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RESEARCH ARTICLE

Ultrasonic-Assisted Extraction of Bioactive Compounds from Elecampagne (*Inula helenium*, Radix Inulae)

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Abstract

In this study, we had developed a method of ultrasound assisted extraction from Elecampagne in order to obtain, identify and the dosing the main bioactive compounds from plant, such as: alantolactone and isoalantolactone, inulin etc. Aqueous/hydroalcoholic extraction assisted by ultrasound, was used in order to obtain plant extracts rich in bioactive compounds, in order to ensure the highest possible extraction yield (exhausting the plant). In the process of extracting bioactive compounds from plant, the acoustic energy of the ultrasound is not absorbed by the molecules and, as such, has an effect of breaking the cell walls and improving the mass transfer. Ultrasound are transmitted in the reaction medium in the form of pressure waves that induce vibrational movements of the molecules, thus the molecular structure of the medium, alternately, it compresses and expands, as a result of a pressure, that varies over time. When the ultrasound intensity is high enough, the expansion cycle can create bubbles or cavities. Cavitation is the process by which bubbles form, grow and undergo implosion. Ultrasound allows the solvent to penetrate the cell walls, and the bubbles produced by the acoustic cavitation favor the breaking of the cell wall and the release of the active compounds, increasing the reaction yield.

A high content from these compounds, make the plant to be used in the treatment of different respiratory diseases (asthma, cold, flu), indigestion, intestinal parasites etc.

Recent studies had shown that the extracts from dried root of Elecampagne have antitumor and antimicrobial effect and also detox and anti-inflammatory action.

Introduction

Elecampagne is sporadically cultivated as a medicinal plant, but often it is found in spontaneous flora, in wet places from meadows, everglades, orchards and vineyards. It is used in traditional medicine for headaches, colds, tuberculosis, stomach diseases. The root of Elecampagne contains Inulin (40–50%), volatile oils, alantolactone, the leaves contain alantopicrine (sour principle), vitamin C, mineral salts and the flowers contain helenina.

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Active principles give it, diuretic, anti-inflammatory, anti-rheumatic properties, and some principles of alantolactone and isoalantolactone give it, general tonic properties and moderate anti-tumor properties.

Elecampagne roots, harvested in the fall, after the 2-3 year of vegetation, are traditionally used for skin conditions, bronchitis, helminthic diseases [1], against asthma, expectorant and insecticide [2], diaphoretic and antibacterial agent [3].

Native Americans used elecampagne decoction to treat lung disorders and tuberculosis [4]. Some specialized articles show that polyphenols and flavones from elecampagne have antioxidant [5], antitumor [6] and immunomodulatory properties [7]. Other articles show that the extracts from elecampagne, in the form of fractions, obtained by column chromatography, have an important activity against Mycobacterium Tuberculosis, in particular, due to the 2 lactones in the composition, alantolactone and isoalantolactone [8]. The structures of alantolactone, isoalantolactone and inulin are presented in figures 1,2. Figure 3 shows images of the elecampagne plant.

In this study we set out to identify and dose the active aqueous/hydroalcoholic extracts, from the root of the elecampagne, the dried, ground and

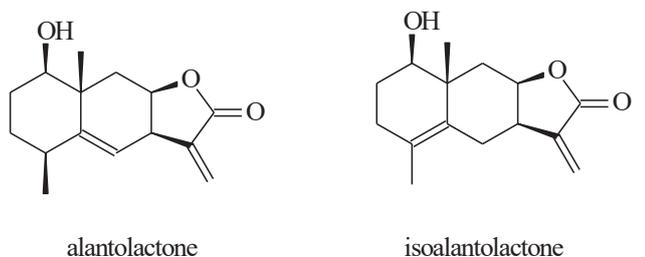


Figure 1 Alantolactone and isoalantolactone structures [9].

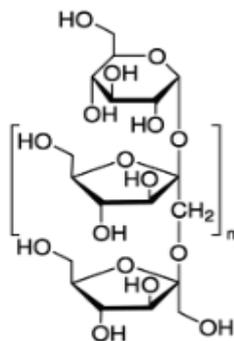


Figure 2 Inulin structure [10].



Figure 3 Inula flower, herb and root.

sieved plant. Aqueous/hydroalcoholic extraction assisted by ultrasound, was used in order to obtain plant extracts rich in bioactive compounds, in order to ensure the highest possible extraction yield (exhausting the plant). In the process of extracting bioactive compounds from plant, the acoustic energy of the ultrasound is not absorbed by the molecules and, as such, has an effect of breaking the cell walls and improving the mass transfer. Ultrasound are transmitted in the reaction medium in the form of pressure waves that induce vibrational movements of the molecules, thus the molecular structure of the medium, alternately, it compresses and expands, as a result of a pressure, that varies over time. When the ultrasound intensity is high enough, the expansion cycle can create bubbles or cavities. *Cavitation* is the process by which bubbles form, grow and undergo implosion [11,12]. Ultrasound allows the solvent to penetrate the cell walls, and the bubbles produced by the acoustic cavitation favor the breaking of the cell wall and the release of the active compounds, increasing the reaction yield [13].

Physico-chemical analysis was performed to determine total polyphenols (expressed in gallic acid) total flavones (expressed in quercetin) as well as the antioxidant capacity, for the extracts used. The volatile compounds were extracted, using the method described in the European Pharmacopoeia [14] starting from 50 g of elecampagne root, and the chromatographic profile of the volatile compounds

from the extracted oils was analyzed by GS-MS, in which alantolactone and isoalantolactone were identified.

Materials and Methods

Materials

The root of Elecampane was harvested, in 2022, from Hofigal SA. This was washed and then dried on artificial dryer Biovita, in controlled regime (at 40°C, for a period of 48 hours). Biovita dryer is a dehydrator with hot air, with trays. After drying, the plant is grinded in the mill GM-200, of laboratory, supplier RETSCH-VERDER-Romania, at granulation 1-2 mm, for dried plant and 1-3 mm for fresh, undried plant.

The reagent Folin-Ciocalteu, Fehling, CuSO₄, Neocuproine, sodium acetate, aluminium chloride, ethanol, methanol and the other used solvents and reagents are of analytical purity (chemical pure, cp or for analyze, pa).

Ultrasonication apparatus is a cuvette ELMASONIC P type, *Elma*, with PULSE application, working frequency 80 KHz, max.1130 W power, with displaying of working temperature and setting of the working time.

Working method

In this study, the extraction assisted by ultrasound of the elecampagne root it was followed, in the presence of water or ethanol (aqueous solutions of 50 or 96.5%) as solvent, according to scheme 1 shown below. For sample 5(P5), in order to obtain an inulin concentrate, I used the method from the literature [15], with 60 g of elecampagne root, in methanol solvent, R = 1/5, in the presence of ultrasound, followed by precipitation of inulin with 96% ethanol.

The elecampagne the fresh root, was harvested in October-November 2022, it was cleaned of soil, washed, dried and ground in laboratory mill, GM 200. The cited fraction, between 315-500 µm, was used for de following five types of extraction (Scheme 1):

Sample processing for analysis (samples 1-4): 1 g of the prepared sample (of the 4 samples - solution) is weighed on the analytical balance into a weighing vial, then placed in a 10mL volumetric flask and adjusted with the same solvent (ultrapure water, HA 50%, or ethanol 96.5%) for each sample in which the plant material was extracted.

Sample 5: For the determination of inulin in the precipitate using the spectrophotometric method, weigh 1.0 gram of the precipitate into a weighing vial on the analytical balance, and crush with 5 mL or 10 mL of ultrapure water at a room temperature of 25°C. Leave for 10 minutes, filter through a Whatman filter with pore size 8-12 µm into a 100 mL volumetric flask and wash the Whatman filter with ultrapure water, repeat the crushing operation several times with small volumes of ultrapure water until the filter remains clean and add to the mark with the same solvent.

Methods of analysis

Total Polyphenol Content (TPC), expressed as gallic acid equivalent: The Total Polyphenol Content (TPC) was assessed using the Folin-Ciocalteu method [16].

The absorbance was measured at 765 nm against ultrapure water with a Jasco V-530 UV/VIS spectrophotometer and the total polyphenol content was determined using the standard gallic acid calibration curve with concentrations ranging from 5 to 100 µg/100 mL and expressed as gallic acid equivalent (mg/g).

Scheme 1: Working method.

Sample	Dried Plant, g (Elecampagne)	Solvent	Treatment
P1	1	50 mL ultrapur water	15 min. grinding + 15 min. US
P2	1	50 mL ethanol 96.5%	idem
P3	1	50 mL ethanol 50%	idem
P4	20	200 mL ethanol 50%	15 min. grinding + one hour US
P5	60	300 mL methanol	Maceration, 7 days, one hour US, concentration, precipitation with ethanol 96% and analysis of precipitate [15].

Note 1: This sample (P5) was studied to obtain, with the help of maceration and ultrasound, an increased dosage yield of inulin from elecampagne root, by extraction in methanol and precipitation with 96% ethanol. Then, by comparing the result with the data from the literature, we will be able to observe the positive effect of applying ultrasound in order to increase the reaction yield.

Note 2: Filter the solution after each ultrasound.

The Total Flavone Content (TFC): Was determined using the method described by Marinova D, et al. [17], namely the spectrophotometric method in the presence of aluminium chloride, using quercetin ($\lambda = 510$ nm) as analytical standard.

This method is based on the formation of a light reddish-brown colored complex following the reaction of flavonoids and aluminium chloride in a weakly acidic medium. The total flavonoid content was expressed as quercetin equivalent (mg/g) [17].

The absorbance was measured at 510 nm against a control prepared with water instead of the extract sample with a Jasco V-530 UV/VIS spectrophotometer, and the TFC was determined using a standard quercetin calibration curve with concentrations ranging from 5 to 100 $\mu\text{g}/\text{mL}$ and expressed as quercetin equivalent per gram.

Total antioxidant capacity (Ac AO) by CUPRAC (CAT-CUPRAC-Reducing Antioxidant Power) method: The CUPRAC method described and modified by Özyürek M, et al [18], is based on the total antioxidant capacity (CAT-CUPRAC-Reducing Antioxidant Power) by reducing the cupric ion Cu^{2+} to the cuprous ion Cu^+ . The antioxidant activity is measured using a Jasco V-530 UV/VIS spectrophotometer, based on a calibration curve using Trolox (antioxidant substance) of known concentrations as standard, at a wavelength $\lambda = 450$ nm [18,19].

Determination of the antioxidant capacity FRAP II $\lambda = 593$ nm (by reducing Fe^{3+} to Fe^{2+}) (Ferric reducing antioxidant power): This Frap II method relies on the reducing power of biologically active compounds associated with their electron-donating capacity, thus reducing ferric ions to ferrous ions, which form an intensely violet-blue colored complex under acidic pH [20,21].

The inulin content (P5) is determined using the spectrophotometric method (UV-VIS): At an absorbance of 413 nm, using perchloric acid, resorcinol and hydrochloric acid as reagents, based on a previously established calibration curve [22,23].

GS-MS identification of the active substances, alantolactone and isoalantolactone: Analyses were performed using the Thermo Scientific Focus GC gas chromatograph with a Macropol 20,000 R column (0.25 μm film thickness, 60 m length and 0.25 mm diameter). The mobile phase used was helium at a flow rate of 1.5 mL/min, while the sample injection volume was 1 μL . The Thermo Scientific DSQII mass spectrometer was used for detection.

Elecampane, raw material, dried, ground and sieved plant is prepared for GS-MS, same as sample 2 (dried plant + ethanol 96.5%, grinding, US, filtration, concentration, uptake in HA 50%). The GS-MS identification of active substances in elecampane is also performed for sample 4, as sample 4 is the extraction method for active substances very common in practice (solvent : plant ratio = 10:1, v/m, US, 1 hour, solvent, ethanol 50%). For both determinations, raw material and sample 4, the sample preparation for GS-MS analysis involves the following procedure:

A volume of 50 mL of the extract prepared as above (HA 50%) is placed in a separating funnel with a volume of 10 mL hexane. After stirring, leave to stand for separation of the two layers (hexane and hydroalcohol). The hydroalcoholic layer is removed from the separation funnel, and the hexane layer is run over anhydrous sodium sulfate to remove traces of water, then filtered through a 0.2 μm filter, and injected into the system.

Statistical methods

All 5 analyzed samples were carried out in triplicate and the data were expressed as mean value \pm SD (Standard Deviation) for triplicate of samples ($n = 3$). All the results were subjected to one-way Analysis of Variance (ANOVA). Statistical analysis of the data was performed using the multiple comparison post hoc t -tests in order to detect the significant statistical differences between the averages of the Total Polyphenol Content (TPC) and Total Flavone Content (TFC) of two or more independent groups. The differences were considered statistically significant at $p < 0.05$ as a minimal level of significance.

Results and Discussion

The results of the above analyses are listed in tables 1,2 below.

Total Polyphenol Content (TPC)

The data analysis in table 1 shows that the solvent is very important, the replacement of water with ethanol (96.5%) causes a significant increase in the amount of polyphenols (about four times compared to the water solvent). When ethanol is used as solvent 50% the amount of polyphenols extracted decreases but remains higher than when only water is used. In terms of plant: solvent ratio, a plant: solvent ratio of 1 to 50 was used in the first three types of extractions. Under these conditions the amount of extracted compounds is higher (expressed in mg/gDM) (sample

Table 1: Values of total polyphenols and flavones, mg/mL and mg/gDM.

Crt. No.	Analysis Description	Concentration Solutions, Scheme 1, mg/mL				Content Solutions, Scheme 1, mg/gDM (extraction yield)			
		P1	P2	P3	P4	P1	P2	P3	P4
1	Total flavones	0.28	0.233	0.639	2.042	14	11.65	31.95	20.42
2.	Total polyphenols	0.103	0.401	0.172	0.665	5.15	20.05	8.6	6.65

Table 2: Antioxidant capacity values, mg/g, CUPRAC and FRAP 2.

Crt. no.	Analysis Description	Obtained Values, mg/g, Antioxidant Capacity, P1, P2, P3, P4, Scheme 1.			
		P1	P2	P3	P4
1.	Antioxidant capacity, CUPRAC, expressed in TROLOX	0.172	0.504	0.376	2.141
2.	Antioxidant capacity, FRAP2expressed in FeSO ₄ xH ₂ O	0.199	0.059	0.375	1.539

3 compared to sample 4) but the concentration of the obtained solutions is too low for commercial uses (expressed in mg/mL). For commercial uses the best conditions are those of experiment 4, where 50% ethanol solution in a S:L ratio of 1:10 is used as solvent and the solutions are sufficiently concentrated to be used in food supplements.

The chart representation of the results in table 1 is shown in figure 4. From the chart shown in figure 4 it appears that sample 4 provides the most concentrated solutions.

It can be concluded that the best method for extracting polyphenols from dried elecampagne root is the US-assisted hydroalcoholic extraction at an optimal plant/solvent ratio, R = 1/10.

Total Flavones Content (TFC)

According to the data in table 1 the best solvent for flavone extraction is 50% ethanol. Again, changing the S:L ratio from 1:50 (experiments 1-3) to 1:10 (experiment 4) leads to a decrease in the amount of extracted flavones (expressed in mg/gDM). However, the fact that a sufficiently high concentration is obtained in solution (expressed in mg/mL) makes this type of extraction suitable for commercial applications.

The chart representation of the data in table 1 is shown in figure 5.

As shown in the chart and data in table 1, concentration of total flavones are 2.3 times higher in the HA 50% hydroalcoholic extract, with R = 1/50, than in P1 and 7.3 times higher in the HA 50% hydroalcoholic extract, with R = 1/10, US, one hour, than in P1. It shows that the method most often used

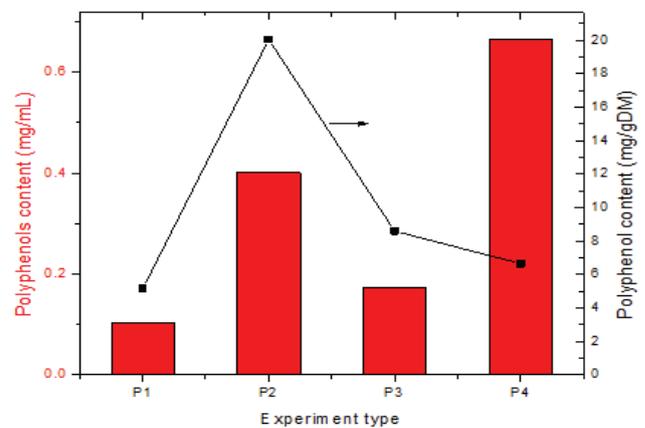


Figure 4 Polyphenol values, mg/g DM, for elecampagne root, US, depending on solvent and plant/solvent ratio: 1). water, R = 1/50, 2). ethanol 96%, R = 1/50, 3). ethanol 50%, R = 1/50, 4). ethanol 50%, R = 1/10, R = m/v.

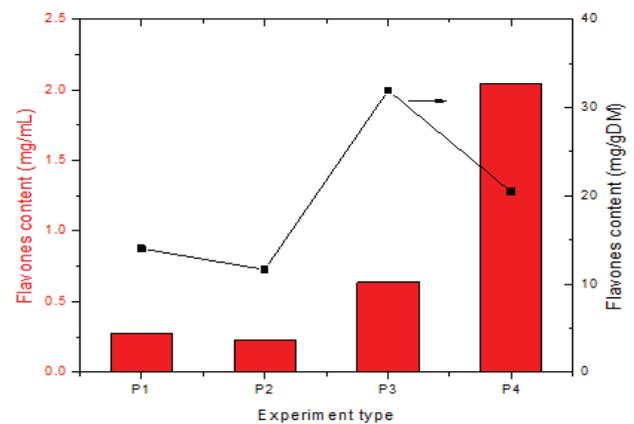


Figure 5 Flavone values, mg/g DM, for elecampagne root, US, as a function of solvent and plant/solvent ratio: 1). water, R = 1/50, 2). ethanol 96%, R = 1/50, 3). ethanol 50%, R = 1/50, 4). ethanol 50%, R = 1/10, R = m/v.

in practice, i.e., ultrasonic-assisted extraction in hydroalcoholic solvent at the optimum plant/solvent ratio, $R = 1/10$, gives the best results. The lowest flavone values of 0.233 mg/mL (for sample 2) indicate lower flavone extraction in concentrated alcohol, 96.5% ethanol.

Antioxidant capacity CUPRAC

The data in table 2 show that the values for CUPRAC antioxidant activity, (expressed as TROLOX, mg/g) for the extracts studied have the following values: 0.172 mg/g for P1, 0.504 mg/g for ethanolic extract 96.5%, 0.376 mg/g for hydroalcoholic extract HA 50%, with plant/solvent ratio $R = 1/50$ and 2.141 mg/g for HA 50%, with $R = 1/10$. The chart representation of the results obtained for CUPRAC is shown in figure 6.

As shown in the chart and the data in table 2, the CUPRAC values are lower in hydroalcoholic solvent, HA 50%, than in ethanol 96.5%, with the same plant/solvent ratio, $R = 1/50$ and the same ultrasonication time, 15 min, but double/triple compared to P1. However, by increasing the ultrasonication time from 15 min. to one hour and by improving the plant/solvent ratio fivefold, at $R = 1/10$, the CUPRAC values also reach values 5.7 times higher (sample 4 compared to sample 3) and 12 times higher than the values obtained in aqueous extraction (P1). The extraction conditions for sample 4, i.e. plant/solvent ratio $R = 1/10$, US, one hour and hydroalcoholic solvent HA 50%, are the most favourable for the extraction of active principles, including CUPRAC, which is also confirmed in industrial practice for the Elecampagne root.

Antioxidant capacity FRAP 2

The data in table 2 show that the antioxidant activity, FRAP 2 (expressed in mg/g $\text{FeSO}_4 \cdot \text{H}_2\text{O}$) has a value of 0.199 mg/g for P1, 0.059 mg/g for ethanolic extraction 96.5%, lower than P1, 0.375 mg/g for hydroalcoholic extraction HA 50% and 1.539 mg/g for sample 4, which meets the most favorable extraction conditions: plant/solvent ratio $R = 1/10$, US, one hour, hydroalcoholic solvent Ha 50%. The chart representation of the data in table 2 for FRAP 2 is shown in figure 7.

From the chart representation and the data in table 2, the FRAP 2 values increase relative to P1 in the hydroalcoholic extracts, twice for $R = 1/50$ and US, 15 min. and reach 7.7 times higher values for the most favorable conditions obtained for sample 4, i.e.: US, one hour, HA 50%, $R = 1/10$.

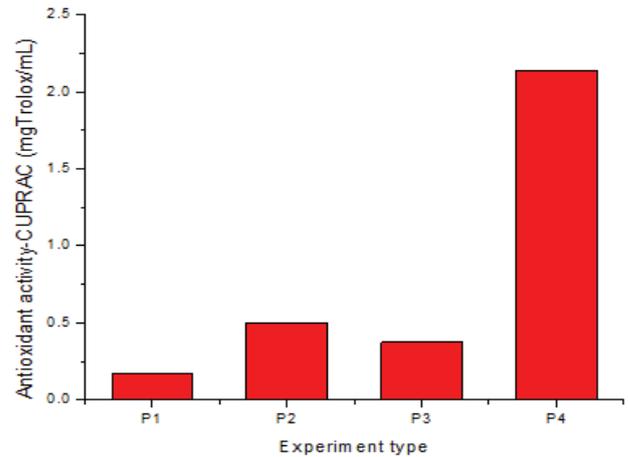


Figure 6 CUPRAC values, mg/g, for elecampagne root, US, as a function of solvent and plant/solvent ratio: 1). water, $R = 1/50$, 2). ethanol 96%, $R = 1/50$, 3). ethanol 50%, $R = 1/50$, 4). ethanol 50%, $R = 1/10$, $R = m/v$.

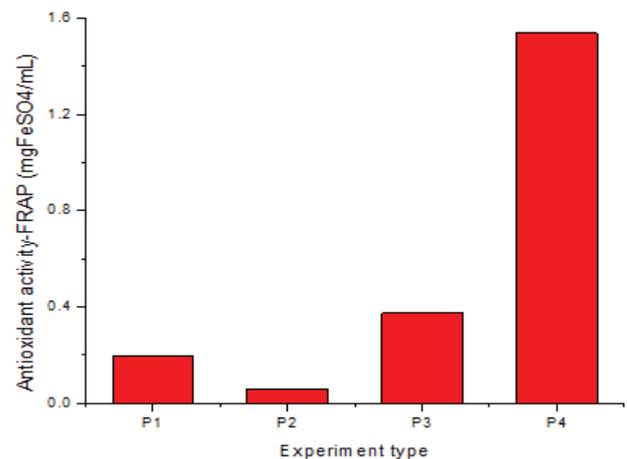


Figure 7 FRAP 2 values mg/g, for elecampagne root, US, as a function of solvent and plant/solvent ratio: 1). water, $R = 1/50$, 2). ethanol 96%, $R = 1/50$, 3). ethanol 50%, $R = 1/50$, 4). ethanol 50%, $R = 1/10$, $R = m/v$.

Regarding the ratio between the antioxidant capacity values, CUPRAC or FRAP 2, they are approximately equal in most of the samples, except for sample 2, where CUPRAC has values 10 times higher than FRAP 2, which shows that the mechanism of FRAP 2 (ferric reducing antioxidant power) mechanism does not occur in 96% ethanol medium.

Taking into account all studied parameters (TPC, flavone content and antioxidant activities) the best results for the studied extractions or obtained for sample 4, with the following conditions: extraction time assisted by US 60 min, solvent HA 50%, $R = 1/10$.

These conditions represent the most favorable way of working encountered in practice.

From the data presented above, it follows that the concentration of polyphenols, flavones and antioxidant activity is positively influenced by the concentration of the solvent, leading to the highest values for ethanol 50%, but equally by the solvent/plant ratio (R) (m/v), for which $R = 10/1$ is the optimal value obtained. This optimal ratio between solvent and plant leads to at least double the values of the bioactive compound concentration (expressed in mg/mL) compared to the other ratio, $R = 50/1$. This has a high economic efficiency by using small amounts of solvent. The optimal ratio, $R = 10/1$, favors the vibrational movements of the molecules so that the molecular structure of the solvent, under the influence of ultrasound, compressed and expands, favoring the penetration of the solvent inside the cells and the release of active compounds, increasing the reaction yield.

The inulin content (P5) analyzed in chapter Methods of analysis, has a value of 383.2 mg/g (38.32%), which corresponds to the literature data

According to Bokov DO, et al. [24], the inulin content should be min. 25%, in the dry plant and according to Petkova NT, et al. [25], the inulin content is 32–33%. With the extraction in methanol, daily stirring, US, 1 hour, followed by precipitation of inulin with 96% ethanol [15], we managed to increase the value of the inulin content dosed from the dried plant to 38.32%.

GS-MS identification of the active substances, alantolactone and isoalantolactone

The identification of the active substances in the elecampagne, alantolactone and isoalantolactone, using the GS-MS was carried out for the dry raw material and for sample 4, an extraction often used in current practice. The resulting chromatograms, following the procedure described in chapter Methods of analysis, are shown below in figures 8,9. The two active substances, alantolactone and isoalantolactone have the largest peak area, making them the main bioactive compounds in elecampagne root, raw material or hydroalcoholic extract, with the value of the cumulative concentrations for the two alantolactones reaching 86%, which is extremely beneficial for the treatment of respiratory diseases and for the antitumor and antimicrobial effect of elecampagne root products.

We had determined by distillation (method from European Pharmacopoeia, [14]) the content of volatile oils from plant, obtaining a value of 0.25 mL per 50 g of the sample taken in the work, i.e. a value of 5 mL per kg of dry plant, and we analyzed, GS-MS, the volatile oil. We identified the existence of alantolactone and isoalantolactone in chemical composition of these oils, using the library of spectra NIST. Below we give GS-MS chromatogram for the volatile oil of Elecampagne, figure 10.

Conclusion

In this study the extraction of the bioactive compounds from the elecampagne root was followed, considering the importance of this plant in the treatment of various ailments. Using the US-assisted extraction of the active compounds from the elecampagne root, we obtained results comparable to those in specialized literature. The most suitable solvent was the hydroalcoholic solution of ethanol in ratio S:L of 1:10.

Ultrasound assisted extraction of bioactive compounds from inula root is highly efficient, requires low energy, small amounts of solvent and short periods of time to carry out the extraction process. It works at ambient temperature, thus avoiding possible damage to active plant substances, an important thing for industry.

Applying this protocol of extraction, we had demonstrated a high efficiency in the separation and the extraction of the compounds from plant, with perspectives of application in pharmaceutical industry, in particular, for obtaining dietary supplements and of dermato-cosmetic products.

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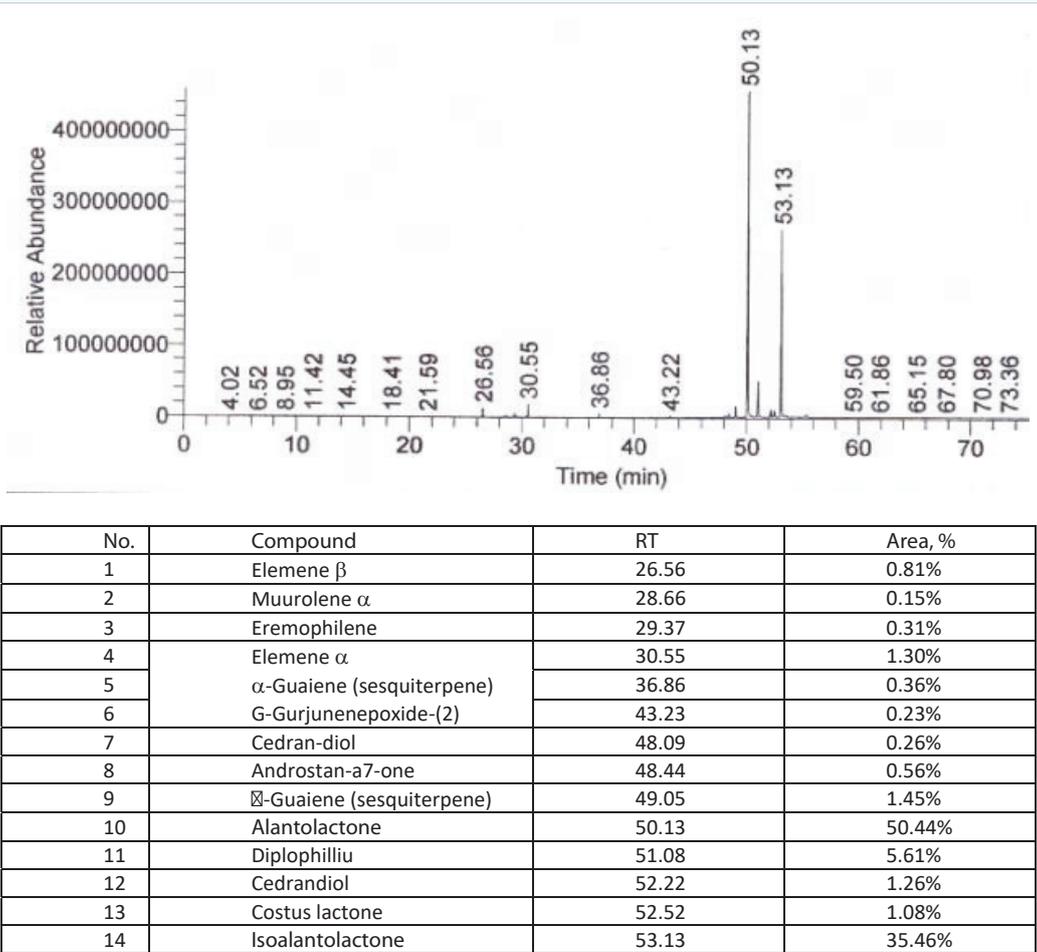


Figure 8 Chromatogram GS-MS, for elecampagne, raw material.

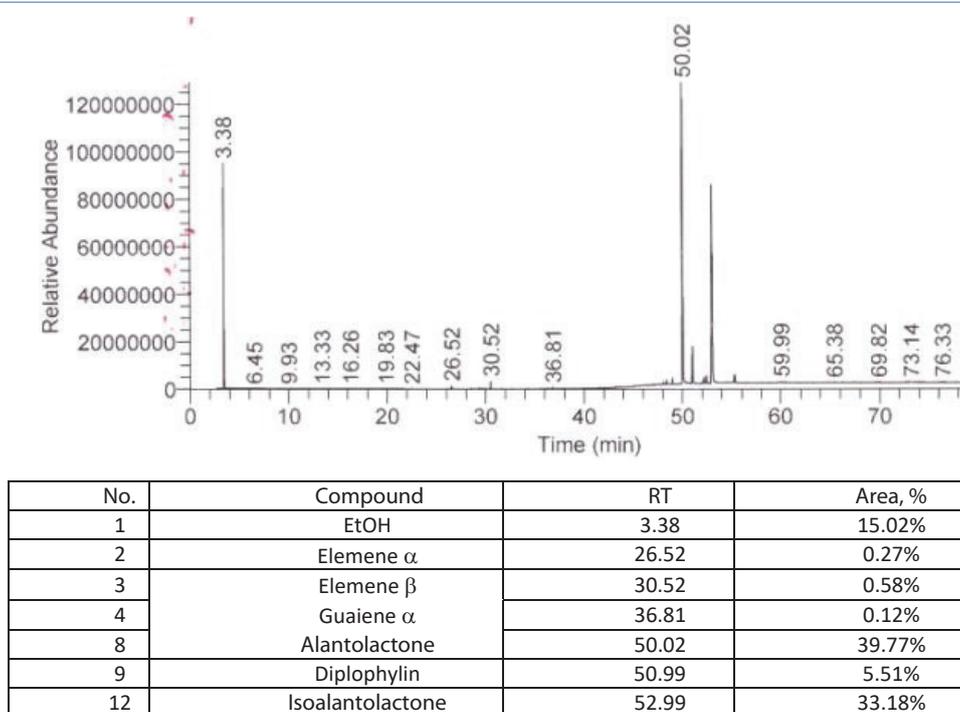
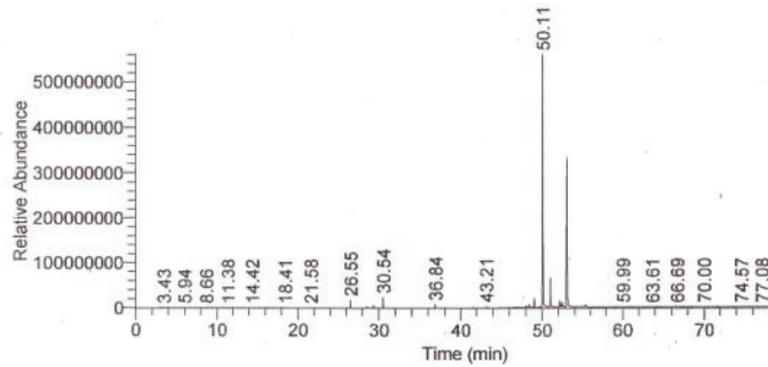


Figure 9 Chromatogram GS-MS, for the elecampagne extract with 50% ethanol, sample 4.



No.	Compound	RT	Area, %
1	α -Elemen	26.55	0.84%
2	γ -Selinene	28.56	0.14%
3	α -Muurolene	28.65	0.15%
4	Eremophilene	29.35	0.31%
5	β -Elemen	30.54	1.15%
6	α -Guaene (sesquiterpene)	36.84	0.36%
7	γ -Gurjuneneepoxide-(2)	43.21	0.21%
8	Androstan-17-one	48.07	0.27%
9	Androsatan-dione	48.42	0.52%
10	β -Guaene (sesquiterpene)	49.03	1.51%
11	Alantolactone	50.11	49.64%
12	Diplophylin	51.05	5.65%
13	Cedran-diol	52.19	1.32%
14	Costus lacone	51.49	1.10%
15	Isoalantotactone	53.10	36.23%

Figure 10 GS-MS, volatile oils.

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