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Comparison of Methods for the Exhaustive Extraction of Hypericins, Flavonoids, and Hyperforin from *Hypericum perforatum* L.

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Renewed interest in plant-derived drugs has led to an increased need for efficient extraction methods. *Hypericum perforatum* L. contains several groups of bioactive compounds with noteworthy pharmacological activities. Direct sonication of *H. perforatum* was investigated and compared with conventional maceration, indirect sonication, Soxhlet extraction, and accelerated solvent extraction (ASE). Highly selective liquid chromatography/tandem mass spectrometry analysis showed that the content of six investigated active compounds (hypericin, pseudohypericin, hyperoside, rutin, quercitrin, and hyperforin) in extracts obtained by direct sonication was significantly higher than in extracts obtained by the other methods. The active compound contents increased on increasing the ultrasonic power from 40 to 60 W when using direct sonication. Conventional maceration gave the lowest amount of analyzed active compounds. Soxhlet extraction gave better results than ASE or indirect sonication.

KEYWORDS: ***Hypericum perforatum* L.; direct sonication; comparison of extraction methods; LC/MS/MS**

INTRODUCTION

While ultrasonically assisted extraction of bioactive compounds from plant material and their constituents is not a new topic, there is a dearth of information on systematic studies in this area (*1*). The process of extraction of active substances from a plant material by means of a solvent generally occurs in two main stages: first, dissolution of material near the surface (so-called washing or fast extraction), and second, diffusion of the solute from the porous plant residue into the solution (so-called slow extraction) (*2*). It has been shown that the extraction processes could be improved with the use of ultrasound (*1*, *3*−*6*). Analysis of the kinetics of extraction of resinoids and hypericins from *Hypericum perforatum* L. (St. John's wort) showed that the period of fast extraction, with intensification of the extraction by ultrasound, was significantly shorter than that required for the extraction without ultrasound (*6*). The possible benefits of ultrasound in extraction processes are mass transfer intensification, cell disruption, improved penetration, and capillary effects (*1*).

H. perforatum has been reported as an antidepressive, an antiviral, an antimicrobial, an antiinflammatory, and a healing agent (*7*). Its main constituents are naphthodianthrones, primarily represented by hypericin (**1**) and pseudohypericin (**2**), flavonoids, such as hyperoside (**3**), rutin (**4**), or quercitrin (**5**), and phloroglucinol derivatives, especially hyperforin (**6**) (*8*) (**Figure 1**). Compounds **1** and **2** have been found to possess antiretroviral activity (*9*, *10*). The flavonoids may possess some antidepressant activity (*11*), as well as antioxidant activity (*12*). Compound **6** might make an important contribution to the antidepressant activity of *Hypericum* extracts (*13*, *14*).

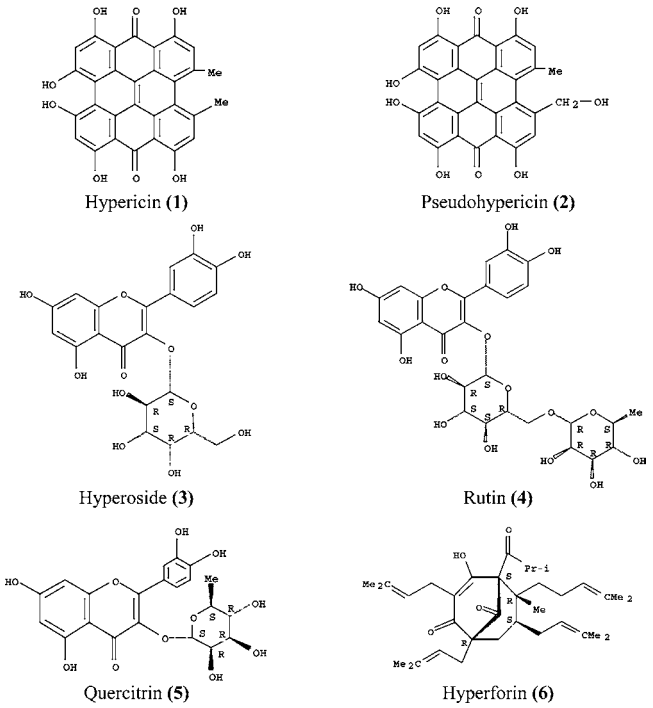

Figure 1. Chemical structures of the compounds under investigation.

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Several methods have been used to extract active compounds from *H. perforatum*, such as maceration, with and without ultrasound (*6*), Soxhlet extraction (*15*), accelerated solvent extraction (ASE) (*15*), pressured water extraction (*16*), and supercritical fluid extraction (*17*−*20*). A review on the ultrasonically assisted extraction of active principles from plants was published (*21*). With regard to improvements in the extraction process using ultrasound in comparison to conventional extraction methods, significant differences are evident between plant species (*5*, *22*). Data on the use of direct sonication for the extraction of active compounds from plants already exist (*3*, *4*, *22*). A comparison between the classical (Soxhlet) and the ultrasonically assisted (indirect and direct sonication) extractions of dill seeds showed that direct sonication gave the highest oil amount (*4*). Salisova et al. (*3*) reported the study of sage (*Salvia officinalis* L.) extraction using ultrasonic assistance. The large improvement in the extraction process was achieved using an ultrasonic horn immersed in a stirred extraction mixture. In the report of Valachovic et al. (*22*), a similar ultrasound device was used in an industrial scale static extraction apparatus. Using direct sonication, greater improvements in the extraction were observed for sage as compared to valerian (*Valeriana officinalis* L.). Data on the comparison of different extraction methods for the extraction of some active compounds from *H. perforatum* have already been reported (*15*). Thus, the content of hypericins from *H. perforatum* obtained by ASE was little higher than that obtained by Soxhlet extraction or indirect sonication. The optimal conditions for ASE extraction were 40 °C and 100 bar, using methanol as the extraction solvent (*15*). These results are in agreement with a study on the optimization of extraction conditions for active components from *H. perforatum* using response surface methodology (*23*). Because of the importance of hypericins and the temperature sensitivity of **6** (*24*), in the present investigation, the extracts were obtained by ASE at 40 °C.

In this paper, the extraction of six active compounds (**1**−**6**) from *H. perforatum* L. using direct sonication was investigated and compared with extraction using conventional maceration, indirect sonication, Soxhlet extraction, and ASE. The composition of extracts was analyzed using a highly selective liquid chromatography/tandem mass spectrometry (LC/MS/MS) method.

MATERIALS AND METHODS

Materials. The plant material (*H. perforatum* L.) was collected at the bloom stage at the end of June 2004 on Rujan mountain (Southern Serbia); a voucher specimen (no. 732) was deposited in the Herbarium Moesicum Doljevac (Serbia). The plant material was dried at room temperature and then milled. The dry plant material was then packed in paper bag and kept in a dark, dry, and cool place.

Compound **1** was purchased from Biomol GmbH (Hamburg, Germany), **2** was purchased from Calbiochem (Darmstadt, Germany), **3** was purchased from Merck (Darmstadt, Germany), **4** was purchased from Acros Organics (Geel, Belgium), **5** was purchased from Sigma (Taufkirchen, Germany), and **6** was purchased from Cayman Chemical (MI). Ammonium acetate, glacial acetic acid, acetonitrile, and methanol (Merck) and ultrapure water (Millipore, Schwalbach, Germany) were all high-performance liquid chromatography (HPLC) grade.

Plant Material Extraction. The plant material (5 g) and methanol (100 mL) were placed in an Erlenmeyer flask (250 mL). Direct and indirect sonications were performed for 5, 10, 15, 20, 30, 45, and 60 min, respectively. Direct sonication was performed using a Branson Sonifier B-12 apparatus (Branson, Heusenstamm, Germany), operating at 20 kHz, on two power values (40 and 60 W). A Bandelin Sonorex Super RK103H apparatus (Bandelin Electronic, Berlin, Germany), operating at 35 kHz, was employed for indirect sonication. Conventional maceration was performed without shaking (5 g of plant material and 100 mL of methanol for a period of 24 h). Soxhlet extraction was performed with 5 g of plant material and 150 mL of methanol for a period of 24 h. ASE was performed with 5 g of plant material using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA) equipped with 33 mL stainless steel cells and 60 mL collection vials. ASE conditions were as follows: extraction solvent, methanol; temperature, 40 °C; pressure, 100 bar; four cycles with a static extraction time of 5 min; flush volume, 60%; and final solvent volume, 128 mL.

At the end of each extraction cycle (direct and indirect sonication and conventional maceration), the extracts were separated from the residual plant material by vacuum filtration (Folded Filters, grade 597 1/2, Schweitzer & Schuell, Germany). The residues were then washed twice with 25 mL aliquots of methanol. The filtrates were combined and concentrated using a rotary vacuum evaporator. Methanol was added to make the volume 100 mL. The extracts were stored in the dark at 4 °C. A 50 mL portion of the extracts was used for the determination of the total extract contents from the plant material, which was calculated from the mass of dry extract and the mass of initial dried plant. Each extraction was done in duplicate. Relative deviations of total extract and single compounds content were in the range of 1.5−7.3%. The average value of relative deviation for all experiments was 4.4%.

Analytical Method. HPLC analysis of the extracts was performed using a Dionex HPLC system, equipped with a Gina 50 autosampler (Dionex, Idstein, Germany). Separations were performed on a Luna C18 100 Å column (150 mm × 2 mm, 3 μm particle size) from Phenomenex (Torrance, CA).

The mobile phase consisted of 10 mM ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid (A) and a 9:1 mixture of acetonitrile and methanol (B). Gradient elution was performed using the following solvent gradient: from 87A/13B in 10 min to 80A/20B, then in 25 min to 10A/90B, and in 5 min to 100B; each run was followed by an equilibration period of 15 min. The flow rate was 0.25 mL/min, and the injection volume was 10 μL. All separations were performed at 22 °C.

Mass spectra were obtained using a Varian 1200L LC/MS (Varian Inc., Palo Alto, CA) equipped with an electrospray ionization (ESI) source operating in negative ionization mode. For ESI, the best conditions were found to be as follows: needle voltage at 4.5 kV, shield at −600 V, and tube lens at 4 V. Nitrogen was employed as both the drying and the nebulizer gas. The drying gas temperature was fixed at 200 °C and a pressure of 18 psi. The collision gas pressure was set at 2 mTorr, and the detector voltage was set at 1060 V.

The calibration curve was constructed by dilution of external standards with methanol to give the desired concentrations. The concentrations of standard solutions were 1, 2.5, 5, 10, 20, 25, and 50 μg/mL for **3**−**5** and 1, 2.5, 5, 10, and 20 μg/mL for **1**, **2**, and **6**. Standard solutions were stored in the dark at 4 °C. Within the range of concentrations injected, the detector response (peak area) was linear. The correlation coefficient for the calibration curve was 0.999 for **1**, 0.995 for **2**, 0.995 for **3**, 0.981 for **4**, 0.973 for **5**, and 0.981 for **6**. All procedures were carried out under light protection.

The relative standard deviation (RSD) of the analytical method was determined by eight injections of an extract. RSDs were in the range of 1.3−6.9% for all compounds under study.

RESULTS AND DISCUSSION

The results of the kinetic investigations of the extraction of dry residue (total extract) from *H. perforatum* by the application of the maceration techniques coupled with ultrasound are shown in **Figure 2**. The total extract contents obtained by direct sonication were higher than those obtained by indirect sonication, in agreement with a study of oil extraction from crushed dill seeds (*4*). Direct sonication performed with ultrasonic power of 60 W gave 20.4−53.6% (dependent from extraction time) higher total extract contents than the experiments performed at 40 W. The total extract contents obtained using an ultrasonic power of 60 W for 1 h were 39.1% higher than those obtained by conventional maceration for 24 h (see **Table 1**).

Organic solvent extraction of plant material leads to innumerable compounds within the extracts. Thus, identification and correct quantification of bioactive substances are difficult tasks requiring highly selective and sensitive analytical methods. LC/MS/MS promises both separation of the analytes from the disturbing matrix and very selective detection. In particular, the multiple reaction monitoring mode in tandem mass spectrometric

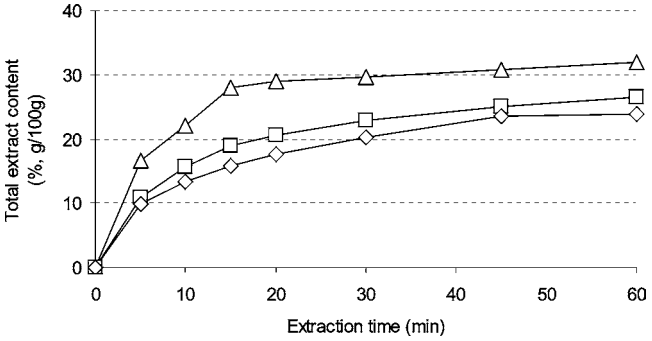

Figure 2. Influence of extraction time on the total extract content using direct and indirect sonication. △, direct sonication [60W]; □, direct sonication [40 W], ◇, indirect sonication.

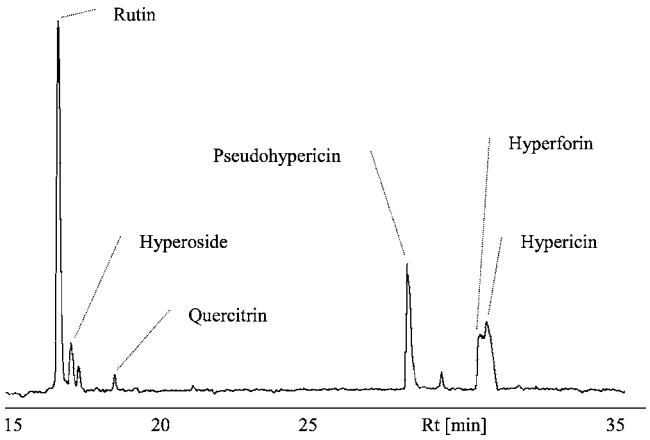

Figure 3. LC/MS/MS reconstructed ion chromatogram of a plant extract; the sum of all SRM chromatograms is displayed.

Table 1. Comparison of Active Compound and Total Extract Contents (mg/g Plant Material) in *H. perforatum* Extracts Obtained Using Different Extraction Methods

extraction method	compound						total extract
	1	**2**	**3**	**4**	**5**	**6**	
direct sonication (60 W, 1 h)	0.20	0.51	2.79	2.95	0.75	1.52	319.3
direct sonication (40 W, 1 h)	0.17	0.37	2.71	2.85	0.75	1.50	265.3
Soxhlet (24 h)	0.15	0.32	1.78	2.29	0.48	0.89	253.4
ASE (40 °C, 100 bar)	0.15	0.28	1.78	2.04	0.42	0.87	240.3
indirect sonication (1 h)	0.15	0.25	1.75	1.89	0.37	0.85	238.9
conventional maceration (24 h)	0.13	0.19	1.62	1.78	0.34	0.85	229.6

analysis helps to avoid false negative results or overrated values. A typical LC/MS/MS chromatogram of the *H. perforatum* extract is presented in **Figure 3**. Here, the sum of all selected reaction monitoring (SRM) chromatograms is given. Every single SRM is very selective, and no disturbance by matrix or other compounds within the corresponding retention time was observed. Retention time, precursor and product ions, and collision energies for the standard compounds are shown in **Table 2**.

The dependencies of the contents of the six active compounds under study (**1**−**6**) on the extraction time are shown in **Figure 4**. The optimum extraction time varies between compound and extraction method. On application of direct sonication, the extraction efficiencies increased significantly when the extraction time increased to about 20 min. On application of both ultrasound techniques (direct and indirect sonication), the extraction kinetics of hyperforin differed from the extraction kinetics of the other compounds through a shorter period of fast extraction (about 5 min).

Table 2. Retention Times, Precursor and Product Ions, and Collision Energies for the Standard Compounds

compound	retention time (min)	precursor ion (*m*/*z*) [M − H]$^-$	product ion (*m*/*z*)	collision energy (V)
4	16.6	609.4	300.0	20.0
3	17.0	463.2	300.0	22.0
5	18.3	447.3	300.0	22.0
2	27.8	519.3	487.0	28.0
1	31.1	503.3	405.0	32.0
6	30.3	535.5	243.0	40.0

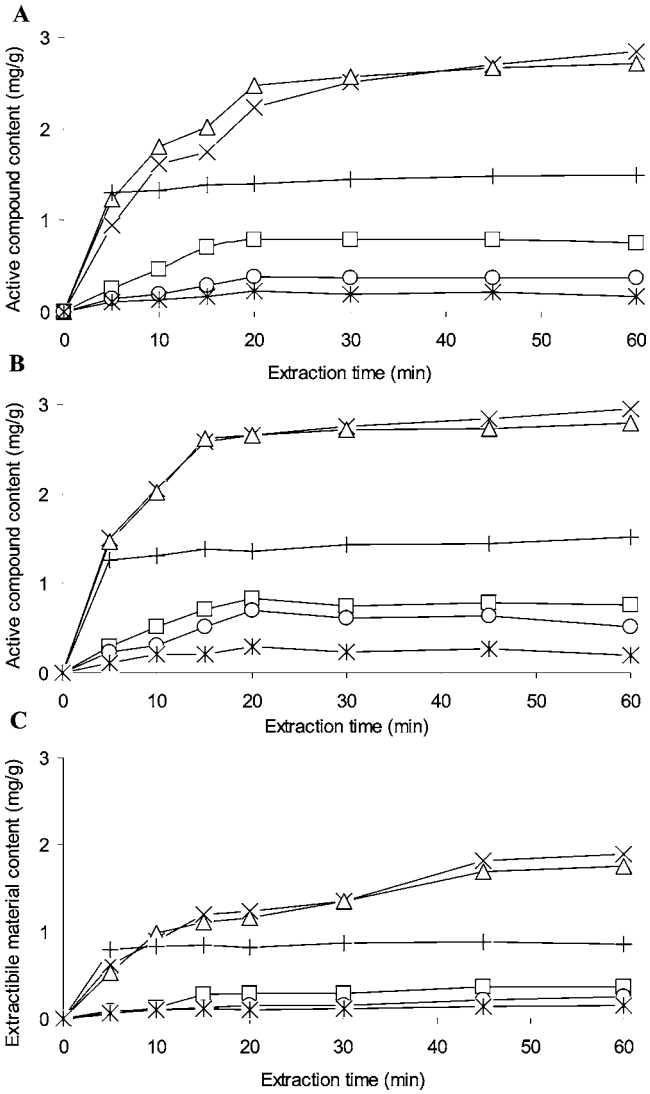

Figure 4. Influence of extraction time on active compound contents using direct sonication at 40 W (**A**), direct sonication at 60 W (**B**), and indirect sonication (**C**). ×, rutin; △, hyperoside; +, hyperforin; □, quercitrin; ○, pseudohypericin; ✱, hypericin.

The active compound and total extract contents, obtained by direct and indirect sonication for a period of 1 h, were compared with those obtained by conventional maceration, Soxhlet extraction, and ASE (**Table 1**). The extraction methods are listed according to the obtained amount of analyzed active compounds. The trend of the extraction is valid for all of the analyzed active compounds. However, the ratios between the active compound contents varied depending on the extraction method. In agreement with our results, Salisova et al. (*3*) reported that the contents of cineole, thujone, and borneol, obtained from the extraction from sage, varied with extraction method, extraction

temperature, and stirring effect. Plant materials are not completely homogeneous, and this may be one reason for the variations in active compound contents in different extracts from the same plant material. The data given in **Table 1** can be used only as a primary screening, as the best extraction time varies from one compound to another (see **Figure 4**). Direct sonication gave the highest amount of analyzed active compounds. The compound contents increased with an increase of ultrasonic power (direct sonication) from 40 to 60 W. Soxhlet extraction gave better results than ASE, in contrast to an earlier study on the extraction of hypericins from *H. perforatum* (*15*). In both reports, small differences between active compound contents obtained by Soxhlet extraction and ASE were observed. However, significantly longer extraction times are necessary in Soxhlet extraction. Comparison of ASE and Soxhlet extraction with conventional maceration showed that the active compound contents were higher using the former two, partly because of the higher temperatures used, which result in accelerated diffusion through the plant material. Liu et al. (*23*) showed that of the three test variables (extraction temperature, extraction time, and solvent concentration), the extraction temperature affected the extraction efficiency of active compounds from *H. perforatum* most significantly. However, higher temperatures may also provoke decomposition of some active compounds from *H. perforatum* (*23*, *24*). Comparison of the data indicates that direct sonication is more effective than indirect sonication because the cavitation effect is stronger, thus causing an intensification of mass transfer.

The main conclusion of this report is that direct sonication could be a powerful tool for extraction of pharmacological useful compounds from *H. perforatum*. In contrast to earlier reports on the extraction of active substances from plants, the ultrasonically assisted extractions have been compared with a greater number of other extraction methods. Thus, in combination with highly reliable LC/MS/MS measurements, a more systematic overview of active components extraction from *H. perforatum* is given.

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