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DPPH RADICAL SCAVENGING ACTIVITY OF IN VITRO REGENERATED *HABERLEA RHODOPENSIS* FRIV. PLANTS

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Summary. The Balkan endemite *Haberlea rhodopensis* Friv. (Gesneriaceae) is a very rare, relict plant species, which grows in restricted humid and shady areas in Bulgaria. The species is among the few plants known as resurrection plants for their vegetative desiccation tolerance. After successful establishment of a protocol for in vitro multiplication earlier, here we report that the in vitro produced plants possess the same resurrection capability as the plants from the nature. Leaf extracts of the plants contain three groups of biologically active substances: flavonoids, tannins and polysaccharides. Leaves of in vitro multiplicated plants produced about 30% more flavonoids than the plants from natural habitats. Total content of flavonoids showed a better correlation than total tannins with the free radical scavenging effect of the extracts measured by means of the DPPH discoloration assay. The extracts of *Haberlea rhodopensis* leaves display strong antioxidative activity, which could be exploited as potential pharmaceuticals.

Key words: *Haberlea rhodopensis* Friv. (Gesneriaceae), resurrection plant, antioxidative activity, flavonoids, DPPH-radical scavenging effect

DPPH РАДИКАЛ-СВЪРЗВАЩА АКТИВНОСТ НА IN VITRO РЕГЕНЕРИРАНИ РАСТЕНИЯ *HABERLEA RHODOPENSIS* FRIV. PLANTS

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Резюме. Балканският ендемит *Haberlea rhodopensis* Friv. (Gesneriaceae) е рядко срещан, дребен растителен вид, растящ в ограничени влажни и сенчести области в България. Видът е сред малкото растения, познати като възкръсващи растения, поради тяхната толерантност към вегетативно изсушаване. След разработване на протокол за размножаване на *Haberlea* in vitro, тук ние съобщаваме, че in vitro размножените растения притежават същата възстановителна способност като растенията в природата. Листни ектракти от този вид съдържат три групи биологичноактивни вещества – флавоноиди, танини и полизахариди. Установено бе, че растенията от in vitro размножаване синтезират 30% повече флавоноиди от събраните от естествени находища. Общото флавоноидно съдържание в *Haberlea rhodopensis* показва по-добра корелация в сравнение с танини по отношение на радикал-свързващата активност, установена чрез DPPH тест. Екстракти от листа на *Haberlea rhodopensis* са с висока антиоксидантна активност, чийто потенциал би могъл да се използва във фармацията.

Ключови думи: *Haberlea rhodopensis* Friv. (Gesneriaceae), възобновяващо се растение, антиоксидантна активност, флавоноиди, DPPH радикал-свързващ ефект

Introduction

A small group of higher plant species known as poikilohydric or resurrection plants possess unique desiccation tolerance. Their mature leaves can lose almost fully their water content and survive long periods of dryness. Upon re-watering, they recover very fast and restore their photosynthetic activity within several hours. Most resurrection plant species are native to arid climates [4]. The possibility to use them as models to study the reaction of plants to extreme conditions has been discussed and protocols for in vitro manipulations have been developed for *Craterostigma plantagineum Ramonda myconi* and *Haberlea rhodopensis* [2, 3, 9].

The Balkan endemite *Haberlea rhodopensis* Friv. (Gesneriaceae) is a very rare plant species. It grows

in restricted humid and shady areas in Bulgaria – the Rhodope and the Balkan mountains. Up to now photosynthesis and metabolism under desiccation and re-hydration have been investigated in *Haberlea rhodopensis* plants taken from the nature [5, 8, 13]. On the other hand, there is a growing body of hard-to-prove data from recent archaeological studies that this relict plant is closely connected to ancient history of the area. Secondary metabolism in the abitoic stress tolerance has been evaluated in some resurrection plants [12]. Little is known about pharmacological application of these plants and their phytochemical composition.

The accumulation of gallothanins, known for their wound-healing properties during dehydra-tion and re-hydration of *Myrothamnus flabelifolia* could explain the use of this shrub in the traditional folklore and medicine in southern Africa [10].

Secondary metabolites and biologically active compounds of Haberlea rhodopensis have been not studied so far. The use of Haberlea rhodopensis leaves for treatment of wounds and diseases of stock in the Rhodope region could have putative ethno-botanical importance.

At present, the few resurrection plant species all over the world are under intensive study for their anti-oxidative behaviour and with the aim to isolate anti-oxidative compounds. As a part of our efforts to find new antioxidative and antiradical active compounds we have investigated the extracts of individual plants from natural habitats and regenerated in vitro *Haberlea rhodopensis* plants, cultivated in greenhouse conditions. The aim of the present study was also a prelimi-nary screening for biologically active substances in *Haberlea rhodopensis* leaves.

Experimental

Plant material and growth conditions

Plant material from Haberlea rhodopensis (natural habitat plants) was collected in Bulgaria near the town of Asenovgrad (Bachkovo region) in July 2007. Voucher specimens are depos-ited in the herbarium of the Faculty of Pharmacy, Medical University of Sofia (FAF 00041).

Plants of *Haberlea rhodopensis* were regenerated and propagated *in vitro* [3]. After surface sterilization of seeds and germination on MS [11] basal medium, plantlets were transferred to Woody Plant Medium (WPM) [7] for further development. Well-rooted plants were potted and cultivated in vivo in greenhouse. After acclimation, the plants grow normally under greenhouse conditions. Well-developed potted plants were used for the study.

Tests for resurrection behavior of in vitro regenerated plants

In vitro propagated Haberlea rhodopensis plants were successfully acclimated under green-house conditions. About 3 months later, 50 plants were subjected to drought by withholding of water along with 10 plants obtained from natural habitats of the species. The irrigation was resumed 3 weeks later. The recovery was scored in both groups.

Extraction, isolation and proof of biologically active compounds

Flavonoids – to 0,20 g of the air dried powdered leaves add 10 ml of methanol. Heat in a water bath under a reflux condenser for 10 min. The methanolic extract was concentrated to 1 ml. Four different glycosides and 4 aglycones after acid hydrolysis using TLC Silica gel plates were identified. The systems: ethyl acetate: ethyl methyl ketone : formic acid : water (5:3:1:1) for glycosides; toluene : formic acid (5:4:1:) for aglycones, spray solution -10 g/l solution of diphenylboric acid aminoethyl ester in methanol and examination on UV light at 365 nm.

Tannins – to 0,10 g of the dried powdered leaves add 10 ml water. Heat in a boiling water bath under a reflux condenser for 5 min. Cool and filter. To the filtrate add 2-3 drops 1% water solution of $FeCl_3$ – a blue-black color is formed.

Proof of polysaccharides – to 0,15 g of the dried powdered leaves add 20 ml water at room temperature and shake for 30 min. After filtering, polysaccharides in the filtrate form a residue, under the influence of 95% ethanol. The residue is separated by centrifuging and dissolves in 10 ml water. To 1 ml of solution add 1 ml 5% solution of phenol and 5 ml concentrated sulphuric acid. Shake immediately – a yellow-brown color is formed.

Quantitative analysis

Assay of flavonoids – to 250 ml flask introduce 0,40 g of dried powdered leaves, 30 ml 90% ethanol, containing 1 % hydrochloric acid and heat in a water bath under reflux condenser for 30 min. After cooling under water, the extract is filtered through a paper filter in the volumetric flask. To the powdered leaves in the flask add 30 ml 90% ethanol and heat in a water bath for 30 min. After cooling the extract is filtered through the same filter in the same volumetric flask. The filters are rinsed with 20 ml 90% ethanol and dilute the filtrate to 100 ml with 90% ethanol (solution I).

Test solution. In a volumetric flask introduce 2 ml of the solution I, 1ml 1 % solution of $AlCl_3$ in 95% ethanol and dilute to 25 ml with 95% ethanol.

Compensation solution. Dilute 2 ml of the solution I to 25 ml with 95% ethanol.

Measure the absorbance of the test solution after 20 min by comparison with compensation solution at 430 nm (WPA UV/VIS spectrophotometer Kyoto, Japan).

Assay of tannins. In a 250 ml flask introduce 0,20 g of the dried powdered leaves, 150 ml water and heat in a boiling water bath for 30 min. Cool the flask at running water. Filter the extract through a paper filter in a volumetric flask. After rinsing of the flask and the filter with 20 ml water, dilute to 250 ml with water (solution I). Dilute 5 ml of solution I to 25 ml with water (solution II). In a volumetric flask introduce 2 ml of solution II, add 1 ml Folin reagent, 10 ml water and dilute to 25 ml with 29% solution of Na_2CO_3 (sodium carbonate).

The absorbance of samples was measured at 760 nm (WPA UV/VIS spectrophotometer Kyoto, Japan) after 30 min, by comparison with compensation solution water and the results were expressed in mg of gallic acid per g (GAE) of dry weight of samples. Concentration of tannins was determined by standard linear as tannin (expressed as gallotannic acid equivalents – GAE).

Preparation of methanol extracts. The air-dried leaves of *Haberlea rhodopensis* (Gesneriaceae) (0.150 g) were powdered in a blender and extracted with MeOH (3 times) at room temperature. The extract was then filtered and evaporated on a rotary vacuum evaporator to give a solid MeOH extract (0.026 g).

DPPH discoloration assay

The antioxidant activity of the methanol extract was measured on the basis of the scavenging activities of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH, Fluca – Germany) free radical. The scavenging of free radicals by extracts was evaluated spectrophotometrically (WPA UV/VIS spectrophotometer, Japan) against the absorbance of the DPPH radical. After 30 min of incubation period in the dark at room temperature, the absorbance was measured against a blank at 517 nm. The percentage of discoloration was calculated as follows:

Percentage inhibition (%) = [(Ablank – Asample)/Ablank)] x 100

Where, Ablank is the absorbance of the control reaction (containing all reagents except test extract), and Asample is the absorbance of the test extract.

Synthetic antioxidant reagents, Butylhydroxytoluen (BHT) and L-ascorbic acid (each from Sigma), were used as positive controls and all tests were carried out in triplicate.

Results and discussion

Various plant species display resurrection capability. They can tolerate and survive extremes of vegetative desiccation, subsequently resuming normal cellular metabolism within a short period after water has become available again [14].

The development of protocol for in vitro multiplication offers great advantages. It makes the use of uniform material, taken from plants grown under controlled conditions, possible. This is extremely important when putative bioactive compounds are investigated. In addition, when the story goes for a rare and endangered species like *Haberlea*, this is obligatory prerequisite to preserve the natural biodiversity.

Resurrection behavior of in vitro regenerated plants

To proof that the in vitro developed *Haberlea* plants have the same "resurrection" capacity, we performed a special trial. After 3 months of acclimatization in pots under greenhouse condi-tions, the plants were subjected to drought by withholding of water along with plants obtained from natural habitats of the same species.

Desiccation in soil of in vitro developed plants proceeded similarly to that of the plants from natural habitats. Light symptoms of drying were visible within the first 10 days of water with-drawal. The severe drying appeared after two weeks in all groups. At the end of the treatment (3 weeks), all plants looked completely desiccated. Immediately after re-watering, the resurrection started and the plants recovered almost fully within one day. Normal growth was fully restored in three days both in the "native" (control) and in all three groups in vitro developed plants.

Screening for biologically active substances

To our knowledge, there is no report so far related to phytochemical investigation of extracts from *Haberlea rhodopensis*. We found that the extract contains three groups biologically active substances: flavonoids and tannins and polysaccharides. The leaves of in vitro developed plants contain about 30% more flavonoids calculated as quercetin than the leaves from natural habitats' plants (Table 1). One main phenol compound is detected in methanolic extract of the leaves which is under further investigation. Total tannins were also higher in the in vitro plants.

Table 1. *Flavonoid and tannin contents in methanol extracts of Haberlea rhodopensis plants and their DPPH activity*

Plant material from *Haberlea rhodopensis*	**Natural habitat plants**	**In vitro propagated cultivated plants**
Content of flavonoids (% Dry wt)*	0,26	0,37
Content of tannins (% Dry wt)*	6,42	7,35
DPPH radical scavenging activity* (%)	91,43	98,28

*Values are reported as means ± SD of three determinations

DPPH activity

Nowadays, a lot of different plant species are used as nutritional additives to add antioxidants to the organism to improve the immunity against many metabolic disorders connected to such diseases as neurodegeneration, cancer or diabetes mellitus [1]. Flavonoids, naturally occurring compounds, have recently been studied extensively for their antioxiant properties [6]. The DPPH radical scavenging method is a standard procedure applied to the evaluation of antiradical activity. Antioxidants react with DPPH, which is a stable free radical, and convert it to 1,1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazine. The degree of discoloration indicates the free-radical scavenging potentials of the antioxidant extracts or compounds. The evaluation of the antioxidative activity of methanol extracts of *Haberlea rhodopensis* showed stronger 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity then reference compounds – butylated hydroxytoluol and L-ascorbic acid (Table 1). The results of our experiments demonstrated that all of the extracts tested possess radical scavenging activity and indicated that in vitro propagated cultivated plants are more active than plants from natural habitats in the DPPH discoloration assay. We found higher correlation between total flavonoid content and free radical scavenging effect of the extracts measured by means of the DPPH discoloration assay than total tannins and scavenging activity.

Using this method, we found that the MeOH extracts of Haberlea rhodopensis leaves display strong antioxidative activity, which could be exploited as potential pharmaceuticals.

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