HPLC METHOD FOR SCREENING OF STEROIDAL SAPONINS AND RUTIN AS BIOLOGICALLY ACTIVE COMPARTMENTS IN TRIBULUS TERRESTRIS L.

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ABSTRACT
An improvement method for extraction of steroidal saponins and rutin of Tribulus terrestris L. (Zygophyllaceae) was evaluated. It is shown that the ultrasound extraction is faster, easier, solvent-saving, more reliable and effective method than the conventional heat reflux extraction method.

Under optimized conditions, the main biologically active compounds protodioscin, prototribestin, dioscin and rutin were extracted from Tribulus terrestris with different origin and analyzed by reversed phase HPLC with UV detector.

Keywords: Tribulus terrestris, ultrasound extraction, steroidal saponin, HPLC

Abbreviations used: HPLC - high performance liquid chromatography, USE – ultrasound extraction

Introduction

Tribulus terrestris L. (Zygophyllaceae) is an annual herb that grows worldwide, especially in the subtropical area, used in the folk medicine in Bulgaria, India, China, southern USA, Turkey, Spain and other countries against sexual impotency, oedemas, abdominal distention and cardiovascular diseases (1, 2). Preparations containing T. terrestris extracts are on sale in Bulgaria, USA as food supplements with claim of a general stimulating action on motor activity, muscle tone and restorative tonic (3, 7, 8). A wide range of compounds has been reported to occur in this plant: saponins, flavonoids, amides and alkaloids (4, 5). Several studies have shown that saponins are among the compounds responsible for the biological activities of T. terrestris extracts. Steroidal saponins isolated from T. terrestris are spirostanol saponins bearing a sugar chain linked to C-3 and furostanol saponins carrying a sugar chain at C-3 and D-glucose residue at C-26.

The previous investigation shows some differences of the content of biologically active components in Tribulus terrestris. The samples from Bulgaria, Turkey, Greece, Serbia, Macedonia, Georgia and Iran exhibited similar chemical profile and only some quantitative difference in the content of protodioscin, prototribestin, dioscin, tribulosin and the flavonoid rutin. The Vietnamese and Indian samples exhibit totally different chemical profile. Tribulosin is the main component, while prototribestin is not present in the samples (5).

The aim of our study is screening for the presence of protodioscin, prototribestin, dioscin and rutin – the main compounds of Bulgarian Tribulus terrestris L. in the plant materials of the same herb from different regions using HPLC.

Our attempts to find an improved method for extraction of steroidal saponins lead to elucidation of modified method of extraction. It is the first important step for recovery and purification of active ingredients of plant materials. Usually, the traditional techniques require long extraction hours and have low efficiency. Recently there have been numerous reports on the application of high intensity or power ultrasound in the extraction of various phytochemicals, such as saponins, alkaloids, flavonoids, polysaccharides, proteins and essential oils from various parts of plant and plant seeds (6). In addition, ultrasonic extraction can be carried out at lower temperatures, avoiding thermal damage to extract.
Materials and Methods

Plant material
The study of the content of steroidal saponins and rutin have been carried out with cultivated plants obtained from commercial seeds of Hungary and Turkey, as well as a plants, natively distributed in Bulgaria, districts of Plovdiv and Vedrare – collected from open sandy sites and from cultivated field with sunflower.

The cultivated plants were propagated from seedlings, sowing in the beginning of May, 2009. The seedlings were transplanted in the beginning of June on the experimental field near Sofia into plant beds. The plants were collected in full blossoming and seed formation in August, when the samples developed 100-120 cm long stems in all directions.

Analytical method
An HPLC system La Chrom Elite consisting of L-2130 pump equipped with gradient controller and UV detector L-2400 was used. The separation was performed on 250 x 4.6 mm i.d., 5 µm, Inertsil ODS-2 column (Tokyo, Japan) with MetaGuard Pursuit direct connect guard column from Varian was used for all separations. The mobile phase which consisted of phosphoric acid buffer with pH-3 (A) and acetonitrile (B) was used for gradient elution. The flow rate was adjusted to 1.0 ml/min. The detection wavelength was at 203 nm. All separations were performed at ambient temperature.

Sample preparation by conventional heat reflux extraction
The dried and powdered plant material (10 g, leaves and fruits, 1:1) was extracted in a succession by chloroform at room temperature (3 x 90 ml x 1 h) and 70% v/v ethanol (reflux at 80°C, 3 x 150 ml x 2 h). The combined ethanol solutions were concentrated under vacuum at 70°C to a small volume ~ 50 ml and extracted in the separatory funnel with n-butanol (3 times 20, 15, 15 ml). The butanol layers were concentrated to dryness giving the crude saponin extract (CSE). The extract was dissolved in 50% aqueous acetonitrile in 200.00 ml volumetric flask.

Sample preparation by ultrasonic extraction
The finely powdered plant material (1 g, leaves and fruits, 1:1) was extracted three times with 5.0 ml of 50% aqueous acetonitrile by sonication for 15 min. The extracts were combined, after filtration in 20.00 ml volumetric flask and the volume was adjusted to 20 ml with the solvent use for extraction.

Prior injection, all samples were filtered through a 0.45-µm Chromafil 0-45/25 Machery-Nagel. Each sample solution was injected in triplicate with injection volume of 20µl.

Calibration
Standard compounds protodioscin (2 mg), prototribestin (2 mg), dioscin (2 mg) and rutin (0.2 mg) were dissolved in 1.00 ml of 50% aqueous acetonitrile (stock solution). Further calibration levels were prepared by diluting the stock solution with the same water/acetonitrile mixture. The resulting range, for the concentration is 1mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml (0.09 mg/ml, 0.067 mg/ml, 0.045 mg/ml, 0.0225 mg/ml, 0.01152 mg/ml for rutin). All calibration levels were injected with injection volume of 20 µl and the calibration data obtained (0.9848 to 0.9882) indicated linearity of the detector response.

Results and Discussion
In the present investigation the steroidal saponins protodioscin, prototribestin, dioscin and rutin (Fig. 1) in various samples of Tribulus terrestris were profiled and quantified using reverse phase HPLC with UV detector.

The conventional heat reflux extraction method was used as a control for comparison with the ultrasound extraction (USE). The optimized experimental conditions of USE are as follows: the extraction solvent (50% aqueous acetonitrile), the extraction time (2 h), the extraction temperature (room temperature). The chromatograms (Fig. 2) show that the yield of main steroidal saponins and rutin was 1.51% in ultrasound extraction, while the yield of 0.92% was achieved during heat reflux extraction (Table 1). The USE method showed an improved efficiency over the conventional one (Fig. 2). It is also faster, easier, solvent-saving, more reliable and effective.

For the quantitative determination of these four compounds it was necessary to prepare standard calibration curves. For this purpose the stock solution was diluted to 5 different concentrations ranging from 0.125 to 1 mg/ml (from 0.01152 to 0.09 mg/ml for rutin). The standard curves showed good linearity, with R² values not less than 0.9848 for all concentration ranges used (Fig. 3).
Fig. 1. Structural formulae of the biologically active compounds in *Tribulus terrestris*

![Structural formulae of biologically active compounds](image)

Protodioscin

Prototribestin

Dioscin

Rutin

Fig. 2. Comparison between two methods of extraction: (1) Heat reflux extraction, (2) Ultrasound extraction; (a) rutin, (b) protodioscin, (c) prototribestin, (d) dioscin

![Comparison of extraction methods](image)
TABLE 1
The yields (wt. %) of the main components extracted from *Tribulus terrestris* by different extraction methods

<table>
<thead>
<tr>
<th></th>
<th>Ultrasound extraction</th>
<th>Heat reflux extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>0.25</td>
<td>0.21</td>
</tr>
<tr>
<td>Protodioscin</td>
<td>0.62</td>
<td>0.36</td>
</tr>
<tr>
<td>Prototribestin</td>
<td>0.51</td>
<td>0.27</td>
</tr>
<tr>
<td>Dioscin</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td>Total</td>
<td>1.51</td>
<td>0.92</td>
</tr>
</tbody>
</table>

The results from the quantitative determination of steroidal saponins and rutin in the analyzed samples are presented in Table 2. The samples from Bulgaria contain high amount of protodioscin and prototribestin, as these furostanol saponins are dominant in the Plovdiv wild growing sample. The concentration of protodioscin and prototribestin in *Tribulus terrestris* from the cultivated plants of Hungary and Turkey is lower than from the Bulgarian one.

The content of rutin from Turkey and Hungary is 10 times more than in the other samples. The content of spirostanol saponin dioscin was found in all samples in this study in commensurable amounts. It is obvious that the concentration of the main biologically active compounds protodioscin and prototribestin in wild growing samples from Vedrare and Plovdiv is higher than the one from Vedrare in the cultivated field with sunflower (Fig. 4).

Fig. 3. Calibration curves of standard compounds

Fig. 4. Chemical profile of biologically active compounds in *Tribulus terrestris*
TABLE 2
Content of biologically active compounds in *Tribulus terrestris* with different origin

<table>
<thead>
<tr>
<th></th>
<th>Hungary cultivated</th>
<th>Turkey cultivated</th>
<th>Vedrare wild growing</th>
<th>Vedrare in culture</th>
<th>Plovdiv wild growing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin (mg/g DW)</td>
<td>1.02±0.11</td>
<td>1.51±0.07</td>
<td>0.34±0.07</td>
<td>0.11±0.08</td>
<td>0.11±0.0</td>
</tr>
<tr>
<td>Protodioscin (mg/g DW)</td>
<td>3.04±0.11</td>
<td>6.43±0.26</td>
<td>17.84±0.59</td>
<td>14.68±0.61</td>
<td>20.48±0.49</td>
</tr>
<tr>
<td>Prototribestin (mg/g DW)</td>
<td>2.28±0.08</td>
<td>4.74±0.64</td>
<td>13.71±0.47</td>
<td>5.15±0.15</td>
<td>15.01±0.51</td>
</tr>
<tr>
<td>Dioscin (mg/g DW)</td>
<td>7.49±0.50</td>
<td>4.72±0.54</td>
<td>7.05±0.47</td>
<td>5.98±0.73</td>
<td>8.52±0.59</td>
</tr>
</tbody>
</table>

Conclusions

The ultrasonic extraction method was proved as:
- more rapid (2 instead of 48 hours);
- solvent-saving;
- more-reliable and effective over the conventional heat reflux extraction.

Acknowledgment

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REFERENCES