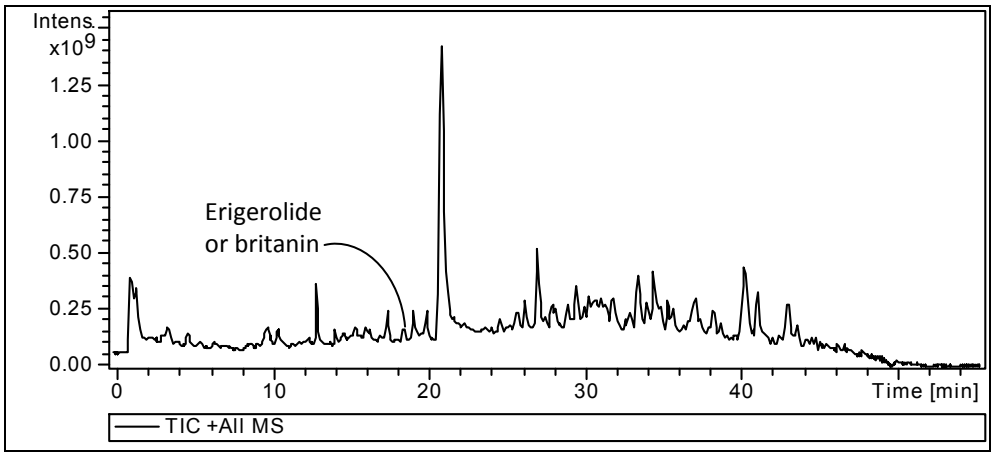

Figure 2. LC-MS chromatogram of root and rhizome extract

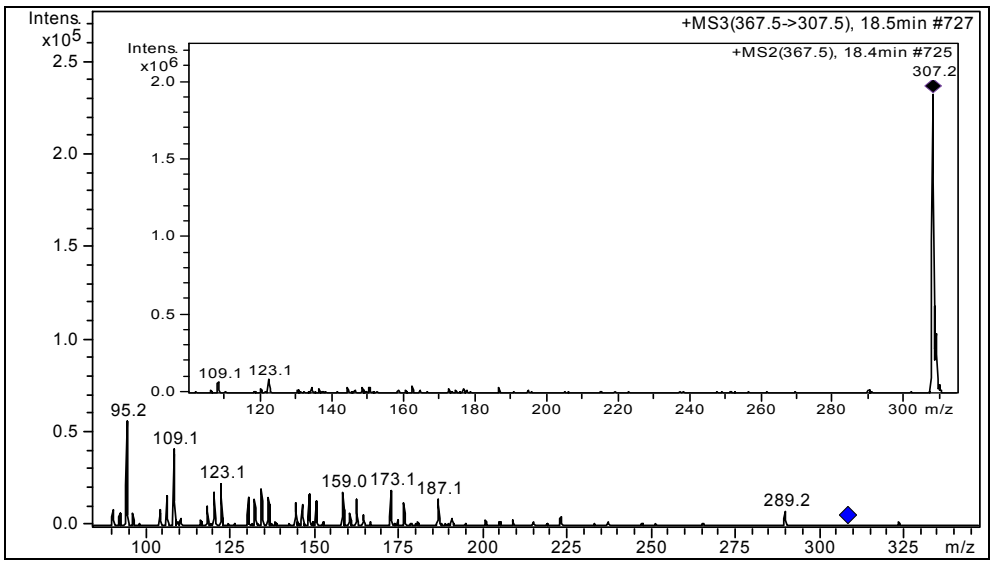

Figure 3. LC-MS^2, LC-MS^3 spectrum of erigerolide or britanin

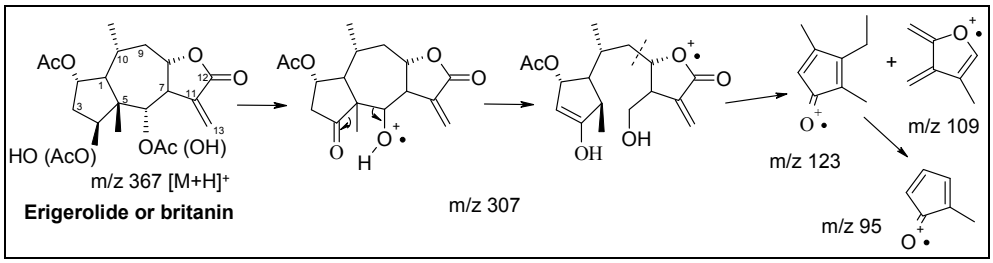

Figure 4. Presumed fragmentation mechanism of erigerolide or britanin

Result of gas chromatographic analysis of root and rhizome extract

Gas chromatography analysis of root and rhizome extract resulted separation and identification of only one germacrene (fig. 5) and one guianolide sesquiterpene named achillin (t_r=11.43 and 13.52 in fig. 6). Other compounds are presumably not volatile or they van be decomposed easy in gas chromatograph injector. Both compounds have m/z 246 $[M]^+$ spectra.

From biological viewpoint, only achillin is known as anti-inflammatory, antifeedant and plant growth inhibitor substance [20, 21]. It has no anticancer activity to RKO and RKO-E6 colon cancer cell lines [22]. Bilogical activity of erigerolide, britanin and identified germacrene compound is not described in literature.

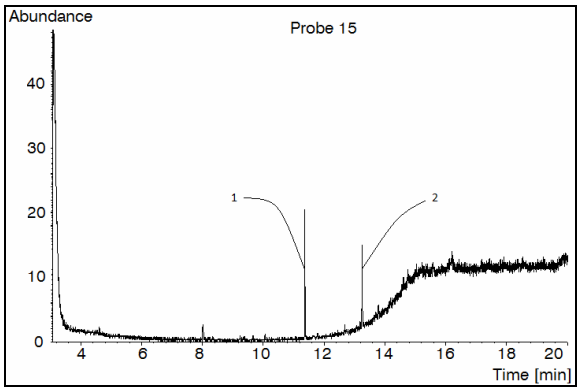

Figure 4. GC-MS chromatogram of extract of the root extracts; one germacrene (1) and achillin (2)

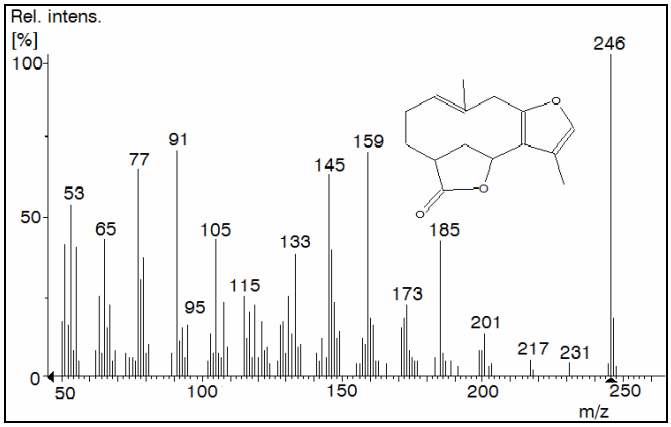

Figure 5. GC-MS spectra of germacrene

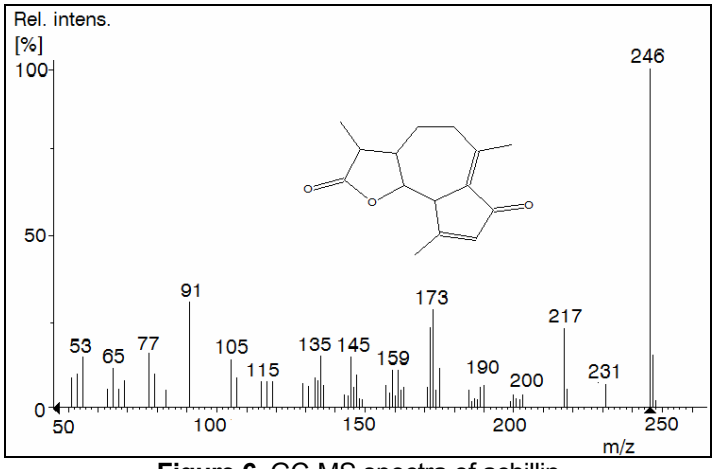

Figure 6. GC-MS spectra of achillin

CONCLUSIONS

Nevertheless, compounds isolated by other authors were not identified in this study. Composition differences between the Romanian and Mongolian species could partly be due to habitats and partly due to genetic properties differences between the two plants. The less successful GC-MS and LC-MS analysis of extracts requires executing further research for extraction, separation, purification and structure elucidation of compounds.

EXPERIMENTAL SECTION

Materials and apparatus

The plant (*L. sibirica*) was collected in September of 2009 in Harghita marsh from Harghita County, Romania and stored in a cool and dry place. All the necessary chemicals (dichlormethane, ammonium hydroxide, anhydrous sodium-sulfate, acetonitrile, methanol and hydrochloric acid) were obtained from Sigma Aldrich Co.

Extraction procedure

Four grams of powdered rhizome and roots were suspended in 1 ml of ammonium-hydroxide solution (25% v/v concentration) and were extracted with 30 ml of dichloromethane at room temperature in ultrasonic bath at 10 minutes. The obtained extract was filtered through a layer of anhydrous sodium-sulfate and stored in dark at 4 °C temperature. Before analysis they were evaporated, resolved in acetonitril-methanol-acetic acid solution (75:25:1) or *n*-hexane and finally filtered with a syringe filter.

For the extraction of leaves we used the same extraction procedure as in the case roots and rhizomes The filtered extract was evaporated under reduced pressure at 40 °C and the residue was dissolved in 30 ml of 2% sulfuric acid. The acid soluble fraction was filtered and neutralized to pH 9-9.5 with ammonium-hydroxide solution, and then was extracted two times with amount of 30 ml of chloroform at room temperature. For LC-MS analysis the extract was evaporated and resolved in acetonitril-methanol-acetic acid mixture (75:25:1).

Liquid chromatography with mass spectrometry detection

The LC separations were performed using Agilent 1100 HPLC system, column: Phenomenex Luna C_{18} (2), (10 cm × 2.0 mm × 2.5 µm), the flow rate was 0.2 ml/min at 25 °C temperature. In all cases the injected volume was 4 µl of acetonitril extract. The mass spectrometry in every case was executed by using one Agilent LC MSD XCT Plus mass spectrometer with positive ionization

mode with 40 psi pressure nebulizer gas, 8 ml/min flow rate dry gas at 350 °C temperature. The eluent composition were 1% aqueous solution of acid acetic (A) and solution of acetonitril-1% and acetic acid (B). The gradient program was the following: from 0 to 55 min: 30 to 100 % B, from 55 to 65 min: 100% B eluent and finally 10 minutes postrun.

Gas chromatography with mass spectrometry detection

GC-MS experiments were executed by Agilent 6890 N Network GC System, Agilent 5975 mass selector detector and Agilent 19091S-602b HP-1MS (25.0 m × 200 μm × 0.33 μm) column. The carrier was He gas at pressure 168.0 kPa and 1.5 ml/min flow rate. Temperature program was started from 150 °C at 0 min with 10 °C/min temperature gradient to 325 °C. 1 μl from the diluted (1:25) sample was injected at 250 °C injector temperature. Detection was performed in scan mode from m/z 50 to 600, at 70 eV. MS Quad and MS source temperatures were set at 150 and 230 °C respectively.

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