

NITRIC OXIDE-SCAVENGING ACTIVITY OF *IN VITRO* CULTURED BALKAN MEDICINAL AND AROMATIC PLANTS

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Abstract

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Methanolic extracts from the aerial and root parts of *in vitro* cultured *Artemisia alba* and the aerials of *in vitro* cultured *Clinopodium vulgare*, *Hypericum tetrapterum*, *H. richeri* and the endemic *H. rumeliacum* were evaluated for their nitric oxide scavenging capacities. Extracts of *H. rumeliacum*, *H. richeri* and *H. tetrapterum* exhibited remarkably higher activity (0.18, 0.17 and 0.97 mg.ml⁻¹, respectively) compared to *C. vulgare* and *A. alba* (SC₅₀ = 3.45, 2.93 and 2.62 mg.ml⁻¹, respectively). Vitamin C (a reference compound), exhibited activity of 0.26 mg.ml⁻¹. The presented results are indicative of the high therapeutic potential of the extracts derived from the *in vitro* cultured plants and are a good basis for further more detailed research.

Key words: *In vitro* culture, medicinal and aromatic plants, *Artemisia*, *Hypericum*, *Clinopodium*, NO-scavenging activity
Abbreviations: NO[•] – nitric oxide, SC – radical scavenging activity, HgCl₂ – mercuric chloride, BA – benzyladenine, MS – Murashige and Skoog, G5 – Gamborg, DW – dry weight, PGR – plant growth regulators, IBA – indole-3-butyric acid, TDZ – thidiazuron, Kin – kinetin, 2iP – 6-(α,α -dimethylallylamino)-purine

Introduction

Nitric oxide (NO[•]) has major involvement in a number of physiological processes such as regulation of blood pressure, platelet adhesion, neutrophil aggregation and synaptic plasticity in brain (Fang, 1997).

When present in excessive concentration, NO[•] combines with other oxidants to form reactive nitrogenous species with damaging effect on cellular DNA and proteins, leading to apoptosis, mutagenesis or carcinogenesis (Bishop and Cashman, 2003). Scavenging potential of preparations from *in vitro* cultured plants is worth studying, because such systems offer a standardized and constant supply of raw material

rich of bioactive components. The aerials of the medicinal plant *Artemisia alba* Turra have been traditionally utilized for treatment of digestive disorders (Rigat et al., 2007). Essential oil of the plant *in situ* (Radulović and Blagojević, 2010 and references cited within) and *in vitro* (Ronse and De Pooter, 1990; Danova et al., 2012a) has been studied for its chemical composition. It possesses strong spasmolytic and antimicrobial activities (Perfumi et al., 1999; Stojanovic et al., 2000). Meanwhile, scarce information exists about the non-volatile components of this species. Concerning their biological properties only anti-inflammatory activity has been reported so far (Stalińska et al., 2005; Talhouk et al., 2007). The wild basil *Clinopodium vulgare* L. is commonly

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used in Bulgarian folk medicine for the treatment of irritated skin, mastitis- and prostatitis-related swelling, as well as for some disorders accompanied by significant degree of inflammation (e.g. gastric ulcers, diabetes, and cancer). In addition, anti-inflammatory, strong free radical scavenging and antitumor activities of the aqueous extract of this plant have also been reported (Dzhambazov et al., 2002; Burk et al., 2009).

Phenolic and flavonoid compounds, naphthodianthrones (hypericin and pseudohypericin) and phloroglucinols (hyperforin and adhyperforin) are amongst the most important biologically active substances of the *Hypericum* species. These constituents possess anti-depressive, antitumor, antiviral and antibiotic activities. Hypericin has been widely studied for its antidepressant, antiviral action, and for its pro-oxidant phototoxic properties in the photodynamic cancer therapy (Karioti and Bilia, 2010). Some representatives of the evolutionally more developed *Hypericum* species possess the potential to produce an increased quantity of hypericin in comparison to the representatives from the more primitive sections of the genus. For example, *H. boissieri*, *H. barbatum*, *H. rumeliacum* (representatives of the more developed *Drosocarpium* section) may contain 2–4 fold higher amounts of hypericins than *H. perforatum* (Karioti and Bilia 2010). Surprisingly, the hypericin productivity *in vitro* has not been explored yet for representatives of other evolutionarily more developed sections of the genus except for the most explored *H. perforatum* and some other closely related species from the *Hypericum* or *Taeniocarpium* sections (Kirakosyan et al., 2004; Coste et al., 2011). Our previous research has led to the development of *in vitro* culture system of *H. rumeliacum* (*Drosocarpium* section), able to produce hypericin and pseudohypericin in higher amounts than other *Hypericum* species *in vitro* (Danova et al., 2012b). The aim of the present work was to perform a screening of the NO[•] scavenging activities of the methanolic extracts of *in vitro* cultivated *A. alba* and *C. vulgare*, chosen on an ethobotanical principle, and three less studied representatives of the widely explored *Hypericum* genus – *H. rumeliacum*, *H. richeri*, and *H. tetrapterum*.

Materials and Methods

Plant material - the *in situ* plant materials were from different origin (Table 1). Shoot cultures were initiated from surface sterilized (1 g.L⁻¹ HgCl₂) stem segments of the five species. After triple washing in sterile distilled water, the segments were placed in MS (1962) salts medium with G5 (1968) vitamins, supplemented with 0.5 mg.L⁻¹ BA, 20 g.L⁻¹ sucrose and 6.5 g.L⁻¹ agar. Then the induced axillary shoots were placed in the MS culture medium formula, supplemented with 30 g.L⁻¹ sucrose. Shoots were maintained at 25°C at a 16 h-photoperiod. Stock

Table 1
Origin of *in situ* plant materials

| Plant species | Location of the origin |
|---|---|
| <i>Artemisia alba</i> , Asteraceae | Commercial cultivar provided by Dr Ljuba Evstatieva |
| <i>Clinopodium vulgare</i> , Lamiaceae | Rila mountain |
| <i>Hypericum rumeliacum</i> , Guttiferae | The Rhodopes mountain |
| <i>H. richeri</i> , Guttiferae | Vitosha mountain |
| <i>H. tetrapterum</i> , Guttiferae | Western Stara Planina |

shoots, maintained under these conditions, were subjected to modifications of vitamin and PGR supplementation in order to achieve optimal growth, to avoid unfavorable hyperhydricity caused by BA supplementation, and to prolong the sub-culture period.

Extraction of the plant material – 150 mg DW of the aerial parts of *A. alba*, *C. vulgare*, *H. rumeliacum*, *H. richeri*, *H. tetrapterum* (*Taeniocarpium* section) and roots of *A. alba* *in vitro* were ground in a mortar with hot (64 °C) methanol and left to macerate for 20 minutes. Then samples were subjected to 30 minute-ultrasonic extraction. After centrifugation of the plant material and addition of fresh portions of methanol, the extraction procedure was repeated until the solvent discolored. The extracts were combined and evaporated under vacuum. The DW was determined after obtaining constant weight of the six extracts.

Determination of the (NO)[•] scavenging activity - the procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent (Ebrahimzadeh et al., 2010). Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions.

For the experiments, the method of Harput et al. (2011) was adapted to a micro-scale. Briefly, a strip plate, containing 100 µl of serial diluted plant dry extract (5 concentrations, n = 3) and 100 µl of freshly prepared 10 mM sodium nitroprusside, in phosphate buffer (0.1 mM, pH 7.4), was irradiated with fluorescent light (36 W) for 15 minutes. Then, 100 µl of fresh Griess reagent were added and the absorption of the resulting mixture was measured at 560 nm using Elisa strip reader STATFAX 303+. The radical scavenging activity (SC) was calculated using the formula:

$$SC [\%] = [(A_0 - A_n)/A_0] \times 100, \text{ where:}$$

A_0 is the absorption of the control sample (without extract); A_n is the absorption of the sample containing n concentration of the extract.

L-ascorbic acid (Vitamin C) was used as a positive control. The NO[•] scavenging activities of the samples were expressed as the concentrations (mg/ml dry extract), able to inhibit 50% of the free radicals (SC₅₀).

Results and Discussion

Establishment of in vitro collection of the studied species - the medium composition was modified to obtain slow growing *in vitro* culture for each of the species (Table 2).

Table 2
Maintenance conditions for the studied plants at the *in vitro* collection of the Institute of Organic Chemistry with Centre of Phytochemistry, BAS

| Plant species | Growth conditions for slow growing cultures, sub-culture period |
|-----------------------|---|
| <i>A. alba</i> | G5 vitamins, 30 g.L ⁻¹ sucrose, 3 months (m) |
| <i>C. vulgare</i> | 30 g.L ⁻¹ sucrose, 12 m |
| <i>H. rumeliacum</i> | 30 g.L ⁻¹ sucrose, 3 m |
| <i>H. richeri</i> | MS medium formula, 0.2 mg.L ⁻¹ BA, 0.1 mg.L ⁻¹ IBA, 30 g.L ⁻¹ sucrose, 3 m |
| <i>H. tetrapterum</i> | MS medium formula, 30 g.L ⁻¹ sucrose, 7 m |

All media are based on the MS salts composition

To our knowledge, this is the first report about tissue culture studies on *C. vulgare*. A sub-culture period of 12 months was possible for this species in the basic MS formula, thus achieving slow growth maintenance without the need of other modifications. *H. tetrapterum* was the second species with a longer sub-culture period in the same medium. Surprisingly, unlike the two other *Hypericum* species and when compared with *A. alba* and *C. vulgare*, supplementation of PGR to the culture medium was necessary to achieve and maintain growth and development of *H. richeri in vitro* (Table 2). Inoculation of the stem segments in PGR-lacking media (disregarding the MS or G5 vitamin supplementation) led to retarded growth, lack of axillary shoots formation and subsequent necrosis of the whole explants with prolonging cultivation period over a month. In a quite recent work (Coste et al., 2012) the authors report for the first time on *in vitro* culture initiation, micro-propagation and cryopreservation of *H. richeri* ssp. *transsilvanicum* (endemic to Romania). The authors established that BA was superior for *in vitro* multiplication as compared with TDZ, Kin and 2iP. Though the lack of cytokinins resulted in significantly slower growth *in vitro*, unlike the results of the present report, the authors achieved multiplication also in the PGR-free MS medium within 6 week period.

NO[•] scavenging activity of the obtained methanolic extracts - as far as we are concerned, the NO[•] scavenging activity

Table 3
NO[•] scavenging activity of the methanolic extracts

| Tissue culture sample | SC ₅₀ ^a mg/ml |
|------------------------------------|-------------------------------------|
| <i>C. vulgare</i> aerial parts | 3.45 ^c |
| <i>A. alba</i> aerial parts | 2.93 ^{bc} |
| <i>A. alba</i> roots | 2.62 ^b |
| <i>H. rumeliacum</i> aerial parts | 0.18 ^a |
| <i>H. richeri</i> aerial parts | 0.17 ^a |
| <i>H. tetrapterum</i> aerial parts | 0.97 |
| Vitamin C | 0.26 |

Same letters denote non-significant differences ($p < 0.01$)

of tissue culture material derived from the studied species has not been studied before. Among the analyzed extracts, those obtained from the *Hypericum* species displayed remarkable activity (Table 3). Particularly, the samples from *H. rumeliacum* and *H. richeri* showed scavenging capability 1.5-fold higher than that of the referent compound, vitamin C. Meantime, the activity of the sample from *H. tetrapterum* was almost 4 times lower than that of vitamin C, but still higher regarding the rest of the studied samples (from *C. vulgare* and *A. alba*). Usually, radical scavenging activities of plant extracts are attributed to their total amounts of phenolics and flavonoid compounds (Prakash et al., 2007). However, we have already reported that methanolic extracts obtained from *H. rumeliacum* (the species with high NO[•] scavenging activity) and *H. tetrapterum* (the species with moderate activity) *in vitro* possess similar quantities of total phenols and flavonoids (Danova et al., 2012b). Hence, the difference in their NO[•] scavenging potential may be due to some particular components, for example the hypericins. Their level in the sample of *H. rumeliacum* is strikingly higher as compared with that of *H. tetrapterum* (Danova et al., 2012b). Further research on the phytochemical composition of the extracts of the studied *Hypericum* species will elucidate the potential synergism and/or antagonism between the different components, underlying the differences in NO[•] scavenging of the high and low hypericin producing species. In conclusion, our results show that more detailed study of the methanolic extracts obtained from *H. rumeliacum* and *H. richeri* is needed that should be directed to identification of individual components or groups of them responsible for the NO[•] scavenging potential. Also, the samples of all the species should be evaluated for their ability to scavenge other radicals spread in the living organisms.

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