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HERBAL FORMULATIONS OF *THYMUS SERPYLLUM* L. AND *HYPERICUM PERFORATUM* L. FROM SOUTHERN ITALY: PREPARATION AND CHEMICAL CHARACTERIZATION

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Abstract

The present study was designed to characterize the phenolic compounds of *Thymus serpyllum* L. and *Hypericum perforatum* L. The extraction process was carried out through the use of a Kumagawa extractor. The solvents (hexane, diethyl ether, acetate ethyl and methanol) were chosen in order of increasing polarity. The methanol soluble fractions were analyzed by liquid chromatography–mass spectrometry (LC-MS). High performance liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method was used for the phenolic compounds analysis. 14 phenolic compounds were identified in the *Thymus serpyllum* L., five of which were phenolic acids, nine flavan-3-ols. Moreover, this methodology allowed the separation of up to 14 constituents in methanolic *Hypericum perforatum* L. extract, covering various classes of plant secondary metabolites, namely hydroxycinnamic acids, flavanols including flavonol glycosides and flavonol aglyca, biflavone and a naphthodianthrone.

Keywords: *Thymus serpyllum L., Hypericum perforatum L., Phenolic compounds, Kumagawa extractor, LC-MS, Herbal formulations*

Introduction

It is known that folk medicine has always used plants for therapeutic treatment. Medicinal and aromatic plants (MAPs) are employed in the form of crude drugs or their extracts. For the isolation of active substances native plant species growing wild or cultivated may be used. It is estimated from the World Health Organization (WHO) that there are about 20.000 species of plants from which medicinal products are obtained [1].

Hypericum perforatum L. (Hypericaceae), or commonly known as St. John's Wort has been used for pharmacological activities as wound healing, antiviral, antimicrobial, antidepressant, anxiolytic. It is native to Europe and Asia, the plant features yellow flowers, rounded leaves, and oblong petals populated with a number of brown-black glandular dots, giving the plant its eponymous "perforated" appearance. Topical St. John's wort preparations such as oils or tinctures is obtained by maceration of the fresh flowering tops of *H. perforatum* for 40 days [2,3].

Oleum Hyperici reduces wound size and healing time. The antimicrobial activity against some Gram-positive bacteria is due to the presence of two components such as the naphthodianthrone hypericin and phloroglucinol hyperforin. *H. perforatum* has been characterized chemically: many secondary metabolites have been identified, such as flavonoid glycosides (hyperoside), phloroglucinols (hyperforin), naphthodianthrones (hypericin and pseudohyperycin), biflavones, and anthocyanidins [4]. Hypericin has been assigned an antidepressant activity, in fact it is found in some commercially available hyperic products [5].

Another widely used plant in the medical field is the *Thymus serpyllum*. Its name "*serpyllum*" is derived from the Greek word meaning "to creep". It is a perennial shrub, native to regions of northern and central Europe. It flowers from May to September. The plant is used in preparations of natural remedies, such as infusions, syrups, tea, tinctures, decoctions, and oil with proprieties antiseptic, diaphoretic, anthelmintic, tonic, disinfectant, antispasmodic, deodorant, sedative, and expectorant [6].

The main components present in the essential oil of *T. serpyllum* are: carvacrol, α-pinene, citronellal, borneol, caryophyllene, 1,8-cineole, *p*-cymene, citral, citronellol, isobutyl acetate geraniol, linalool, γ-terpinene, α-terpineol, terpinyl acetate, and thymol in quite high concentrations. Monoterpenic phenols such as carvacrol and thymol (isomers) are associated with potent antiseptic properties [7].

Inhibitory activity of aqueous extracts of *T. serpyllum* against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* has been demonstrated [8]. Essential oil has shown more inhibitory activity than only thymol and total phenols, thanks to the synergistic effects of the other components of the essential oil [7]. Ahmad *et al.* in 2006 [9] reported that wild thyme essential oil has bactericidal effects against species *Escherichia coli*, *Salmonella typhi*, *Shigella ferarie*, *Bacillus megaterium*, *Bacillus subtilis*, *Lactobacillus acidophilus*, *Micrococcus luteus*, *Staphylococcus albus*, *Staphylococcus aureus*, and *Vibrio cholera.*

The inhibitory activity of essential oils depends on the conditions and duration of incubation, so a greater inhibitory effect is achieved thanks to the synergistic and cumulative effects of the other components of the essential oil.

From the scientific literature, it is clear that the composition of the extract plays a fundamental role with respect to the properties that are found for the various biological activities.

There are many factors that influence the concentration of the various components present such as: geographical origin of the plant, exposure to sunlight, group of wild cultivated plants, harvest time, polarity and temperature of the extraction solvent and final formulation, time and conditions of conservation.

The aim of this research has been to study two types of medicinal plants easily available in Southern Italy, the *Hypericum Perforatum* L. and *Thymus serpyllum* L., defining the quantities of extracts in different solvents with increasing polarity and the chemical composition of the soluble fraction in methanol using LC-MS/MS analyses. We developed LC-MS/MS method, which was validated and allowed the separation of a broad range of plant secondary metabolites.

Methods

Solvents were purchased from Sigma-Aldrich S.r.l. and were used as received. The extractions were conducted using a Kumagawa extractor.

Extractions of Hypericum perforatum L. *and Thymus serpyllum* L.

Stem, leaves and flowers have been shredded and weighed: for *Hypericum perforatum* 3,175 g, whereas for *Thymus serpyllum* 2,578 g. The quantities weighed were placed in the thimble of cellulose and extracted by Kumagawa extractor. Were used in the order: hexane, diethyl ether, acetate ethyl and methanol (approximately 130 ml of solvent was used). At the end of each extraction, the soluble fraction has been transferred to a flask to remove the solvent in a vacuum. The quantities recovered were:

- *Hypericum perforatum*, fraction from: hexane 0,0255 g, diethyl ether 0,0646 g, acetate ethyl 0,152 g, methanol 0,205 g;
- *Thymus serpyllum*, fraction from: hexane 0,0992 g, diethyl ether 0,0761 g, acetate ethyl 0,0679 g, methanol 0,296 g.

Liquid chromatography–mass spectrometry (LC-MS) analyses.

LC-MS/MS analyses were performed on a quadrupole time-offlight (Q-TOF) Premier instrument (Waters, Milford, MA), equipped with an electrospray ion source and coupled to a 2690 Alliance HPLC. Instrument tuning and mass calibration of the spectrometer were performed using a-solanine ($[M+H]^+$ *m/z* 868.51) as standard. Chromatographic separations were conducted using the same conditions and chromatographic column described above.

Analyses were carried out using a Luna 2.5 µm C18(2) column (100 Å Phenomenex, 100 x 2 mm) at a flow rate of 0.2 mL/min, and injecting 10 µL of sample. Gradient elution was as follows: 0 min 90% buffer A (H2O with 0.1% formic acid) and 10% buffer B (100% acetonitrile), 0-30 min 10-55% buffer B, 30-35 min 55-95% buffer B, 35-40 min 95% buffer B, 40-41 min 95-10% buffer B and 41-50 min 10% buffer B. Structural identification of individual metabolites and in extracts was performed by associating the HPLC peaks of each compound with the corresponding MSn spectra comparison to those of standards. Moreover, all metabolites identified by HPLC in extracts were also verified with co-injection of the related internal standard. High Resolution MSn detection was used to obtain information on the structural features and the conjugated forms of phenolic compounds. Mass analyses were carried out in positive and negative ion mode, using a MS/MS dependent function. The following instrumental parameters were used: source temperature 90 °C, desolvation temperature 150 °C, cone gas flow rate 50 L/h, and desolvation 300 L/h. Capillary voltage was set at 3,5 kV, cone voltage 30 V, extraction cone 5 V, while fragmentation voltage was set at 5 V. The full mass scan ranged between m/z 200 and 800 [10-12]. Identification of compounds was based on retention times, accurate mass measurements, MS/MS data, exploration of specific spectral libraries and public repositories for MS-based metabolomic analysis [13] and comparison with data reported in the literature [1, 14-19].

Data were acquired using MassLynx 4.1 software (Waters), and for fragmentation studies a data dependent scan experiment was carried out selecting precursor ions as the most intensive peak in LC-MS analysis.

Results and Discussion

Chemical Characterization of: Hypericum perforatum and Thymus serpyllum of Southern Italy

The investigation was conducted by analyzing two species of plants. The collection took place in year 2019 in flowering period at the end of June. The plant material was collected in its entirety: stem, roots, leaves and flowers were chopped up and weighed. Few grams of vegetable product were subjected to exhaustive extractions in the suitable solvents.

The extraction process was carried out through the use of a Kumagawa extractor. The solvents (hexane, diethyl ether, acetate ethyl and methanol) were chosen in order of increasing polarity. We proceeded by varying the solvents at the end of each extraction. After each extraction, the solvent loaded with its solutes was taken from the

extractor flask Kumagawa. It was established as a time limit for extraction the time taken by the solvent to perform 200 extraction cycles. The extracts has been obtained removing the solvent to the rotavapor.

In table 1 were reported the quantity of extract obtained for *Hypericum perforatum*.

The best extraction yield was obtained from methanol (6,45% w/w), while the extract from hexane was obtained with the lowest yield (0,80% w/w).

In table 2 were reported the quantity of extract obtained for *Thymus serpyllum*.

Also for *Thymus serpyllum*, as for *Hypericum perforatum*, the best extraction yield was obtained from methanol (11,4% w/w), while for the other extracts the percentages vary from 2,63% w/w for acetate ethyl to 3,85% w/w for hexane. Soluble fractions recovered from less polar solvents, such as hexane and diethyl ether, are significantly lower than those obtained with more polar solvents. The methanol soluble fractions were subjected to LC-MS/MS analyses.

Qualitative analysis of metabolite content

Based on retention time, MS spectra and fragmentation patterns, individual compounds were assigned by comparison with literature data and analytical reference standards [1, 17-21].

The phenolic profile of *T. serpyllum*, obtained after alcoholic extraction and mass spectral data for compounds identified in negative and positive ionization modes were listed in Table 3.

A total of 14 phenolic compounds were identified, five of which were phenolic acids, nine flavan-3-ols. As for the phenolic compounds: protocatechuic acid-hexoside (1S), rosmarinic acid-glucoside (4S), rosmarinic acid (10S), chlorogenic acid (13S) and neochlorogenic acid (14S). The other phenolic compounds identified in the analyzed sample belong to the groups of flavones, flavonols and flavanones: Apigenin 6,8-di-C-glucoside (2S), Rutin (3S), Luteolin 7-*O*-glucoside (5S), Luteolin 7-*O*-glucuronide (6S), Kaempferol *O*-glucuronide (7S), Naringin (8S), Apigenin *O*-glucuronide (9S), Methyl kaempferol *O*-rutinoside (11S) e Luteolin (12S).

LC-ESI-MS/MS chromatograms of phenolic compounds extracted from *T. serpyllum* was given in Figure 1.

The data carried out on the *T. serpyllum* extract by LC-MS/ MS technique are in accordance with what is reported in the literature as regards the relative abundance of the different analytes identified (where the majority compounds are Luteolin 7-*O*-glucoside, Rosmarinic acid, Luteolin and Kaempferol *O*-glucuronide) [1].

The phenolic profile of *H. perforatum*, obtained after alcoholic extraction and mass spectral data for compounds identified in negative and positive ionization modes were listed in Table 4.

In methanolic extracts of *H. perforatum* whole plant, 14 constituents were detected (Fig. 2), covering various classes of plant secondary metabolites, namely hydroxycinnamic acids (compounds 2H and 11H), flavanol (compound 1H), flavonol glycosides (compounds 4H, 5H, 7H-9H) and flavonol aglyca (compounds 3H and 6H), biflavone (compound 12H), naphthodianthrones (compounds 10H, 13H and 14H).

LC-ESI-MS/MS chromatograms of phenolic compounds extracted from *H. perforatum* was given in Fig. 2.

The data carried out on the *H. perforatum* extract by LC-MS/ MS technique are in accordance with what is reported in the literature as regards the relative abundance of the different analytes identified [where the majority compounds are Quercetin, Quercetin-3-*O*-galactoside (hyperoside), Quercetin-3-*O*-glucuronide (miquelianin), Protohypericin e Chlorogenic acid] [17].

References

1. Sonmezdag, A.S., Kelebek, H., Selli, S., Characterization of aroma-active and phenolic profiles of wild thyme (*Thymus serpyllum*) by GC-MS Olfactometry and LC-ESI-MS/MS. J Food Sci Technol 2016;53:1957–1965.

2. Sarić, M., Medicinal Plants of SR Serbia, 1st ed.; SASA: Belgrade, Serbia, 1989;315-317.

3. Stjepanović-Veselčić, L., Hypericaceae. In Flora of Serbia, 1st ed.; Josifović, M., Ed.; SANU: Belgrade, Serbia 1972;3: 104-125.

4. Porzel, A., Farag, M.A., Mülbradt, J., Wessjohann, L.A., Metabolite profiling and fingerprinting of Hypericum species: a comparison of MS and NMR metabolomics. Metabolomics 2014;10:574–588.

5. Chatterjee, S.S., Bhattacharya, S.K., Wonnemann, M., Singer, A., Müller, W. E., Hyperforin as a possible antidepressant component of hypericum extracts. Life Sci 1998; 63: 499–510.

6. Chevallier, A., The Encyclopedia of Medicinal Plants, DK Publishing, University of Michigan, 1996.

7. Jarić, S., Mitrović, M., Pavlović, P., Review of ethnobotanical, phytochemical, and pharmacological study of *Thymus Serpyllum* L. Evid Based Complement Alternat Med 2015:1-10.

8. Kavita, G., Santosh, K., Reeta, S., Evaluation of antibacterial activity of aerial parts of *Thymus serpyllum* L. J Pharm Res 2011; 4: 641–642.

9. Ahmad, A.M., Khokhar, I., Ahmad, I., Kashmiri, M.A., Adnan, A., Ahmad, M., Study of antimicrobial activity and composition by GC/MS spectroscopic analysis of the essential oil of *Thymus serphyllum*. J Food Safety 2006; 5: 56–60.

10. Aversano, R., Contaldi, F., Adelfi, M.G., D'Amelia, V., Diretto, G., De Tommasi, N., Vaccaro, C., Vassallo, A., Carputo, D., Comparative metabolite and genome analysis of tuber-bearing potato species. Phytochemistry 2017;137:42–51.

11. Esposito, A., Malafronte, N., Sanogo, R., Vassallo, A., D'Ambola, M., Severino L., Sesquiterpene Lactones from *Vernonia nigritiana*. Nat Prod Commun 2016; 11(5):583–584.

12. Malafronte N., Sanogo R., Vassallo A., De Tommasi N., Bifulco G., Dal Piaz F., Androstanes and pregnanes from Trichilia emetica ssp. suberosa J.J. de Wilde. Phytochemistry 2013;96:437-442.

13. Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., et al., MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom;45:703-714.

14. Cherchar H., Faraone I., D'Ambola M., Sinisgalli C., Dal Piaz F., Oliva P., Kabouche A., Kabouche Z., Milella L., Vassallo A., Phytochemistry and Antioxidant Activity of Aerial Parts of *Phagnalon sordidum* L. Planta Med 2019;85(11-12):1008-1015.

15. Tebboub O., Cotugno R., Oke-Altuntas F., Bouheroum M., Demirtas Í., D'Ambola M., Malafronte N., Vassallo A., Antioxidant Potential of Herbal Preparations and Components from *Galactites elegans* (All.) Nyman ex Soldano. Evid Based Complement Alternat Med 2018;2018:Article ID 9294358.

16. Gualtieri M.J., Malafronte N., Vassallo A., Braca A., Cotugno R., Vasaturo M., De Tommasi N., Dal Piaz F., Bioactive Limonoids from the Leaves of *Azadirachta indica* (Neem). Nat Prod Commun 2014; 77(3):596-602.

17. Heinrich, M., Daniels, R., Stintzing, F.C., Kammerer, D.R., Comprehensive phytochemical characterization of St. John's wort (*Hypericum perforatum* L.) oil macerates obtained by different extraction protocols via analytical tools applicable in routine control. Pharmazie 2017;72(3):131-138.

18. Lelario F., Scrano L., De Franchi S., Bonomo M.G., Salzano G., Milan S., Milella L., Bufo S.A. (2018). Identification and antimicrobial activity of most representative secondary metabolites from different plant species. Chemical and Biological Technologies in Agriculture 5:13.

19. Saturnino C., Caruso A., Iacopetta D., Rosano C., Ceramella J., Muià N., Mariconda A., Bonomo M.G., Ponassi M., Rosace G., Sinicropi M.S., Longo P. (2018). Inhibition of human Topoisomerase II by new N,N,N-trimethylethanammonium iodide alkylcarbazole derivatives. ChemMedChem, 13, 2635-2643.

20. Cafaro C., Bonomo M.G., Guerrieri A., Crispo F., Ciriello R., Salzano G. (2016). Assessment of the genetic polymorphism and physiological characterization of indigenous *Oenococcus oeni* strains isolated from Aglianico del Vulture red wine. *Folia Microbiologica*, 61, 1-10.

21. Bonomo M.G., Cafaro C., Guerrieri G., Crispo F., Milella L., Calabrone L., Salzano G. (2017). Flow cytometry and capillary electrophoresis analyses in ethanol-stressed *Oenococcus oeni* strains and changes assessment of membrane fatty acids composition. Journal of Applied Microbiology, 122 (6), 1615-1626.

Table 1. Extracted fractions of *Hypericum perforatum*.

Extraction solvent	QUANTITY OF EXTRACT (g)	YIELD[a] (%)
Hexane	0,0255	0,80
Diethyl ether	0,0646	2,03
Acetate ethyl	0,152	4,80
Methanol	0,205	6,45

[a]Percentage calculated as: quantity of extract/ initial total grams (3,175 g)

Table 2. Extracted fractions of *Thymus serpyllum*

Extraction solvent	QUANTITY OF EXTRACT (g)	YIELD[a] (%)
Hexane	0,0992	3,85
Diethyl ether	0,0761	2,95
Acetate ethyl	0,0679	2,63
Methanol	0,296	11,4

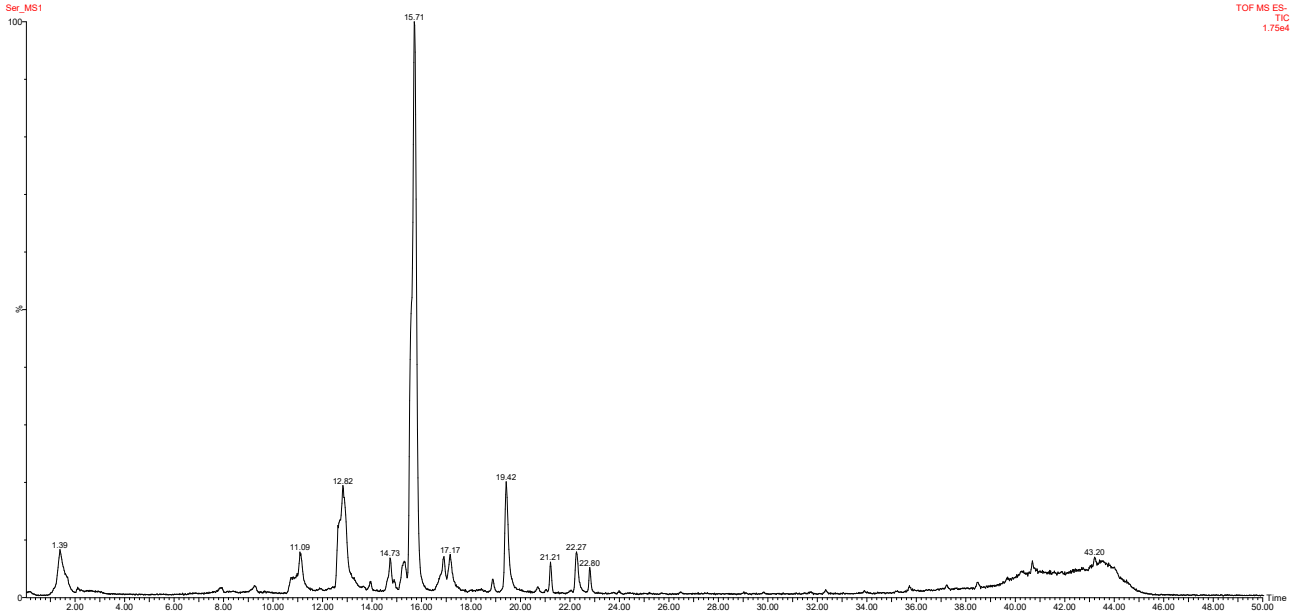
[a]Percentage calculated as: quantity of extract/ initial total grams (2,578 g)

Table 3. Compounds identified by liquid chromatography/mass spectrometry in methanolic extract solution of *T. serpyllum*

	Retention time (minutes)	$[M-H]^-$	MS/MS	Compounds
1S	2.72	315	153	Protocatechuic acid-hexoside
2S	8.80	593	503	Apigenin 6,8-di-C-glucoside
3S	11.88	609	301	Rutin
4S	12.47	521	359	Rosmarinic acid-glucoside
5S	12.82	447	285	Luteolin 7-*O*-glucoside
6S	13.12	461	285	Luteolin 7-*O*-glucuronide
7S	13.32	461	285	Kaempferol *O*-glucuronide
8S	13.95	579	459	Naringin
9S	15.55	445	269	Apigenin *O*-glucuronide
10S	15.71	359	197	Rosmarinic acid
11S	16.59	607	299	Methyl kaempferol *O*-rutinoside
12S	19.42	285	199	Luteolin
13S	19.53	353	191	Chlorogenic acid
14S	22.26	353	179	Neochlorogenic acid

Figure 1. ESI (-) ESI (+) LC-MS of methanolic extract solution of *T. serpyllum*

LC-MS profile of *Thymus serpyllum* by Qtof in negative mode



LC-MS profile of *Thymus serpyllum* by Qtof in positive mode

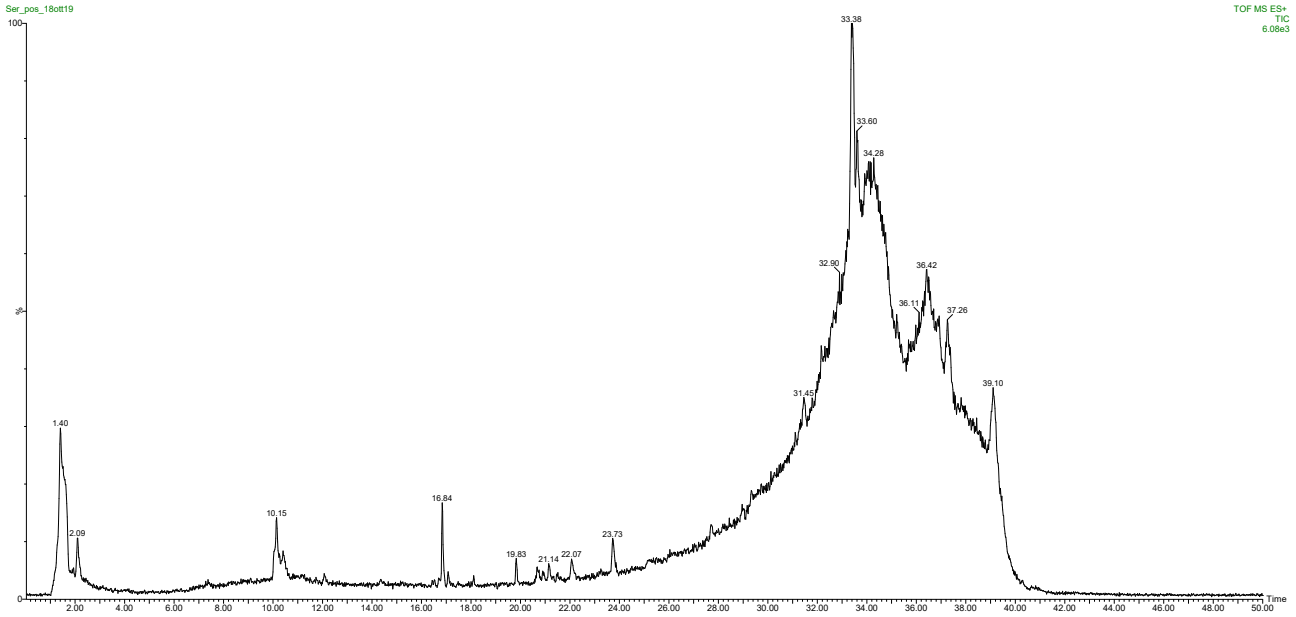
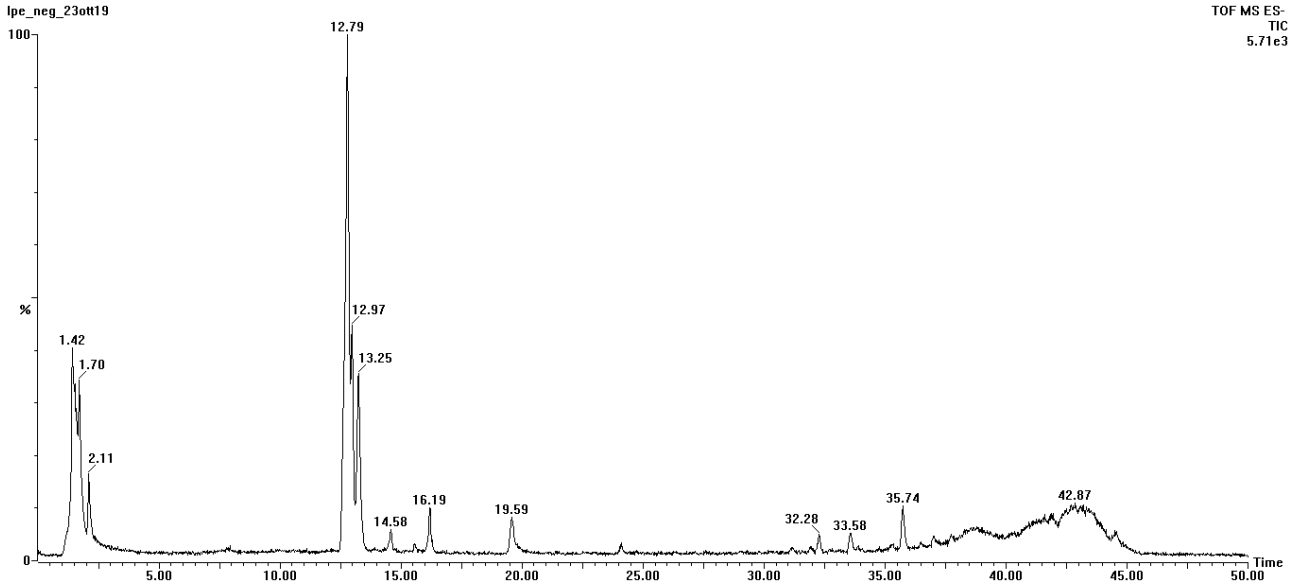


Table 4. Compounds identified by liquid chromatography/mass spectrometry in methanolic extract solution of *Hypericum perforatum*

	Retention time (minutes)	$[M-H]^-$	$[M-H]^+$	MS/MS	Compounds
1H	1.68	289	/	245	Catechin
2H	6.08	337	/	191	3-p-Coumaroylquinic acid
3H	12.77	301	303	179	Quercetin
4H	12.79	463	465	179, 151	Quercetin-3-*O*-galactoside (hyperoside)
5H	13.25	477	479	301, 179	Quercetin-3-*O*-glucuronide (miquelianin)
6H	13.90	285	287	151	Kaempferol
7H	14.08	609	/	301,151	Quercetin-3-*O*-glucorhamnoside (rutoside)
8H	14.34	433	/	301, 271	Quercetin-3-*O*-arabinoside (guaijaverin)
9H	14.60	447	/	301, 179	Quercetin-3-*O*-rhamnoside (quercitrin)
10H	16.18	505	/	461, 407	Protohypericin
11H	19.55	353	355	191	Chlorogenic acid
12H	24.10	537	539	443	Amentoflavone
13H	32.29	519	/	477	Pseudohypericin
14H	33.58	503	/	459	Hypericin

Figure 2. ESI (-) ESI (+) LC-MS of methanolic extract solution of *Hypericum perforatum*

LCMS profile of *Hypericum perforatum* by Qtof in negative mode



LCMS profile of *Hypericum perforatum* by Qtof in positive mode