ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 22(2) 2023, 45–59
https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 https://doi.org/10.24326/asphc.2023.4817
ORIGINAL PAPER
Accepted: 20.09.2022
First published online: 24.04.2023
Issue published: 28.04.2023

THE OPTIMIZATION GROWTH OF *Dracocephalum forrestii* IN RITA® BIOREACTOR, AND PRELIMINARY SCREENING OF THE BIOLOGICAL ACTIVITY OF THE POLYPHENOL RICH EXTRACT

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ABSTRACT

Dracocephalum forrestii is a medicinal plant growing in China. The aim of the present study was to large-scale cultivation of *D. forrestii* transformed shoots in a temporary immersion system based on previously-optimized Murashige and Skoog (MS) medium supplemented with 0.5 mg/L N-benzyl-9-(2-tetrahydropyranyl)-adenine (BPA) and 0.2 mg/L indole-3-acetic acid (IAA) and physical (under blue LED) conditions. Shoot proliferation, and biomass and secondary metabolite accumulation in the shoots were assessed after a three-week growth period in a RITA® bioreactor. The levels of polyphenols in four types of extract (hydromethanolic extracts – mixtures with a 20%, 50%, and 80% methanol content and infusion) were determined using high-performance liquid chromatography (HPLC). Within three weeks, the culture increased its biomass 283-fold, with a proliferation ratio of 40.5 shoots or/and buds per explants. The most efficient solvent for extraction of phenolic compounds from raw material turned out to be 80% methanol solution; the highest polyphenol content was 40 mg/g DW (dry weight) with acacetin rhamnosyl-trihexoside (12.97 mg/g DW) and rosmarinic acid (10.68 mg/g DW) predominating. The intensive growth of the biomass of the culture allowed 570 mg of polyphenolic compounds to be obtained per liter of the medium. The antioxidant potential of extract of *D. forrestii* shoots was evaluated using three free radical-scavenging tests, and the inhibition of lipid peroxidation assay. In the study, the cytotoxic, antibacterial and antifungal potentials of the extract were also determined.

Key words: acacetin and apigenin glucoside, biological potential, large-scale cultivation, rosmarinic acid, RITA® bioreactor, transformed root culture

INTRODUCTION

Dracocephalum forrestii W.W. Smith, a member of the Lamiaceae, is a perennial plant growing in Yunnan (China) at around 2300–3500 m above sea level. The aerial parts of the plant have long been used in Tibetan folk medicine for its astringent, diuretic, and antipyretic properties [Li et al. 2007, Li et al. 2009]. *D. forrestii* extract contains a wide spectrum of bioactive compounds including phenolic acids (chlorogenic acid, rosmarinic acid, salvianolic acid B), flavonoids (luteolin, apigenin, narirutin) and their glucosides, as

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well as triterpenoids, lignans, and arylglycerol glucosides [Li et al. 2009, Li et al. 2006, Weremczuk-Jeżyna et al. 2018]. Many of the phenolic compounds present in *D. forrestii* shoots, including rosmarinic acid and salvianolic acid B, possess strong antioxidant and anti-inflammatory properties. These active metabolites contain hydroxyl groups that can exert free radical-scavenging activity by donating hydrogen atoms. Additionally, it has been reported that rosmarinic acid and salvianolic acid B exert their antioxidant activity by regulating the expression of antioxidant enzymes [Nadeem et al. 2019, Xiao et al 2020]. Rosmarinic acid has been found to inhibit tumour formation by interfering with the signaling pathways involved in the up-regulation of metastasis such as extracellular signal-regulated kinase (ERK) or kinase microtubule-affinity regulating kinase (MARK)-4, thus reducing cancer cell growth and inducing apoptosis [Anwar et al. 2020, Scheckl et al. 2008]. It is also known that rosmarinic acid and salvianolic acid B inhibit the growth of various Gram-negative and Gram-positive bacteria and some pathogenic fungi [Abedini et al. 2013, Zhang et al. 2018b]. Other compounds from *D. forrestii* also demonstrate biological potential. Many reports showed that apigenin and acacetin and their glucosides possess antioxidant properties, inhibit tumour cell migration and induce the immune response [Yan et al. 2017, Kang et al. 2020, Vassallo et al. 2016]. Despite the interesting pharmacological potential, a small number of studies on the medical properties of *D. forrestii* have been reported so far. The rarity of this plant makes it impossible to harvest it on a large scale for testing.

This problem can be overcome by the use of *in vitro* plant cultures. In recent years, studies have shown that, after the optimization of growth conditions, a large number of homogenous plants capable of producing bioactive metabolites can be obtained in a short time by micropropagation [Espinosa-Leal et al. 2018]. However, for *in vitro* cultivation to be profitable, it must be carried out on a large scale using bioreactor systems. Many reports have shown that the growth conditions offered by plant bioreactors e.g., transfer of nutrients or better gas exchange allow higher multiplication rates and greater biomass growth [Georgiev et al. 2014a, Zhang et al. 2018a]. For shoot cultures sensitive to continuous immersion and mechanical mixing, the most commonly-used type of bioreactor is the temporary immersion system (TIS). TISs offer periodical immersion of the culture and provide adequate oxygen transfer, thus minimalizing stress conditions [Georgiev et al. 2014b]. Although culture in various bioreactor types (Plantform, Rita and nutrient sprinkle bioreactor) significantly increased the growth of *D. forrestii* shoot cultures compared with tube culture, the RITA® (Récipient à Immersion Temporiaire Automatique, VITROPIC, France) bioreactor offered the best conditions for shoot growth and phenolic compound production, especially rosmarinic acid [Weremczuk-Jeżyna et al. 2020].

Several factors, including nutrients and physical factors, influence the accumulation of biomass and secondary metabolites *in vitro*. Our previous studies showed that the key factor in the accumulation of flavonoid glucosides in transformed *D. forrestii* shoots was the choice of light conditions. Among the various tested light sources, blue LEDs significantly increased the concentration of flavonoid glucosides (by about 89%) and phenolic acids (by about 20%) compared to traditionally-used fluorescent lamps [Weremczuk-Jeżyna et al. 2021]. However, the type and content of compound regulators in the medium was also found to be important in optimizing the productivity of the *D. forrestii* shoot culture [Weremczuk-Jeżyna et al. 2019].

The current study aimed at optimizing the production of phenolic compounds in *D. forrestii* shoots in large-scale *in vitro* cultivation conditions. As the study partially follows on previous findings [Weremczuk-Jeżyna et al. 2019, 2020, 2021], the cultures were carried out in RITA® bioreactor under blue LED conditions. The present experiment, for the first time, compares the phytochemical profile of various types of extract (hydromethanolic mixtures with 20%, 50%, and 80% alcohol content and infusion) to obtain the most efficient polyphenol extraction procedure. *D. forrestii* is used in traditional medicine, and so far there are no report on biological studies for this species. Therefore, the selected extract of plant material obtained from this culture was also assessed for its antioxidant, antimicrobial and cytotoxic activity.

MATERIAL AND METHODS

The transformed shoot culture in bioreactor. The transformed shoots of *D. forrestii* were cultivated in a RITA® temporary immersion system. The bioreactor contained 250 mL of MS nutrient medium [Murashige and Skoog 1962] with 0.5 mg/L BPA, and 0.2 mg/L IAA. Dosage of the liquid medium was controlled using a DT4.4 pressure pump (Becker, Germany) with a capacity of 4.2 m^3/h and a pressure of 1000 mbar. In the bioreactor, a growth medium was supplied to the shoot culture for 10 min every 80 min. Shoot fragments (7–8) about 4 cm in length with 3–4 nodal segments were used as explants. The biomass of inoculum was cir. 0.1 g of fresh weight (FW), i.e., 0.002 g of dry weight (DW). The transformed shoots of *D. forrestii* were cultured in the growth chamber at 26 ±2°C under a 16 h photoperiod provided by LED lamps emitting blue light (430 nm). Spectral characterization of the tested LED lamps was made using a BTS256-LED Tester (Gigahertz-Optik, Germany). After three weeks, the proliferation ratio, FW and DW of the culture (g/bioreactor) were determined. The growth index (GI) of FW and DW was calculated according to the equation below:

$$GI = \frac{\text{final biomass} - \text{initial biomass}}{\text{initial biomass}}$$

Extraction and estimation of phenolic compounds by HPLC. Lyophilized and pulverized plant material (100 mg) were first extracted with 15 mL chloroform using a UD-20 ultrasonic disintegrator (15 mL). After filtration, the defatted samples were extracted for 15 min with a mixture of methanol and water at methanol concentrations of 20%, 50%, and 80%. Extractions were performed for 15 min at 40°C in an ultrasonic bath with 25 mL of solvent and then twice in 10 mL. The infusion was prepared by flooding the plant material with 25 mL of water at 85–90°C and allowing it to cool (the experiment was repeated three times). The obtained extracts were combined and evaporated to dryness, and the residue was dissolved in methanol (2 mL) and centrifuged (18 000 rpm, 3 min). The supernatant was analyzed quantitatively by HPLC. For the analysis, a modified method described by Grzegorczyk-Karolak et al. [2022] was used.

The HPLC analyses were performed on Waters equipped with a binary HPLC pump (Waters 2545), a diode array detector (Waters 2998), and an autosampler (Waters 2767). The separations were carried out on a C18 OBD column (4.6 × 100 mm) with a particle size of 5 µm. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) with an elution profile as follows: 0–1 min (5% B); 1–10 min (5–10% B); 10–11 min (10–15% B); 11–40 min (15–20% B); 40–41 min (20–50% B); 41–42 min (50–5% B), 42–48 min (5% B). All gradients were linear. The flow rate was 1.6 mL/min, and the injection volume was 4 µL. The UV spectra were recorded over a range of 190–700 nm, chromatograms were acquired at 325 nm. The compounds were identified by comparison of their retention times and UV spectra with those of the standard compounds. The standard phenolic compounds including chlorogenic acid, caffeic acid, and rosmarinic acid (RA) were purchased from Sigma-Aldrich (Germany) and apigenin-7-*O*-glucoside from Extrasynthese (France). The compounds lacking pure standards were quantified according to the calibration curve of similar standards. The results are present in milligram per gram (mg/g) DW and calculate into productivity, which is expressed in milligram per liter (L) of growth medium.

Investigation of biological properties. Antioxidant analysis. The activity of the hydromethanolic extract against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to Grzegorczyk-Karolak and Kiss [2018]. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay was carried out as described by Grzegorczyk-Karolak et al. [2015], while superoxide radical scavenging was based on the capacity of the plant extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) [Grzegorczyk-Karolak and Kiss 2018]. The results of all antiradical assays were presented as EC_{50} (µg/mL), i.e., the concentration of the sample which inhibits free radicals by 50%.

The extent of lipid oxidation was determined according to the 2-thiobarbituric acid reactive substance (TBARS) assay, as described by Grzegorczyk-Karolak and Kiss [2018]. The plant extract was added to the reaction mixture at a concentration of 100 µg/mL. The absorbance of samples was recorded at 532 nm.

TBARS antioxidant activity was described by percentage inhibition, defined as:

$$\% \text{ inhibition} = \left(\frac{\text{control absorb.} - \text{sample absorb.} - \text{extract absorb.}}{\text{control absorb.} - \text{extract absorb.}}\right) \times 10$$

In all antioxidant assays, the absorbance was measured spectrophotometrically using a vis Spectrophotometer (Beijing Rayleigh Corp., Beijing, China). Butylated hydroxyl toluene (BHT) was used as the reference.

Cytotoxicity analysis. The mouse fibroblasts L929 (LGC Standards, Middlesex, UK), human HeLa (CCL-2, ATCC, Manassas, USA) cervix adenocarcinoma epithelial cells, human AGS (CRL-1739, ATCC, Rockville, MD) gastric adenocarcinoma epithelial cells and human LoVo (ATCC, Manassas, USA) colon cancer epithelial cells were cultivated in 25 cm^2 tissue culture flasks in RPMI-1640 medium (Biowest, France) supplemented with 10% heat-inactivated fetal bovine serum – FBS (Biowest, France) and antibiotics: penicillin (100 U/mL) and streptomycin (100 µg/mL, Biowest, France) under standard conditions (37°C, 5% CO_2). The cell viability was calculated for four experiments, including three repeats for each compound. Complete RMPI-1640 medium (cRPMI) was used as a positive control (C+) of cell viability (100% viable cells) and 0.03% H_2O_2 as a negative control (C−) of cell viability (100% dead inactive cells). The metabolic activity of the L929, AGS, HeLa, and LoVo cells was tested after the application of extracts obtained from *D. forestii* extract at the concentration of 0.1, 0.25, 0.5, 1.25 2.5, and 5 mg/L according to Krzemińska et al. [2022]. Cell metabolism was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay recommended by the Food and Drug Administration (FDA) and the International Organization for Standardization (IOS), as described previously [Kamizela et al. 2019].

Antimicrobial activity. The antimicrobial assay was performed using reference bacterial strains from the American Type Culture Collection (ATCC, Manassas, USA), including the Gram-positive *Staphylococcus aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 12228, the Gram-negative strains *Escherichia coli* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853, as well as the fungal strains *Candida albicans* ATCC 10231 and *Candida glabrata* ATCC 2001. Antimicrobial properties were determined by broth microdilution assay according to European Committee on Antimicrobial Susceptibility (EUCAST) recommendations, as described previously [Weremczuk-Jeżyna et al. 2021]. The antimicrobial activity was expressed in milligram per milliliter (mg/mL) of DW extract used. The activities of samples were evaluated based on their minimal inhibitory concentrations (MIC), minimal bactericidal concentrations (MBC), or minimal fungicidal concentrations (MFC). MIC was defined as the lowest concentration resulting in total growth inhibition. MBC was determined by collecting 10 µL of the culture from each well with no visible growth of microorganisms and plating this onto the surface of the culture medium; a lack of bacterial growth following incubation for 24 h at 37°C indicated bactericidal activity. All tests were performed in three independent experiments. Amphotericin B and gentamicin were used as standard antimicrobials.

Statistical analysis. All comparisons were performed using the Kruskal-Wallis test. Data are presented as mean values ± standards error (SE). Statistical significance was accepted at a p-value < 0.05. All statistical analyses were performed using STATISTICA 12 PL software (Stat Soft, Poland).

RESULTS

Cultivation of transformed shoots in bioreactor. In this study, transformed shoots of *D. forrestii* were cultured in MS liquid medium containing 0.5 mg/L N-benzyl-9-(2-tetrahydropyranyl)-adenine (BPA) and 0.2 mg/L indole-3-acetic acid (IAA) under blue LEDs. These conditions have been found to offer optimal growth and secondary metabolite production by *D. forrestii* shoot culture [Weremczuk-Jeżyna et al. 2019, 2021]. For increasing the scale of propagation, the RITA® bioreactor system was used.

After three weeks of growth of *D. forrestii* shoots in the RITA® bioreactor,under blue LED light, a high multiplication rate was observed, with a mean number of 40.5 shoots or/and buds formed per explant. The biomass of obtained shoots was mean of 28.34 g fresh weight (FW) and 3.54 g dry weight (DW) per bioreactor (Fig. 1A). Also, the *D. forrestii* shoots demonstrated

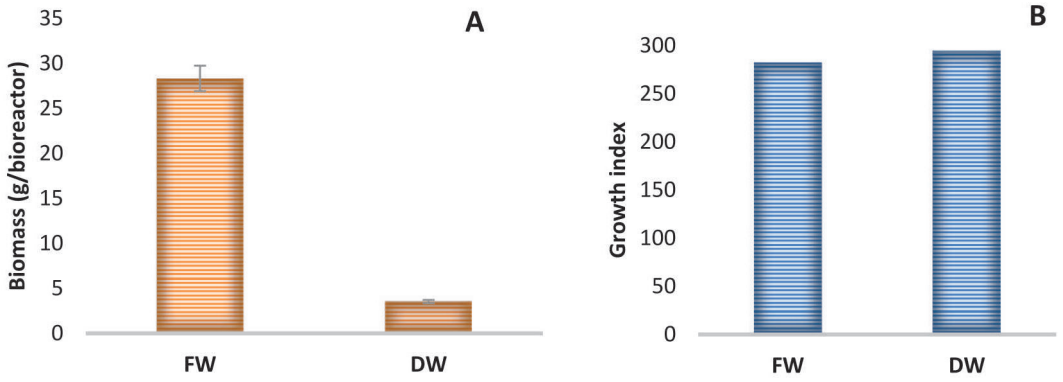

Fig. 1. Growth parameter of *D. forrestii* transformed shoots cultured for three weeks in the RITA® bioreactor in MS medium with BPA 0.5 mg/L and IAA 0.2 mg/L under blue LEDs. The data is given as the mean values of three independent experiments ±SE. FW – fresh weight, DW – dry weight

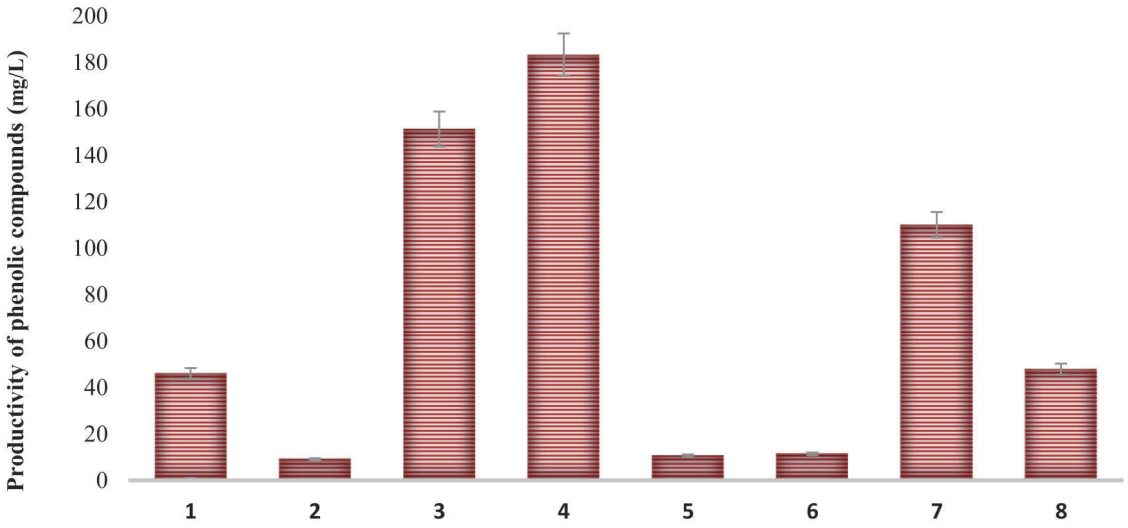

Fig. 2. Productivity of phenolic compounds (mg/L) in *D. forrestii* transformed shoot cultured for three weeks in RITA® bioreactor in MS medium with BPA 0.5 mg/L and IAA 0.2 mg/L under blue LEDs. Results obtained for the best variant of extraction i.e.: with MeOH : H2O 8:2 (v/v). Compound: 1) chlorogenic acid, 2) dicaffeoylquinic acid, 3) rosmarinic acid, 4) acacetin rhamnosyl--trihexoside, 5) apigenin caffeoyl-rhamnoside 6) apigenin p-coumaroyl-rhamnoside (I), 7) acacetin acetyl-rhamnosyl-trihexoside, 8) apigenin p-coumaroyl-rhamnoside (II). The data are the mean of the three independent experiments ±SE

high growth index (GI) i.e., 282.4 for fresh weight and 294.9 for the dry weight (Fig. 1B).

The effect of the solvent used on the extraction efficiency. The transformed *D. forrestii* shoots accumulate chlorogenic acid, dicaffeoylquinic acid and rosmarinic acid (phenolic acids), and acacetin rhamnosyl-trihexoside, apigenin caffeoyl-rhamnoside, apigenin caffeoyl-rhamnoside, apigenin p-coumaroyl--rhamnoside (I and II), acacetin acetyl-rhamnosyl-trihexoside (flavonoid glucosides) [Weremczuk-Jeżyna et al. 2021]. The present study evaluated the efficiency of aqueous methanol solutions of 20%, 50% and 80% alcohol as extraction solvents. These were accompanied by traditional infusion of boiling water.

Table 1. Phenolic compound content in different extracts of *D. forrestii* transformed shoots grown for three weeks in the RITA® bioreactor in MS liquid medium with BPA 0.5 mg/L and IAA 0.2 mg/L under blue LEDs

Compounds (mg/g DW)	Extracts			
	MeOH:H_2O (4:1 *v/v*)	MeOH:H_2O (1:1 *v/v*)	MeOH:H_2O (1:4 *v/v*)	infusion
chlorogenic acid	3.25 ±0.16^{a}	3.34 ±0.13^{a}	2.90 ±0.09^{b}	3.11 ±0.09^{a,b}
dicaffeoylquinic acid	0.64 ±0.09^{a}	0.54 ±0.06^{a}	0.53 ±0.02^{a}	0.39 ±0.07^{b}
rosmarinic acid	10.68 ±0.69^{a}	7.21 ±0.50^{b}	8.11 ±0.38^{b}	4.76 ±0.35^{c}
acacetin rhamnosyl-trihexoside	12.97 ±0.81^{a}	9.05 ±0.57^{b}	4.74 ±0.23^{d}	5.6 ±0.38^{c}
apigenin caffeoyl-rhamnoside	0.75 ±0.02^{a}	0.33 ±0.02^{c}	0.49 ±0.04^{b}	0.19 ±0.02^{d}
apigenin *p*-coumaroyl-rhamnoside (I)	0.78 ±0.05^{a}	0.34 ±0.05^{b}	0.24 ±0.01^{c}	0.19 ±0.01^{d}
acacetin acetyl-rhamnosyl-trihexoside	7.77 ±0.93^{a}	6.04 ±0.24^{b}	6.46 ±0.24^{b}	4.16 ±0.25^{c}
apigenin *p*-coumaroyl-rhamnoside (II)	3.38 ±0.20^{a}	3.56 ±0.24^{a}	1.79 ±0.12^{b}	1.96 ±0.25^{b}
total phenolic	39.17 ±1.11^{a}	30.85 ±1.92^{b}	22.39 ±1.14^{c}	20.31 ±1.37^{c}

The results are expressed as means of three replicates ±SE. Means followed by various letters were significantly different according to the Kruskall-Wallis test ($p < 0.05$)

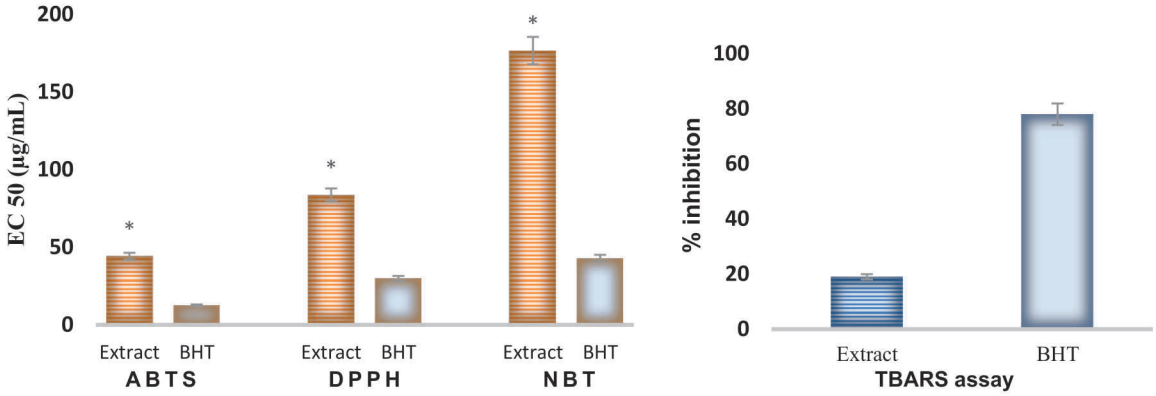

Fig. 3. Antioxidant activity of the hydromethanolic extract of *D. forrestii* transformed shoots in ABTS, DPPH, NBT, and TBARS assays. The results are expressed as means of three replicates ±SE. Statistical significance: * $p < 0.05$ extract vs. BHT (synthetic antioxidant)

The same compounds were detected in the extracts for all the solvents used. However, the greatest efficiency for extracting phenolic acids from transformed *D. forrestii* shoots was observed for MeOH:H_2O 4:1 (v/v), i.e., the solution with the highest methanol content. The complete ranking is as follows: MeOH:H_2O 4:1 (v/v) > MeOH:H_2O 1:1 (v/v) > MeOH:H_2O 1:4 (v/v) > infusion. The detailed polyphenol levels in all extracts are shown in Table 1. In the tested samples, the predominant compounds among the flavonoids were acacetin glucosides. The highest concentrations, i.e., 12.97 mg/g DW of acacetin rhamnosyl-trihexoside and 7.77 mg/g DW of acacetin acetyl-rhamnosyl-trihexoside, were found in the hydromethanolic extract containing 80% of MeOH (Tab. 1). Among the phenolic acids, the main compound was rosmarinic acid, whose highest level (10.68 mg/g DW) was also found for extraction with 80% MeOH. The levels of all identified phenolic compounds decreased as the water content in the extraction solution increased. The total polyphenol level in the water infusion was half that observed in MeOH:H_2O 4:1 (v/v) extract; however, such difference was not observed for chlorogenic acid (Tab. 1).

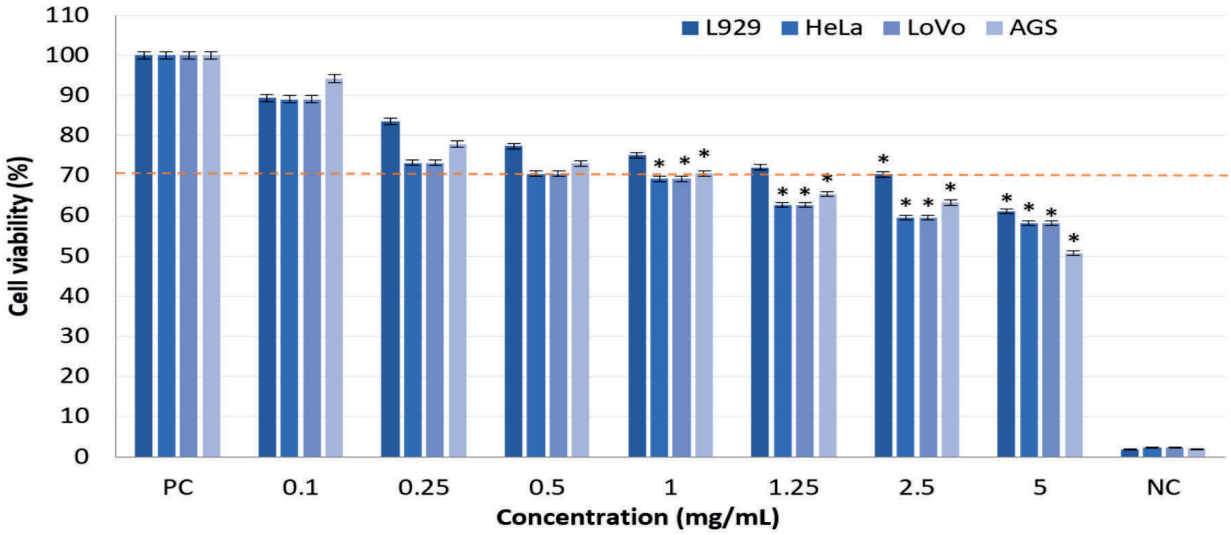

Fig. 4. Cytotoxicity of hydromethanolic extract of *D. forrestii* transformed shoots against four cell lines: L929, HeLa, LoVo, AGS. The cytotoxicity was assessed by MTT reduction assay. The red line indicates the minimal percentage of viable cells (70%) required to confirm the extracts as non-cytotoxic *in vitro*. The results are shown as mean values of cell viability experiments in three replicates for each experimental variant ±(SE). Statistical significance: * $p < 0.05$; untreated cells vs. cells treated with tested plant extracts. Positive control (PC), cells grown in culture medium alone; negative control (NC), cells treated with 0.03% H_2O_2

Productivity of polyphenols in shoots grown in RITA® bioreactor. The RITA® bioreactor was found to be an efficient system for the large-scale accumulation of bioactive compounds by transformed *D. forrestii* shoots. After the growth period during 3 weeks, 138.7 mg polyphenols were accumulated, i.e., 0.55 g phenolic compounds per 1 L growth medium. The levels of the predominant flavonoids in the extract were 183.7 mg/L for acacetin rhamnosyl-trihexoside and 110.1 mg/L for acacetin acetyl-rhamnosyl-trihexoside and a level of main phenolic acid i.e. rosmarinic acid was 151.2 mg/L (Fig. 2).

Evaluation of biological properties. The hydromethanolic extract of *D. forrestii* shoots demonstrated high ABTS radical scavenging activity, with an EC_{50} value of 44.3 µg/mL and in binding DPPH radicals, with EC_{50} = 83.7 µg/mL (Fig. 3). The *D. forrestii* extract was also a moderate scavenger of superoxide radicals generated in the NBT test (EC_{50} 176.7 µg/mL) – Fig. 3. Additionally, the hydromethanolic extract of *D. forrestii* (100 mg/g DW of extract) inhibited lipid peroxidation by 19%, according to the TBARS assay (Fig. 3).

It was found that the extract was safe for normal L929 cells at concentrations below 2.5 mg/mL; it reduced propagation of L929 fibroblast compared to controls (30–40% dead cells) at concentrations of 2.5 and 5 mg/mL, respectively (Fig. 4).

For all the cancer lines used in the present study, viz. HeLa (cervix adenocarcinoma), AGS (gastric adenocarcinoma epithelial cells), and LoVo (colon epithelial cells), a decrease in cell viability was observed at a concentration of 0.25 mg/mL, and a statistically significant effect was observed at 1 mg/mL (about 30% of dead cells) (Fig. 4). Furthermore, this extract in the range 1.25–5.0 mg/mL diminished growth of HeLa, AGS and LoVo below biocompatibility norm (Fig. 4).

The microbiological study showed that tested hydromethanolic extract has antibacterial potential. The minimal inhibitory concentration of extract against the tested bacterial strains was estimated at 5–7.5 mg/mL, with a minimal bactericidal concentration of 10 mg/mL. The *D. forrestii* extract demonstrated slightly greater activity against fungi of the genus *Candida* with a MIC of 2.5 mg/mL and MFC of 10 mg/L (Tab. 2).

Table 2. Antimicrobial activity of hydromethanolic extract from *D. forrestii* transformed shoots, expressed as minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) or minimal fungicidal concentrations (MFC)

Microorganism	*D. forrestii* extract		Gentamicin	Amphotericin B
	MIC (mg/mL)	MBC or MFC (mg/mL)	MIC = MBC = MFC (mg/mL)	
	Gram-negative bacteria			
Pseudomonas aeruginosa ATCC 27853	7.5	10	<0.008	–
Escherichia coli ATCC 25922	5	10	<0.004	–
	Gram-positive bacteria			
Staphylococcus aureus ATCC 29213	5	10	<0.002	–
Staphylococcus epidermidis ATCC 12228	7.5	10	<0.002	–
	fungi			
Candida albicans ATTC 10231	2.5	10	–	<0.001
Candida glabrata ATCC 2001	2.5	10	–	<0.001

Gentamicin and amphotericin B, board-spectrum antibiotics, used as antibacterial and antifungal reference substances, respectively; (–) not tested

DISCUSSION

In vitro culture can provide an alternative for acquiring pharmacologically-important plant species that are rare or grow in poorly-accessible areas, such as those growing in some areas of India or China [Espinosa-Leal et al. 2018]. One such species is *D. forrestii*, which has long been used in traditional Tibetan medicine [Li et al. 2006].

In our experiment, under tested growth conditions, a high multiplication rate of *D. forrestii* shoots was observed (40.5 shoots or/and buds per explant); this result was eight-fold higher than that observed for the same shoots cultivated for four weeks in tubes under blue LED light (5.2 shoots/buds per explant) [Weremczuk-Jeżyna et al. 2021]. The transfer of non-transformed *D. forrestii* shoots from tubes to a nutrient sprinkle bioreactor also resulted in an almost five-fold increase in the proliferation ratio [Weremczuk-Jeżyna et al. 2019]. A similar effect was reported by Businge et al. [2017] for cultures of *Eucalyptus* and *Betula pendula* grown in TISs, with 5.8-fold and 1.8-fold higher multiplication ratios observed compared to shoots cultivated on a solid medium.

The *D. forrestii* shoot culture grown in the RITA® bioreactor under blue LED light yielded a high, about 283-fold, biomass increase. It was significantly greater than that observed for the solid medium culture in the same light conditions, i.e., about 136-fold [Weremczuk-Jeżyna et al. 2021]. Similarly, *S. viridis* shoots cultured in Plantform bioreactor demonstrated higher biomass gains (33-fold increase) compared to agar medium (20-fold), as noted by Grzegorczyk-Karolak et al. [2022]. These differences in proliferation ratio and growth between bioreactor culture and tube culture may be caused by a change in the consistency of the medium from solid to liquid. It is known that a liquid medium provides better accessibility and nutrient absorption compared to a solid medium [Georgiev et al. 2014a]. Additionally, TISs

constantly promote the aeration of containers; this ventilation system allows precise control of the oxygen supply and the removal of volatile compounds such as ethylene. Moreover, this controlled aeration promotes the recirculation of carbon dioxide necessary for photosynthesis [Lyam et al. 2012, Aragón et al. 2014]. Bioreactor culture also increases shoot proliferation by eliminating apical dominance and stimulating the growth of lateral buds [Ahmadian et al. 2017].

In the present study, the key factor influencing shoot biomass was the use of blue LEDs. The *D. forrestii* shoots from the RITA® bioreactor grown under blue LEDs demonstrated several times higher growth index compared with those for shoots grown in the same TIS but under fluorescent light (GI = 52.01 for FW and GI = 55.7 for DW) [Weremczuk-Jeżyna et al. 2020]. However, the RITA®-grown shoots kept under blue LEDs demonstrated a lower multiplication ratio (40.5 shoots and buds per explants) than those under fluorescent light (47.6 shoots or/and buds per explants). This may be due to the fact that the shoots obtained in the blue LED conditions were more massive, with more numerous, and larger, leaves. Similar effects on leaf anatomy have been observed in several other species e.g., *Ficus beniamina* and *Sinningia speciosa* [Zheng et al. 2017].

The type of solvent plays a key role in phenolic compound extraction, and even slight changes can influence the final metabolite content in the extract. Hashim et al. [2016] report that the total phenolic and rosmarinic acid concentrations in *Orthosiphon stamineus* extract depended significantly on the content of methanol in the solvent used for extraction: the most efficient mixture for polyphenol extraction was found to be a 1 : 1 methanol : water solution. Various hydrophilic solvents based on 35–90% methanol or ethanol are used for extracting phenolic acids and flavonoids, apigenin and luteolin derivatives, from the Lamiaceae. The optimal alcohol: water ratio is determined experimentally depending on the extraction conditions and chemical structure of the extracted compounds [Chaves et al. 2020, Picos-Solas et al. 2021]. Similar to our study, 80% methanolic solution was found to be appropriate for extracting flavonoid glucosides and rosmarinic acid from *Salvia verbenaca* or *Rabdosia rubescens* [Kostić et al. 2015, Meng et al. 2019]. However, 50% hydromethanolic solvent was more efficient for extracting apigenin-*O*-glucosides and other flavonoid glucosides from *Ocimum sanctum* leaves [Chaudhary et al. 2020].

Contrary to our findings, several studies report that hot water alone could be comparable or even more effective for polyphenol extraction than hydromethanolic solution. This was found to give the best results for extracting apigenin glucosides from the leaves of *Thymus vulgaris* and *Thymus pallescens* [Sonmezdag et al. 2018, Ziani et al. 2018]. These differences can be due to the polarity of specific derivatives and the methods used for extraction. In conventional extraction, such as infusion, the efficiency of extraction depends mainly on the solubility of the chemical compound in the solvent; however, better results can be obtained by ultrasound extraction, where strong high-frequency sound waves are used to increase the release of extractable compounds and enhance the transport of solvent from the continuous phase into plant cells [Dhanani et al. 2017].

The productivity of the plant culture is reflected in the accumulation of compounds in the bioactive material and the accumulation of its biomass. The RITA® bioreactor was found to be an efficient system for the large-scale production of biomass and bioactive compounds by transformed *D. forrestii* shoots. On the other hand, the individual secondary metabolites were found to be present in a smaller amount per shoot dry weight in comparison to shoots propagated on solid medium, with the same medium composition and light conditions [Weremczuk-Jeżyna et al. 2021]. This same effect was observed during bioreactor cultivation of *D. forrestii* shoots under fluorescent lamps [Weremczuk-Jeżyna et al. 2020]. Also, lower levels of bioactive compounds were observed for *Lycium barbarum* and *Dreosera communis* shoots cultivated in TISs compared to those on semi-solid media [Kunakhonnuruk et al. 2019]. These differences may result from changes in the culture system, emphasizing the need for accurate optimization of growth conditions of shoots in the bioreactor. One such set of parameters comprises the frequency and duration of immersion; several reports indicate that these parameters significantly influence proliferation ratio, biomass, and secondary metabolite accumulation. For example, Grzegorczyk-Karolak et al. [2022] observed that prolonging the culture duration caused a two-fold increase in biomass and

metabolite accumulation in *Salvia viridis* shoots cultivated in a Planform bioreactor, with the bioactive compound content depending on the immersion cycle. Whereas, in a shoot culture of *Pancratium maritimum* grown in TIS, about 58% and 54% higher alkaloid accumulation was noted at an immersion frequency of 15 min/12 h compared to 15 min/6 h and 15 min/24 h [Georgiev et al. 2014a].

In the present study, after growth period the levels of accumulated phenolic compounds (per 1 L growth medium) were about twice those of the total phenolic compounds in the shoots cultivated under the same conditions but with fluorescent light (59.9 mg/bioreactor and 0.24 g/L of culture) [Werem czuk-Jeżyna et al. 2020]. Whereas, the levels of the predominant flavonoids i.e. acacetin rhamnosyl-trihexoside (183.7 mg/L) and acacetin acetyl-rhamnosyl-trihexoside (110.1 mg/L) in the extract were, respectively, 14-fold and almost four-fold higher than those accumulated in shoots under fluorescent lamps [Weremczuk-Jeżyna et al. 2020]. In the previous research, *D. forrestii* shoots grown on agar medium under blue LEDs demonstrated changes in flavonoid level accumulating almost 20-fold higher acacetin rhamnosyl-trihexoside and 4-fold higher other flavonoid glucosides than those under fluorescent lamps [Weremczuk-Jeżyna et al. 2019, 2021]. Other species, such as *Perilla frutescens* or *Rehmannia glutinosa* also accumulated higher flavonoid levels under blue LEDs than those grown under other light conditions [Lee et al. 2014, Manivannan et al. 2015].

However, blue light did not appear to have any effect on the accumulation of rosmarinic acid. The obtained level of this compound (151.2 mg/L) was similar to the level observed under fluorescent lighting [Weremczuk-Jeżyna et al. 2020]. Similarly, the type of lighting did not significantly modify the RA content in *D. forrestii* shoots grown on a smaller scale in test tubes [Weremczuk-Jeżyna et al. 2019, 2021].

A for many years, both plant and their metabolites have been regarded as natural agents against civilization disease. Among them, many species of *Dracocephalum* are known for their protective effects against oxidative stress [Aprotosaie et al. 2016]. Therefore, we also decided to evaluated the antioxidant potential of *D. forrestii* shoots cultured in the RITA® bioreactor. The hydromethanolic extract was subjected to ABTS, DPPH and superoxide anion radical scavenging assays, as well as the inhibition of lipid peroxidation test. These antioxidant assays are often used to measure the antioxidant potential of plant extracts [Munteanu et al. 2021].

In our research observed the high ABTS radical scavenging activity of hydrometanolic extract of *D. forrestii* (EC_{50} 44.3 µg/mL). Similar results were observed for the hydromethanolic extract from shoots of six-month-old *D. moldavica* plants grown in field conditions (42.8 µg/mL) [Weremczuk-Jeżyna et al. 2017]. However, these values are considerably higher than that of the ethanolic extract from the shoots of *Dracocephalum palmatum* (EC_{50} 6.4 µg/mL) [Olennikov et al. 2013].

The tested of *D. forrestii* extract was also effective in binding DPPH radicals (EC_{50} 83.7 µg/mL). A slightly lower EC_{50} value was observed for the alcoholic extract of *D. kotschyi* shoots (51.5–60.7 µg/mL) [Moradi et al. 2020], while the methanolic extract from aerial parts of *D. heterophyllum* (37 µg/mL) found to be significantly stronger [Ray et al. 2009].

In the nitro blue tetrazolium (NBT) test the *D. forrestii* extract was a moderate scavenger of superoxide radicals generated (EC_{50} 176.7 µg/mL). In contrast, *D. palmatum* demonstrated a higher antioxidant activity value (EC_{50} of 37 µg/mL) [Olennikov et al. 2013], and *D. moldavica* shoots a lower one (EC_{50} of 445.5 µg/mL) [Aprotosaie et al. 2016].

In present study the hydromethanolic extract of *D. forrestii* inhibited lipid peroxidation by 19%. By contrast, Weremczuk-Jeżyna et al. [2017] report that *D. moldavica* extract from shoots of field-grown plants demonstrated 14% inhibition, and suspension and callus culture both 4%. Interestingly, RA, known to demonstrate high antioxidant potential, was present at higher levels (13.5–25 mg/g DW) in *D. moldavica* extracts than in *D. forrestii* shoots [Aprotosaie et al. 2016, Moradi et al. 2020]. This suggests that the flavonoids present in the transformed shoots of *D. forrestii* may have a synergistic, or at least additive, effect on antioxidant activity however, further detailed research is needed to confirm this. However, to date, most reports have mainly pointed to RA, a secondary metabolite whose presence is correlated with the antioxidant properties of extracts from some Lamiaceae family

species such as *S. viridis*, *S. verbenaca* or *O. sanctum* [Grzegorczyk-Karolak et al. 2022, Kostić et al. 2015, Sonmezdag et al. 2018].

Many studies have examined the relationship between the antioxidant properties of plant extracts and their antitumour activity [Cai et al. 2004, Muniyandi et al. 2017]. Indeed, some members of *Dracocephalum* have been found to possess cytotoxic effects against some cancer cell lines. For example, Shaabani et al. [2020] reported that 0.2 mg/mL ethanolic extract from *D. kotschyi* aerial parts caused the death of 40% *Glioblastoma multiforme* U87 cells.

In our study, *D. forrestii* extract found to decrease cell viability of HeLa, AGS and LoVo cells at a concentration of 0.25 mg/mL, but only in the range 1.25–5.0 mg/mL diminished growth of all used cancer cell lines below biocompatibility norm. On the other hand, only concentrations of extract below 2.5 mg/mL could be used as a cytotoxic agent, because higher concentration was toxic for normal cells in the ISO 10993-5 [2009] biocompatibility test.

The cytotoxic activity of *D. forrestii* shoots could be connected with the activity of RA and the flavonoid compounds present in the extracts. Indeed, RA has been found to induce apoptosis and inhibit colon tumour cell proliferation (HCT15 and CO115) [Scheckel et al. 2008, Moore et al. 2016], while acacetin and apigenin as well as their glucosides inhibited the growth of colon cancer and human gastric carcinoma lines [DeRango-Adem and Blay 2021, Pan et al. 2005]. Therefore, in the future, the studies should also focus on the isolation of individual compounds from *D. forrestii* shoots and determining their properties, and indicating which metabolites are especially responsible for the raw material activity. Moreover, it might be important to assess the pharmacodynamic synergism of the tested extracts/isolated compounds with the standards used in conventional therapy. Such support for classical therapy would reduce the dose of used chemotherapeutic agents and achieves the desired treatment effect while increasing the safety of the therapy.

Because some species of *Dracocephalum*, e.g., *D. kotschyii*, have been used for treating infectious diseases [Heydari et al. 2019], the hydromethanolic extract from the tested *D. forrestii* shoots was screened for antimicrobial properties. The reference strains included Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus* and *S. epidermidis*), and two strains of fungi (*Candida albicans* and *Candida glabrata*).

MIC values against various pathogenic microorganisms revealed for hydromethanolic extract from *D. forrestii* was ranged obtained for extracts from *D. kotschyi* and *D. moldavica* which demonstrated minimal inhibitory concentration values between 1.5–20 mg/L [Kamali et al. 2015, Khadije et al. 2017]. Similar, antibacterial potential was observed for *D. polyachetum* against various *S. aureus* strains (MIC values of 0.78–25 mg/mL) [Yaghoobi et al. 2018]. It is known that main metabolites occurring in *D. forrestii* extract have antibacterial effects. For example rosmarinic acid demonstrated inhibitory activity against *S. aureus* strains at a concentration of 0.8–10 mg/mL and against *P. aureginosa* at 2.5 mg/mL [Abedini et al. 2013, Ekambram et al. 2016]. This compound has also showed synergism with different antibiotics, resulting in a stronger antimicrobial effect; this allows a lower concentration of antibiotic to be used, and thus greater safety [Ekambram et al. 2016]. The *D. forrestii* extract demonstrated slightly greater activity against fungi of the genus *Candida*. This compares favorably with other species of the Lamiaceae known for their antifungal activity such as *Rosmarinus officinalis* (2.34 mg/mL) and *Origanum vulgare* (1.56 mg/mL) [Blank et al. 2020]. The antifungal activity of the *D. forrestii* extract probably results from the presence of flavonoid glucosides, particularly apigenin derivatives, which are known as strong antifungal agents. For example, the anticandidal inhibitory concentration of apigenin-7-*O*-glucoside was 0.05–0.15 mg/mL, and apigenin-5-*O*-glucoside – 0.03 mg/mL [Smiljkovic et al. 2017, Elansary et al. 2020].

CONCLUSIONS

Secondary metabolites of pharmacological interest are usually acquired through the exploitation of natural plant resources, including rare endemic species. However, recent advances in plant biotechnology have made it possible to reduce this overuse. Our findings indicate that *D. forrestii* shoots cultivated under

optimized conditions in the RITA® bioreactor could be a rich source of bioactive compounds, yielding over 0.5 g of polyphenols per liter of the medium within three weeks (including 151 mg/L rosmarinic acid, 183 mg/L acacetin rhamnosyl-trihexoside and 110 mg/L acacetin acetyl-rhamnosyl-trihexoside). Moreover, our *in vitro* studies indicate that the hydromethanolic extract obtained from the bioreactor-cultured *D. forrestii* shoots has antioxidant and antimicrobial potential. This extract also significantly diminished growth of HeLa, AGS and LoVo tumor cells in the range 1.25–5.0 mg/mL, whereas it was safe for normal mammalian L929 cells at concentrations below 2.5 mg/mL.

SOURCE OF FUNDING

This study was supported by the Medical University of Lodz, a Grant No. 503/3-012-01/503-31-001-19-00.

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