Rosmarinic Acid Accumulation and Antioxidant Potential of *Dracocephalum moldavica* L. Cell Suspension Culture

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Abstract

*Dracocephalum moldavica* L. (Lamiaceae) is known for its medicinal properties; however greater yields can potentially be achieved by in vitro cultivation. A cell suspension culture of *D. moldavica* L. (Lamiaceae) derived from root-derived callus was established in liquid MS medium supplemented with 2,4-D 0.5 mg/l and BAP 0.2 mg/l. The biomass and rosmarinic acid (RA) content were analyzed during the 15-day growth cycle of the culture. The highest fresh and dry weight (14.29 g/flask and 1.14 g/flask, respectively) and RA level (27.2 mg/g DW) were reached at day 12 of culture. Methanolic extracts of the culture were assayed for total phenolic content using the Folin-Ciocalteau method, and antioxidant activities using three in vitro tests: ABTS radical scavenging, ferric ion reduction (FRAP) and lipid peroxidation (LPO). RA content and antioxidant potential were found to be higher in cell suspension culture than in root-derived callus. The cell suspension culture also exhibited higher concentrations of RA and ABTS radical scavenging activity than those of the aerial parts of six-month-old field-grown plants of *D. moldavica*. The overall results show a significant correlation between antioxidant activity, total phenolic content and RA content of the examined extracts. The study presents for the first time the use of cell cultures of *D. moldavica* for production of therapeutically-valuable metabolites. Our results suggest that the obtained culture could be considered as a potential source of rosmarinic acid, a compound known for its strong antioxidant activity.

Keywords: antioxidant activity, *D. moldavica*, phenolic content, undifferentiated in vitro culture, UPLC

Introduction

*Dracocephalum moldavica* L. is an annual aromatic plant of the Lamiceae family. This plant is native to Siberian and Central Asia, it also grows in Egypt, China and Mongolia at altitudes of up to 2700-3100 m above sea level (Nikitina et al., 2008). *D. moldavica* is naturalized in Eastern and Central Europe (Kakasy et al., 2006). Its aerial parts have been widely utilized in traditional medicine for the treatment of stomach and liver disorders as well as headache and toothache (Dastmalchi et al., 2007). Studies indicate that *D. moldavica* extracts have anti-*Helicobacter pylori* activity (Ghannadi et al., 2004) as well as sedative, analgesic and wound-healing properties (Sultan et al., 2008). The main substances responsible for the therapeutic effects of the *D. moldavica* aerial parts are phenolic acids (mainly rosmarinic, caffeic and ferulic acids), flavonoids and essential oil components (Kakasy et al., 2006; Popova et al., 2008; Sultan et al., 2008). Flavonoids and phenolic acids are known to be beneficial to human health and disease prevention thanks to their strong antioxidant activity, capacity to scavenge free radicals and ability to neutralize reactive oxygen species (Saxena et al., 2012). One of the most prominent phenolic compounds in *D. moldavica* is rosmarinic acid. RA is known to have antioxidant, antimicrobial, anti-inflammatory and antiviral properties (Bais et al., 2002; Ly et al., 2006). There are reports on RA activity against *Herpes simplex* virus (Sanches-Medina et al., 2007) and *Human immunodeficiency* virus (HIV-1) (Astani et al., 2012). Other authors have reported RA to have hepatoprotective (Lima et al., 2006) and cardioprotective properties (Psotova et al., 2005) and for it to play a role in the prevention of Alzheimer’s disease (Hamaguchi et al., 2009). There is a need to produce large...
amounts of the valuable compound, and biotechnological methods represent attractive and cost-effective alternatives to field cultivation. Rosmarinic acid has been synthesized in callus and cell suspension cultures from a range of plant species including Ocimum sanctum (Hakkim et al., 2011), Coleus blumei (Qian et al., 2009) or Salvia miltiorrhiza (Wu et al., 2016).

The present study describes the establishment of a D. moldavica cell-suspension culture and determines its ability to biosynthesize RA. It compares RA production in cell suspension culture, callus culture and in field-grown D. moldavica plants. The antioxidant potential of these plant materials was investigated in vitro using three different tests. The total content of phenolic compounds, expressed in milligrams gallic acid equivalents per gram dry weight of methanolic extract in D. moldavica plant materials, was also determined.

Materials and Methods

Plant material

Dracocephalum moldavica seeds were obtained from the Botanical Garden of Lublin (Poland). They were sterilized with 2% sodium hypochlorite solution for two minutes and after rinsing with sterile distilled water, the seeds were germinated in the dark at 26 °C on MS (Murashige and Skoog, 1962) agar (0.7%) medium supplemented with sucrose (30 g/l), 0.02 mg/l kinetin and 1.0 mg/l giberellic acid. After germination, the seedlings were transferred into light conditions (16 hour light / 8 hour dark; cool fluorescent lamps; 40 µm m⁻²/s⁻¹). The roots of two-week-old seedlings were used for callus induction.

The seeds were also used to obtain field-grown D. moldavica plants. After six months, their aerial parts were harvested and used in this study. Voucher specimens have been deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź.

Establishment of callus and cell suspension culture

Callus of D. moldavica from the roots of two-week-old seedlings was obtained on solid agar (0.7%) MS medium with 0.5 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.2 mg/l BAP (6-benzylaminopurine). The callus was subcultured at two-week intervals. For initiation of callus suspension culture, about 2 g fresh weight (FW) of callus from passage 10 was transferred into 80 ml of MS liquid medium supplemented with the same concentration of growth regulators as in the solid medium. The culture was maintained in 300 ml Erленmeyer flasks on a rotary shaker (100 rpm) and subcultured every 14 days using inoculum of about 500 mg fresh weight per flask. The cultures were kept at 26 ± 2 °C under a 16-hour photoperiod provided by cool white fluorescent lamps (approximately 40 µm m⁻²/s⁻¹).

Analysis of cell suspension culture on growth and RA content

Every three days for 15 days, three flasks of cell culture were harvested, and the fresh and dry weights (DW) and RA content were determined. All measurements were repeated three times in three successive subcultures (16-18).

Extraction and determination of RA

Lyophilized and powdered plant materials (i.e. callus, cells from suspension culture and aerial parts of D. moldavica six-month-old plants grown in the field; 150 mg for UPLC analysis and 1 g for antioxidant studies), were extracted three times with 70% MeOH (25 ml) for 10 minutes at room temperature using an ultrasonic bath (Janicaks et al., 1999). After filtration, the extracts were combined and evaporated to dryness under reduced pressure.

UPLC analysis of RA

Analysis was performed using an Acquity Ultra Performance Liquid Chromatograph (Waters) equipped with a photodiode array, a UV–visible detector and an autoinjector, according to Lamien-Meda et al. (2010). Separation was performed on a Shield RP C18-column (2.19 × 100 mm, 1.7 lm pore size) at a column temperature of 35 °C. The solvent gradient used in this study comprised 1% acetic acid : acetonitrile 85:15 v/v as solvent A, and methanol as solvent B. The flow rate was 0.43 ml/min and wavelength used for RA detection was 360 nm. RA was identified by comparing its UV spectrum, retention time (3.8 min) and LC-MS/MS (liquid chromatography-mass spectrometry) data with those of a standard compound (ChromaDexTM). The detailed procedure for the identification and quantification of RA is described in a previous paper (Weremczuk-Jeżyna et al., 2013).

Total phenolic content

Total phenolic content was estimated using a colorimetric method based on Folin–Ciocalteu (POCH) reagent, as described by Singleton et al. (1965). Absorbance at 765 nm was measured after 30 minutes of incubation at room temperature. Quantification was performed against a standard calibration curve for gallic acid. The results were expressed as gallic acid (Sigma-Aldrich) mg equivalents per gram of dry extract (GAE mg/g DW of extract).

Antioxidant Assays

FRAP (ferric ion reducing activity)

The FRAP was determined according to Pulido et al. (2000) methods with some modifications. The absorbance was measured at 595 nm. The antioxidant activity was evaluated against a calibration curve of 0-2000 µM for a known FRAP value: ferrous sulphate. The detailed procedure is described by Grzegorczyk-Karolak et al. (2015a). Ferric reducing power was expressed in µM Fe(II)/g/DW of extract.

ABTS radical scavenging

The antioxidant activity was also determined using the ABTS radical cation decolourisation test as described by Grzegorczyk-Karolak et al. (2015b). The absorbance was measured after 10 minutes at 734 nm. The results were expressed as EC₅₀, the concentration of sample at which 50% of maximum scavenging activity was recorded.

Linoleic acid peroxidation inhibition

The experiment was carried out according to Choi et al. (2002) with some modifications. The absorbance of the reaction mixture was measured at 532 nm. The percentage of linoleic acid peroxidation inhibition was calculated using the following equation: % inhibition = Abscontrol–Abssample/Abscontrol)×100/Abssample, where Abscontrol includes methanol instead of sample, Abssample – absorbance of sample where linoleic acid solution was replaced by methanol.
Statistical analysis
All results were calculated as means ± standard error. The means were compared using the Kruskall-Wallis test, EC50, and the correlation coefficients (r) between the antioxidant assay, the total phenolic content and RA content were calculated using MS-Excel software. Any differences were regarded as significant at p < 0.05.

Results and Discussion

Root-derived friable callus was used to initiate the D. moldavica of cell suspension culture. The cell culture was grown in MS liquid medium supplemented with 2,4-D 0.5 mg/l and BAP 0.2 mg/l. UPLC examination found rosmarinic acid to be the major phenolic compound (retention time 3.8 min) of the methanolic extract. RA content and biomass on the basis of fresh and dry weight were recorded at intervals of three days during the 15-day culture period. As shown in Fig. 1a, the culture was in a lag phase for the first three days, before growing exponentially until day 12 of the culture period. Both fresh and dry biomass demonstrated similar growth curves. The highest fresh weight (14.29 g/flask; 28 times the inoculated value) and dry weight (1.14 g/flask; 23 times), were achieved on day 12. Following this, biomass began to decline and brown, with cell death observed at day 15, which may be due to the oxidation of phenolic compounds, the consumption of nutrients and lack of oxygen in the medium (Kajani et al., 2012; Saharoo et al., 2016). The level of RA in the cell suspension culture was not affected for the first nine days of cultivation (Fig. 1b). A fast increase in RA content was observed between days 9 and 12 of the culture period, when the compound concentration enhanced from 8.4 mg/g to 27.2 mg/g DW; about 3.7-fold greater than the inoculum value. The results presented in Fig. 1 indicate a correlation between biomass and RA production because both maximum growth and RA content were reached at the same time of growth cycle (day 12). Other authors have also shown similar patterns of RA accumulation in plant cell cultures. For example, in cell suspension of Eringium planum, the highest RA content was observed at the end of the linear growth phase, and this decreased when the culture reached the stationary phase (Kikowska et al., 2012). Similar observations were made for cell suspension cultures of Mentha piperita (Krzyżanowska et al., 2012).

As shown Fig. 2, the RA content in the D. moldavica cell suspension culture was 48% higher than in callus culture, grown on agar solidified MS medium with the same composition. The difference may be due to the consistency of medium and shear stress during agitations (Zhong et al., 1995). RA level in suspension culture was also found to be 26% higher than in the aerial parts of intact six-month-old field-grown D. moldavica and about twice the RA content detected by Fattahi et al. (2013) in D. kotschyi plants. The production of high amounts of rosmarinic acid has been described earlier in cell cultures of plants belonging to the Lamiaceae family, for example with suspension cultures of S. officinalis accumulating about 20 mg/g DW (Grzegorzczk et al., 2005) and S. miltiorrhiza about 28 mg/g DW of RA (Wu et al., 2016). High rosmarinic acid content was also found in O. basilicum cell culture (10 mg/g DW), this level being 10 times greater than that observed in the callus of the plant (Kintzios et al., 2003).

Antioxidant activity and total phenolic contents
A comparative analysis of the antioxidant potential of methanolic extracts from cell suspension, callus cultures and shoots of intact plants of D. moldavica is presented in Table 1. Three in vitro tests based on hydrogen donation or electron donation were used to fully characterize the antioxidant properties of the analyzed extracts. The free radical scavenging
activity was evaluated by ABTS assay and results were expressed as \( EC_{50} \) values. The methanolic extract of \( D.\ moldavica \) suspension culture was found to have stronger antiradical activity \( (EC_{50} = 39.64 \ \mu g/ml) \) than extracts of the cell suspension \( (EC_{50} = 61.75 \ \mu g/ml) \) or aerial parts \( (EC_{50} = 42.82 \ \mu g/ml) \). The FRAP assay was used to measure the capacity of the extracts to reduce iron ions \( (Fe^{2+} \rightarrow Fe^{3+}) \), with the results expressed as \( \mu mol \) ferrous ion equivalents per gram of extract dry weight. The extract from the cell suspension culture was found to have greater reductive potential than the callus extract. However, the greatest reducing activity was found for the extract from the aerial parts of \( D.\ moldavica \) plants (Table 1).

The inhibition of lipid peroxidation test (LPO) was also performed to evaluate the antioxidant properties of the \( D.\ moldavica \) extracts. Although the callus extract was found to have the least ability to inhibit oxidation of linoleic acid, the other extracts were also weak inhibitors of LPO peroxidation (max. 14%) (Table 1). These results suggest that the analysed \( D.\ moldavica \) extracts utilize rather the SET (single electron transfer) reaction mechanism than HAT (hydrogen atom transfer). The antioxidant activity of methanolic extracts from hairy roots of \( D.\ moldavica \) has been examined in a previous work (Weremczuk-Jeżyna et al., 2013). However, as different tests were used, it is difficult to directly compare the two sets of results. Various phenolic compounds, including RA, are known to take part in antioxidant activities such as free radical scavenging, acting as reducing agents and protecting cells against lipid peroxidation (Mushtaq et al., 2014). The greatest total phenolic contents (TPC) of \( D.\ moldavica \) extracts, as determined by the Folin-Ciocalteau method and expressed in milligram gallic acid equivalents per gram dry weight of sample, were found in the extract from aerial parts of field-grown \( D.\ moldavica \) plants (110.1 mg GAE/g DW); this amount being about 13% higher than that found in cell suspension culture extract and 41% higher than in callus culture extract (Fig. 2). The correlation coefficients \( r \) between the antioxidant activity of the analysed extracts, total phenolic \( (0.89 \) for ABTS, 0.95 for FRAP and 0.99 for LPO test) and RA contents \( (0.86 \) for ABTS, 0.56 for FRAP and 0.94 for LPO assay) indicate that phenolic compounds and RA are the most active components of \( D.\ moldavica \) extract with regard to its antioxidant potential. The antioxidant activity of RA can be attributed to its chemical structure, characterized by the presence of two catechol rings connected with phenolic acid (Brewer, 2011).

### Conclusions

Our results indicate that it is possible to obtain a large biomass \( (178.6 \ \text{g/l}) \) with a high level of RA \( (27.2 \ \text{mg/g DW}) \) in a relatively short time \( (12 \ \text{days}) \) using \( D.\ moldavica \) cell suspension culture. This cell suspension culture is therefore a good candidate for \textit{in vitro} RA production. In addition, the ABTS assay of the methanolic extract of the \( D.\ moldavica \) cell suspension culture found it to have significant \textit{in vitro} radical scavenging potential, suggesting that it may have beneficial value in the elimination of free radicals and could play a significant role in protecting cells from oxidative stress.

### References


### Table 1. Antioxidant capacities of \( D.\ moldavica \) methanolic extracts

<table>
<thead>
<tr>
<th>Assay</th>
<th>Callus culture</th>
<th>Suspension culture</th>
<th>Aerial parts of plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ABTS (EC50 ( \mu g/ml ))</td>
<td>61.75 ± 8.08a</td>
<td>39.64 ± 5.55b</td>
<td>42.82 ± 0.68b</td>
</tr>
<tr>
<td>2FRAP (( \mu mol ) Fe(II)/g DW)</td>
<td>1018.66 ± 30.92a</td>
<td>1617.96 ± 18.72b</td>
<td>1739.36 ± 54.37c</td>
</tr>
<tr>
<td>3LA peroxidation (% inhibition)</td>
<td>4.14 ± 0.02a</td>
<td>13.69 ± 0.03b</td>
<td>13.69 ± 0.04b</td>
</tr>
</tbody>
</table>

The means with the same letter do not differ statistically according to Kruskall-Wallis test (p ≤ 0.05).

1 \( EC_{50} \) – amount of extract needed to decrease of the initial ABTS concentration by 50%

2 Ferric reducing antioxidant power

3 Inhibition ratio of LA peroxidation after incubation with extract concentration of 100 \( \mu g/ml \)


