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ABSTRACT: The genus Hypericum includes over 500 widely distributed species. The main representative is St. John’s wort (Hypericum perforatum L. (1753), Hypericaceae), the only approved biological source of Hyperici herba by WHO and EMEA monographs. It is frequently used in the form of oil macerate for treatment of burns, scars, eczema and gastrointestinal disorders, as well as in the form of water and alcoholic extracts as clinically proved antidepressant. Available data suggest that the amounts of secondary metabolites in the plant vary depending on ecological factors of the habitat, and consequently affect the quality of herbal drug. The reports show that other species of the genus have similar chemical profile as H. perforatum. But, there are also Hypericum species in which some of the secondary metabolites of interest occur in higher quantities than in H. perforatum. As previous data suggest, Hypericum hirsutum L. 1753, could be such example. Therefore, the aim of this study was to chemically characterize water-alcoholic extracts of H. hirsutum samples, collected at four localities in Vojvodina (Republic of Serbia) by liquid chromatography (HPLC-DAD). The obtained results suggest a good match (in a term of a presence of investigated compounds) of previously published results describing chemical profile of H. perforatum water-alcoholic extracts with examined H. hirsutum extracts. Also, chemotaxonomic analysis showed variations in quantity of secondary metabolites in the examined extracts. This opens the door to further investigation of H. hirsutum as a new source of bioactive secondary metabolites and additional markers in Hypericum chemotaxonomy.

KEYWORDS: chemical characterization, chemotaxonomy, HPLC – DAD, Hypericum hirsutum, PCA, secondary metabolites

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INTRODUCTION

St. John’s wort (Hypericum perforatum L. /1753/, Hypericaceae) is the main representative of the genus Hypericum with long history of use in traditional medicine. Usually, it is administered in the form of oil macerate externally for treatments of burns, bruises, eczema, and internally for treatment of gastrointestinal and gallbladder disorders, inflammation of respiratory and urogenital tract (Bombardelli and Morazzoni 1995; Božin et al., 2013). Different water and water-alcoholic extracts exhibit clinically proved antidepressant activity, especially significant in patients suffering from mild to moderate form of depression, comparable to synthetic antidepressants (Brattström 2009). The main active principles of the herbal drug (Hyperici herba) are naphtodiantrones (hypericin, pseudohypericin), phloroglucinols (hyperforin, adhyperforin), flavonoids (rutin, quercetin, quercitrin, hyperoside, amentoflavone), phenolic acids and a small amount of essential oil (Kladar et al., 2015b). For registration of phytopreparations 6% of phloroglucinols (hyperforin), 0.1–0.3% of naphtodiantrones (hypericin) and 2–4% of flavonoids (hyperoside) are required (Blumenthal et al., 1998). However, the content of secondary metabolites present in final preparations is directly related to the quality of herbal drug. It is known that the amounts of active principles in plants vary depending of abiotic factors specific for plant habitat (Kladar et al., 2015a). The genus Hypericum includes over 500 widely distributed species (Crockett and Robson 2011; Kladar et al., 2015b; Robson 1981). Following the recommendations of WHO and EMEA monographs only H. perforatum is marked as the biological source of Hyperici herba (European Medicines Agency 2009; World Health Organization 2002). Available studies to date suggest that other representatives of the genus also possess similar profile of chemical constituents as H. perforatum. Therefore, a question is whether these species could represent a substitute for the specifically defined biological source of Hyperici herba. Some of these representatives contain higher levels of metabolites of significance than H. perforatum, which opens a potential door to new biological and pharmacological applications (Kladar et al., 2015a). One of these species could be Hypericum hirsutum (Hypericaceae) – hairy St. John’s wort. The aim of this study was to chemically characterize water alcoholic extracts of H. hirsutum collected at four locations with specific sets of ecological factors in Vojvodina, Republic of Serbia. Chemotaxonomic evaluation of the examined samples was applied, to inspect the possible variations in the investigated species, which could reflect healing properties.

MATERIAL AND METHODS

The samples of Hypericum hirsutum were collected at four locations in Vojvodina, Republic of Serbia (Table 1). Vouchers are identified and deposited in Herbarium BUNS at the Department of Biology and Ecology, Faculty of
Sciences, University of Novi Sad (Greuter et al., 1986; Holmgren and Holmgren 2003; Robson 1981). Extraction with ethanol (70%, w/w) for 72h was used for obtaining the samples intended for further chemical characterization (European Directorate for the Quality of Medicines & Health Care 2007). After the evaporation of the solvent, the amount of dry extract (d. e.) was quantified and extracts were dissolved in methanol prior to chromatographic analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Location</th>
<th>GPS coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Veternik, Novi Sad</td>
<td>45°13′55.4″N, 19°45′27.9″E</td>
</tr>
<tr>
<td>2</td>
<td>Fruška Gora Mt.</td>
<td>45°09′07.2″N, 19°42′54.1″E</td>
</tr>
<tr>
<td>3</td>
<td>Zasavica, Ravnje – Banovo Polje road</td>
<td>44°55′48.9″N, 19°26′20.4″E</td>
</tr>
<tr>
<td>4</td>
<td>Hrtkovci</td>
<td>44°53′39.6″N, 19°46′01.2″E</td>
</tr>
</tbody>
</table>

Two methods of liquid chromatography (HPLC-DAD) were used for quantification of selected compounds in the examined extracts (Picture 1). The separation was performed on Zorbax CB-C18 column (4.6×150 mm, i.e., 5 μm particle size) held at 25 °C. Method I was used for determination of hypericin (Hpc), hyperforin (Hpf), apigenin (Ap), naringenin (NA), and amentoflavone (Am) (Božin et al., 2013). A modified method by Ziaková and Brandšteterová (2003) was used as Method II for determination of quercetin (Qe), rutin (R), epicatechin (Ec), caffeic (CA), chlorogenic (CHA), ferulic (FA), gallic (GA), and p-hydroxybenzoic acid (PHB). Solvent A was 0.1% solution of acetic acid in water and solvent B was 0.1% solution of acetic acid in acetonitrile. The mobile phase was delivered in gradient mode (3.25 min. – 0% B; 8 min. – 12% B, 15 min. – 25% B, 15.8 min. – 30% B, 25 min. – 90% B, 25.4 min. – 100% B) with flow rate of 1 mL/min and detection at wavelength of 280 nm. Before the injection of extracts, calibration curves of chemical standards of quantified compounds were obtained.
Principales componentes analysis (PCA) performed by PAST software package was applied for chemotaxonomic analysis and evaluation of differences in the chemical composition of the examined samples (Hammer et al., 2001).

RESULTS AND DISCUSSION

Naphtodiantrones, phloroglucinols, phenolic acids and flavonoids are classes of biologically active compounds present in Hypericum species (Brolis et al., 1998). The results of quantification of selected active principles in the extracts of *H. hirsutum*, as well as the amounts of dry extract are given in Table 2. It can be noticed that the amounts of extractable compounds vary between samples (13.73–19.06 %) which could be directly related to the insolation of plant habitat since sample 1 was collected from the habitat with the most sunlight exposure, and sample 2 from the shadowy habitat. Furthermore, differences between the samples in the content of all compounds except amentoflavone, caffeic, ferulic and para-hydroxybenzoic acid were noticed. The detected levels of hypericin were significantly higher than in the study conducted by Maggi et al. (2004) and Smelcerovic et al. (2006) which is related to the usage of different extraction procedures and solvents. However,
similarities were noticed with the results in studies by Kitanov (2001) and Smelcerovic et al. (2008) in which the plant material was extracted with ethanol, as in the current study. Hypericin was proved to possess antiviral, antidepressant and photodynamic activity (Bombardelli and Morazzoni 1995; Kladar et al., 2015b), and is recognized as one of the most important bioactive compounds present in the St. John’s wort. Hyperforin was another biologically active compound quantified in the examined extracts. In contrast to study where hyperforin was not detected (Maggi et al., 2004), the levels in the current study reached as high as 0.86 mg/g of dry herb. This represents significantly higher level than reported in a study conducted by Smelcerovic et al. (2006), but comparable to the results of another research by Smelcerovic et al. (2008). It is noticeable that the amounts of hypericin and hyperforin in the examined H. hirsutum extracts are generally lower than previously reported data for H. perforatum where the same extraction procedure as in this study was used (Božin et al., 2013). However, it is important to stress that the amount of hypericin in the examined extracts corresponds to the extracts of H. maculatum, which is in some pharmacopoeias listed together with H. perforatum as biological source of Hyperici herba (Kladar et al., 2015a). The quantified amounts of rutin and quercetin were significantly higher than in a study by Maggi et al. (2004). More similarities were noticed with the results reported by Smelcerovic et al. (2008), in which the content of rutin corresponded, but the amounts of quercetin in the examined extracts were significantly lower. Furthermore, the estimated amounts of rutin were generally higher than those reported for H. perforatum (Božin et al., 2013), and comparable to those reported for H. maculatum (Kladar et al., 2015a). The quantified levels of amentoflavone, which is by some authors (Baureithel et al., 1997) mainly responsible for antidepressant activity of Hyperici herba, were significantly lower than those reported for H. perforatum (Filippini et al., 2010). No previous data describing the amounts of apigenin, naringenin, amentoflavone, epicatechine, caffeic, chlorogenic, ferullic, gallic, and para-hydroxybenyoic acid in H. hirsutum were found. Therefore, this represents the first report of quantification of these compounds in the hairy St. John’s wort.

Table 2. Chemical composition of the examined H. hirsutum water-alcoholic extracts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Compounds</th>
<th>Hpc</th>
<th>Hpf</th>
<th>Ap</th>
<th>NA</th>
<th>Am</th>
<th>Qr</th>
<th>R</th>
<th>Ec</th>
<th>CA</th>
<th>CHA</th>
<th>FA</th>
<th>GA</th>
<th>PREB</th>
<th>% of d. w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Mean value</td>
<td>0.1383</td>
<td>0.1626</td>
<td>0.0031</td>
<td>n.d.</td>
<td>0.0153</td>
<td>0.2019</td>
<td>1.1853</td>
<td>0.7501</td>
<td>0.0585</td>
<td>0.2227</td>
<td>0.0222</td>
<td>0.1596</td>
<td>0.1487</td>
<td>19.06</td>
<td></td>
</tr>
<tr>
<td>1 SD</td>
<td>0.0662</td>
<td>0.0119</td>
<td>0.0001</td>
<td>n.d.</td>
<td>0.0014</td>
<td>0.0018</td>
<td>0.0099</td>
<td>0.0054</td>
<td>0.0041</td>
<td>0.0013</td>
<td>0.0025</td>
<td>0.0017</td>
<td>0.0018</td>
<td>0.0025</td>
<td></td>
</tr>
<tr>
<td>2 Mean value</td>
<td>0.1619</td>
<td>0.0049</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.0226</td>
<td>0.1548</td>
<td>0.2737</td>
<td>0.8775</td>
<td>0.0447</td>
<td>0.1115</td>
<td>0.0256</td>
<td>0.0596</td>
<td>0.1401</td>
<td>13.73</td>
<td></td>
</tr>
<tr>
<td>2 SD</td>
<td>0.0119</td>
<td>0.0001</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.0007</td>
<td>0.0083</td>
<td>0.0089</td>
<td>0.0060</td>
<td>0.0033</td>
<td>0.0047</td>
<td>0.0002</td>
<td>0.0056</td>
<td>0.0079</td>
<td>13.73</td>
<td></td>
</tr>
<tr>
<td>3 Mean value</td>
<td>0.3565</td>
<td>0.8689</td>
<td>n.d.</td>
<td>0.1629</td>
<td>0.0116</td>
<td>0.0414</td>
<td>0.6929</td>
<td>0.3702</td>
<td>0.0384</td>
<td>0.0760</td>
<td>0.1033</td>
<td>n.d.</td>
<td>0.1114</td>
<td>15.22</td>
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<tr>
<td>3 SD</td>
<td>0.0342</td>
<td>0.0066</td>
<td>n.d.</td>
<td>0.0105</td>
<td>0.0010</td>
<td>0.0038</td>
<td>0.0451</td>
<td>0.0020</td>
<td>0.0006</td>
<td>0.0064</td>
<td>0.0003</td>
<td>n.d.</td>
<td>0.0068</td>
<td>15.22</td>
<td></td>
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<tr>
<td>4 Mean value</td>
<td>0.2523</td>
<td>0.0027</td>
<td>0.0098</td>
<td>n.d.</td>
<td>0.0019</td>
<td>0.0066</td>
<td>0.0179</td>
<td>0.0010</td>
<td>0.0007</td>
<td>0.0025</td>
<td>0.0017</td>
<td>0.0012</td>
<td>0.0012</td>
<td>0.0097</td>
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<tr>
<td>4 SD</td>
<td>0.0040</td>
<td>0.0002</td>
<td>0.0001</td>
<td>n.d.</td>
<td>0.0014</td>
<td>0.0016</td>
<td>0.0086</td>
<td>0.0045</td>
<td>0.0007</td>
<td>0.0056</td>
<td>0.0004</td>
<td>0.0002</td>
<td>0.0012</td>
<td>0.0097</td>
<td></td>
</tr>
</tbody>
</table>

*n. d. – not detected

Performed PCA reveals that the value of the first component is strong, explaining 62.26 % of variance, and together with the second component covers more than 98 % of variance (Figure 2c). This implicates that PCA is a good
and sufficient method for presentation of the size and shape of variations of examined variables and their grouping based on variation resemblance. The load values of the first and second component (Figure 2a and 2b) show that the main compounds responsible for the separation of the samples are the amounts of hyperforin, hypericin and rutin. It is obvious that the drastic separation of sample 3 (Figure 2d) based on the first component is the result of a presence of notably higher amount of hyperforin. The resemblance of samples 1 and 4 is the result of quantities of rutin which are significantly higher than in sample 2. Samples 1, 2 and 4 show similarity in quantity of epicatechin, which occurs in significantly lower amount in sample 3. The amounts of the rest of the quantified secondary metabolites in the examined samples are relatively stable and do not significantly affect the separation. This might indicate that the levels of synthesized hyperforin, hypericin, epicatechin and rutin in *H. hirsutum* are most affected by the sets of ecological factors specific for plant habitat.

*Figure 2.* PCA based on the compounds detected in examined *H. hirsutum* extracts (2a and 2b – the load values of the first and second component, respectively, 2c – variance covered by the first and second component, 2d – position of the analyzed variables in the space of the first and second PCA axes.
Consequently, this leads to the conclusion that the origin of plant material is important for the quality of herbal drug since all of these compounds, except epicatechin, are essential for pharmacological effects of St. John’s wort.

CONCLUSIONS

The chemical composition of the *H. hirsutum* examined extracts in most of the cases corresponded to those previously reported. When compared to the *H. perforatum* extracts, significantly lower amounts of hypericin and hyperforin were found. However, higher levels of rutin, which is a well-known biologically active compound, were reported. This opens a question of potentially new biological activities of *H. hirsutum*. Furthermore, of particular significance is a resemblance with chemical profile of *H. maculatum*, which is in some pharmacopoeias also listed as a biological source of *Hyperici herba*. Analyses suggested that the main compounds responsible for separation of the samples were hyperforin, hypericin, rutin and epicatechin, which implicates that the production of these secondary metabolites in the plant is highly affected by ecological factors characteristic for plant habitat. This emphasizes the significance of plant collecting locations since all of the mentioned compounds (except epicatechin) are of high importance for so far established pharmacological effects of *Hypericum* species.

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ХЕМИЈСКА КАРАКТЕРИЗАЦИЈА И ХЕМОТАКСОНОМИЈА
Hypericum hirsutum ИЗ ВОЈВОДИНЕ

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РЕЗИМЕ: Род Hypericum убухвата преко 500 широко распрострањених врста. Главни представник рода и према Монографијама СЗО и ЕМЕА, једини биолошки извор биљне дроге је кантарион (Hypericum perforatum, Hypericaceae). Кантарион се често користи у облику уљаних мацерата за трећирање опекотина, ожиљака, екзема и гастроинтестиналних поремећаја, али и у облику водених или алкохолних екстраката као клинички доказан антидепресив. Према доступним подацима, количине секундарних метаболита у биљкама варирају зависно од еколошкх фактора везаних за станиште и последично утичу на квалитет биљне дроге. Такође, претходна истраживања показују да и други припадници рода Hypericum поседују сличан профил хемијског састава као и H. perforatum. Међутим, постоје примери да су одређени биомолекули присутни у већим количинама него код H. perforatum. Један од таквих примера би могао бити H. hirsutum.

Из тог разлога, циљ истраживања био је хемијска карактеризација водено-алкохолних екстраката H. hirsutum прикупљеног са четири локалитета у Војводини методом течне хроматографије (HPLC-DAD). Добијени резултати хемотаксономске анализе указују на одређене разлике у садржају секундарних метаболита међу испитаним екстрактима. Такође, примећене су сличности профила хемијског састава у водено-алкохолних екстраката H. perforatum и H. hirsutum чиме се отвараитање даљих истраживања H. hirsutum као потенцијалног извора нових биолошки активних секундарних метаболити и додатних хемотаксономских маркера рода Hypericum.

КЉУЧНЕ РЕЧИ: хемијска карактеризација, хемотаксономија, HPLC-DAD, Hypericum hirsutum, PCA, секундарни метаболити