IN VITRO CULTURES FROM ASTRAGALUS THRACICUS AND THEIR RADICAL-SCAVENGING ACTIVITY

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Abstract. Aim of this research is to determine radical-scavenging potential of extracts from intact plants and in vitro cultures, gained form Astragalus thracicus Griseb. and to prove the presence of the flavonoid orientin by the HPLC spiking method in them.

In this research is used the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The activity of the gained extracts is measured as an equivalent of ascorbic acid (EqAA) and hyperoside (EqH).

Aerial parts from intact plants possess the highest level of activity, evaluated to 1094.01 µg EqAA and 1166.82 µg EqH. Similar activity is found in extract from plants, grown on MS media (968.77 µg EqAA and 1036.50 µg EqH).

The presence of the flavone orientin by the means of HPLC spiking method is proved in all of the investigated aerial extracts, as it is determined in insignificantly small amounts in the roots.

Keywords: Astragalus thracicus, in vitro cultures, radical-scavenging activity, DPPH

Introduction

Genus Astragalus (Fabaceae) comprises of about 2500 species, distributed mainly in the Northern hemisphere. Species of genus Astragalus are used as diuretics, tonics, laxatives, etc [1,2]. In Bulgaria genus Astragalus is represented by 29 species [3].

The object of this research – Astragalus thracicus – is a Balkan endemic species with a conservation status – vulnerable. It is a Tertiary relict plant, distributed around the Bulgarian towns Sliven, Yambol and Haskovo, as well as in some parts of Greece, Former Republic of Yugoslavia and European part of Turkey.

Some of the Bulgarian Astragalus species have been studied for their saponin and flavonoid content, as well as their biological activity [4-7]. Many species of this genus show diversity in their pharmacological properties, and that is the reason why they are widely used in the Folk Medicine of different Asian and European countries. Elucidated structures of the flavonoid compounds from this genus belong to the group of flavons, flavonols, isoflavons, isoflavans and pterocarpans. For a comparison with the great diversity of the flavon- and flavonol- glycosides, the number of isoflavonoid glycosides is pretty small.

Kaempferol and quercitin are widely distributed among the flavonols, whereas astragalin and rutin are the most commonly found glycosides. A. thracicus itself has not been studied yet in phytochemical aspect. This motivates us to make a comparison of the radical-scavenging activity of extracts from hypogeaal and aerial parts of the intact plant and to compare it with in vitro cultures. This will help us to determine the accumulation of radical-scavenging molecules in in vitro cultures.

The increasing demand of raw herbal plant material nowadays, makes preferred biotechnological approaches for production. The major reason for that is the ability to control growth conditions of the cultures. It is also very convenient if the species of interest is rare or protected. Plant tissue culture biotechnology approach shows its big advantage as an appropriate alternative method for standardized production of phytochemicals.

Materials and Methods

Plant material

Plant material – aerial parts, roots, and seeds – are collected from Bakadzhitsite hills near Yambol in
Orientin is a water-soluble flavon, 8-C-glucoside of flavonoid aglicon luteolin, also known as luteolin-8-C-glucoside or lutexin.

**Data analysis**

Values were reported as Means ± SD of three parallel measurements. The obtained results were analytically analyzed with the computer program GraphPad Prism 6.01.

**HPLC investigation of the extracts for presence of orientin**

Analytical HPLC analysis is conducted on a chromatographic system YL 9100 HPLC System, with YL 9110 Quaternary Pump, injector with 20 µl loop, YL 9160 PDA Detector, collected information is in the range 200 – 900 nm. The apparatus has a column compartment YL 9131 Column Compartment adjusted to 35°C and a YL 9101 Vacuum Degaser. Used column: Ascentis® RP-C18, 250 x 4.6 mm (Supelco Analytical, USA). Total flow 1 ml/min. Used mobile phases: A – 100% H₂O + 0.03% TFA, and B –100% CH₃CN. The following linear gradient program is used: 0-2 min 10% B, 2-30 min 10-60% B, 30-33 min 60-100% B.

**DPPH radical-scavenging activity**

DPPH (1,1-diphenyl-2-picrylhydrazyl) is characterized as a stable free radical, by virtue of the delocalization of the spare electron over the molecule as a whole. The violet color of the methanolic solution of DPPH is a result of the delocalized electron and it possesses absorption maximum at λ=517 nm. If the DPPH solution reacts with molecules with antioxidant activity (as those of flavonoids for example) solution changes its color from intensive violet to bright yellow. During this transition the solution decreases its absorption at λ=517 nm. This is the spectrophotometrically measured change. Method for DPPH radical-scavenging is conducted as it follows: 50 µl of the total extracts with concentration 10 mg/ml is added to 2 ml 0.2 mM solution of DPPH in MeOH. It is stirred with a Vortex® for 30 s and left for 30 min in dark. Measurement is done in λ=517 nm before and 30 min after addition of extract. Pure MeOH was used as a control.

All measurements are done on UV 1100 Spectrophotometer (MAPADA). Quantitative analysis is made with the help of two positive controls: ascorbic acid and hyperoside. Standard curves were prepared with a series of five solutions with accurate concentration. Equations of the curve is $y=2.629x+0.03022$ $r^2= 0.9966$ and $p<0.0001$ for ascorbic acid and $y=2.734x+0.06047$ $r^2=0.9740$ and $p<0.0001$ for hyperoside. Evaluation of radical-scavenging activity is made with interpolation of gained results against standard curve.
Results and Discussion

Investigation of germination ability of seeds from A. thracicus

Method of mechanical scarification is used, because it gives significant increase either of speed or level of germinated seeds. On Fig 2. can be seen, that scarified seeds germinate either faster or in a greater rate in comparison with non-scarified seed. This difference is measured as on the 3-rd day after sterilization 30% of scarified seed germinate, on 5-th day – 70% and after 10-th day maximum germination is measured as 86% of all scarified seeds.

The germination is prolonged for the group of non-scarified seeds. Up to the 10-th day no germinated seeds are observed. On the 12-th day there are only about 15% and just after 25-th day it reaches 66% which is 22% less in comparison with scarified seeds.

From the showed data we can conclude that scarification technique significantly shortens the period for germination and increases germination rate with up to 22%. Reason for this is easier penetration of water in the core of the seed, leading to faster swelling and easier rupture of seed coat.

Determination of radical-scavenging activity of extracts from different in vitro cultures

Table 1 shows correlation between content of growth hormones in the nutrition media and measured radical-scavenging activity of the corresponding extracts. The highest activity is determined for extracts from aerial parts of intact plants, followed by plants grown on basic MS medium with lack of hormones. The lowest radical-scavenging activity is determined in root cultures, grown in liquid MS-Li, containing high level of auxins (5 mg/l 1-Naphthaleneacetic acid). This result proves the ability of auxins to promote root growth, and confirms that synthesis of flavonoids is carried out preferably in epigeous parts of the plants. Comparatively low is the activity of extracts, grown on G56 medium (containing Indole-3-Acetic Acid 5 mg/l, 2,4-Dichlorophenoxyacetic acid 2 mg/l and Kinetin 5 mg/l). Despite HP9 medium is half-strength regarding macro- and micro-elements, plants grown on it, show relatively high radical-scavenging activity.

Radical-scavenging activity is a result of the property of flavonoids to react with free radicals. This is the reason why in our research we use free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The activity of the gained extracts is measured towards an equivalent quantity of ascorbic acid (EqAA) and hyperoside (EqH), by the means of a standard curve. Results show that all the gained extracts possess radical-scavenging activity in different level. Aerial parts from intact plants possess the highest level of activity, equal to 1094.01 µg EqAA and 1166.82 µg EqH. Very close activity shows the extract from plants, grown on MS media (968.77 µg EqAA and 1036.50 µg EqH). Lower activity is observed in cultures, gained on media G56, evaluated as 790.00 µg EqAA and 850.00 µg EqH. Reduced activity can be connected with the high concentration of auxin hormones indol-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in the nutrition media, for both of them we determined, that in high concentrations they indirectly inhibit the flavonoid synthesis. The weakest radical-scavenging activity is for root cultures in liquid MS-Li possessing 408.79 µg EqAA and 454.16 µg EqH.
Table 1. Hormonal content of nutrition media and measured radical-scavenging activity measured as equivalent of ascorbinic acid and hyperoside.

<table>
<thead>
<tr>
<th>№</th>
<th>Investigated extract</th>
<th>Concentration of growth hormones (mg/l) in the composition of the medium</th>
<th>Radical-scavenging activity of the extracts measured as equivalent:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ascorbinic acid (EqAA)</td>
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<td></td>
<td></td>
<td></td>
<td>Hyperoside (EqH)</td>
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<tr>
<td>1.</td>
<td>Aerial parts grown on MS medium</td>
<td>Do not contain growth hormones</td>
<td>968.77 µg</td>
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<td></td>
<td></td>
<td></td>
<td>1036.50 µg</td>
</tr>
<tr>
<td>2.</td>
<td>Root cultures, grown in liquid MS-Li medium</td>
<td>Auxin NAA (5 mg/l)</td>
<td>408.79 µg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>454.16 µg</td>
</tr>
<tr>
<td>3.</td>
<td>Aerial parts, grown on HP9 medium</td>
<td>Half strength MS + Auxin IAA (0.4 mg/l) Cytokinin BAP (0.22 mg/l)</td>
<td>940.73 µg</td>
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<td></td>
<td></td>
<td></td>
<td>1007.34 µg</td>
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<tr>
<td>4.</td>
<td>Aerial parts, grown on G56 nutrition medium</td>
<td>Auxin IAA (5 mg/l) Auxin 2,4-D (2 mg/l) Cytokinin Kinetin (5 mg/l)</td>
<td>790.00 µg</td>
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<td></td>
<td></td>
<td></td>
<td>850.59 µg</td>
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<tr>
<td>5.</td>
<td>Roots, gained from whole plants, grown on MS nutrition medium</td>
<td>Do not contain growth hormones</td>
<td>846.00 µg</td>
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<td></td>
<td></td>
<td></td>
<td>908.00 µg</td>
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</tbody>
</table>

**HPLC investigation of the extracts for presence of orientin**

In the present work is proved presence of orientin in all extracts, used for radical-scavenging activity by the HPLC spiking method. Presence of orientin is determined in all extracts, gained from epigeous parts of in vitro and intact plants. In contrast, in the hypogeal parts its quantity is in traces. The quantity of orientin in significant ranges is found in two media – the basic MS and the HP9. MS is characterized with the lack of any hormones, as HP9 is half-strength MS (regarding micro- and macro-elements) and also contains low concentrations of growth hormones indol-acetic acid (IAA) and 6-benzylaminopurine (BAP). Presence of orientin in *in vitro* cultures proves that biotechnological approaches can be used for flavonoid production.

**Conclusion**

In the present work were created different in vitro cultures from the Balkan endemic species – *A. thracicus*. The determined DPPH, radical-scavenging activity of total extracts shows that highest activity is observed in aerial parts of intact plants, followed by aerial parts of in vitro plants grown on basic MS medium. HPLC analysis of purified total extracts shows that presence of auxins in high concentrations in nutrition media leads to inhibition of flavonoid synthesis and respectively lower radical scavenging activity. The HPLC spiking method confirmed presence of C-glucoside orientin in investigated extracts from epigeous parts of intact and in vitro plants. This proves the possibility for biotechnological production of flavonoids, typical for intact plants.

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**References:**


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