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In vitro Ultraviolet-B radiation mediated antioxidant response of Bulgarian Goldenrod (*Solidago virgaurea L.)* extract

Z. L. Yaneva[1]*, E. B. Simeonov[2], D. G. Ivanova[1],

[1]*Chemistry Unit, Department of Pharmacology, Animal Physiology and Physiological Chemistry, Faculty of Veterinary Medicine, Trakia University, Students Campus, 6000 Stara Zagora, Bulgaria*
[2]*Department of Chemical Engineering, University of Chemical Technology and Metallurgy, 8, Kl. Ohridski Blvd., 1756 Sofia, Bulgaria*

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Damaging Ultraviolet B (UVB) radiation is an important part of the solar electromagnetic spectrum, which seasonal and long-term levels are likely to remain elevated for decades to come. Due to the high sensitivity of plants to UVB radiation, they need to react quickly to minimize surface reactive oxygen species (ROS) mediation. The increased levels of ROS, at cellular level, causes oxidation of proteins, lipids, and other biomolecules, thus jeopardizing enzymes and cell membranes functionality and integrity.

The present study for the first time reported the radical-scavenging, antioxidant-protective properties and stable structure of *Solidago virgaurea L.* extract before and after exposure to UVB radiation. The composition of the chemically pure (97 %) extract was established in the untreated and in the UVB-treated samples by RP-HPLC. By direct EPR spectroscopy, single symmetrical EPR signals were established in both untreated samples ($g = 2.0043 \pm 0.0003$) and UVB-treated samples ($g = 2.0054 \pm 0.0002$). The intensities of the EPR signals of both extracts demonstrated a possible formation of stable radical structures. It should be pointed out that stable radical structures were registered in the extract 2 and 6 months after the UVB-induced oxidation. Furthermore, both untreated and UVB-irradiated *S. virgaurea L.* extracts showed well-expressed radical-scavenging abilities and antioxidant-protective properties against reactive species such as nitric oxide (NO), reducing agents, non-enzymatic 2,2-azinobis (ABTS•+) structures, superoxide anion and DPPH stable radicals. The current results characterize the *S. virgaurea L.* extract as a promising ROS-scavenging antioxidant with UVB-protective properties, and outline its future applicability for the development of new photo-medications.

Keywords: *S. virgaurea L.*, RP-HPLC, EPR spectroscopy, antioxidant, UVB-protection

INTRODUCTION

Ultraviolet radiation (UVR; 100-400 nm) is an important part of the electromagnetic spectrum naturally emitted by the sunlight necessary for the normal physiological functions of medicinal plants [1]. Traditionally, the UVR is divided into three spectral regions: UVC (100-290 nm), UVB (290-320 nm) and UVA (320-400 nm) [1, 2]. The UVB light has received attention in the last two decades because the thinning of the ozone layer leads to an increase in the amount of UVB-irradiation of medicinal plants [3]. In general, many investigators have reported that UVB-irradiation has harmful effects on the growth, leaf expansion, physiology and productivity of different plants species [3, 4]. Moreover, UVB radiation has been found to increase plant membrane changes, protein destruction, hormone inactivation, biomass reductions and epidermal deformation [1, 2]. UVB induced generation of reactive oxygen (ROS) and nitrogen (RNS) species by direct and indirect pathological mechanisms through which the antioxidant defence systems decreased, and intracellular oxidative stress could generate plant cell damages [5]. The reactive species such as superoxide anion radicals (O_2^{-}), hydroxyl radicals (HO·), hydrogen peroxide (H_2O_2), peroxyl radicals (RCOO·) and nitric oxide (NO·/ $NOOO^{-}$) radicals are active components that are detoxified by the endogenous antioxidant system [6]. Sunlight, specifically UVB photons lead to the progression of the replication on the damaged DNA molecules and accumulation of proteins mutation and lipids peroxidation [5, 7]. More specifically, UVB light induces ROS by affecting the antioxidant enzymes, decreases protein kinase C (PKC) expression and increases nitric oxide synthase (NOS) synthesis [5, 7-9]. Studies indicate that synthesis of specific flavonoids and other phenolic compounds is regulated in response to UVB light [3, 10]. Flavonoids as medicinal plant antioxidants (e.g., B-ring substitution) lend beneficial place in the UV-oxidative stress protection and regulation [10]. Previous studies have shown that higher plants have independent photoprotective systems [3] and exposure to UVB radiation stimulated higher levels of flavonoids biosynthesis, and ROS removal [10, 11].

* To whom all correspondence should be sent:
E-mail: z.yaneva@abv.bg

Solidago virgaurea L. (Bulgarian Goldenrod) is an herbaceous perennial plant (family Asteraceae), which is widespread across Europe, North Africa and northern, central, and southwestern Asia. The aerial parts have been applied as spasmolytic, anti-inflammatory and diuretic agents in traditional medicine, as a urological remedy in kidney and bladder inflammation, urolithiasis, cystitis [12]. Studies proved that the plant extracts contain flavonoids (quercetin glycosides), salicylic acid derivatives, chlorogenic acid, caffeic acid, triterpene saponins, tannins, and essential oils. Besides, some of the proven bioactivities of the medicinal plant are due to synergic action of its bioactive substances [13].

In this regard, the aim of the present study was to determine the contents of catechins in *S. virgaurea* L. ethanol extract and to investigate and compare the EPR structural changes and *in vitro* antioxidant activity of untreated and UVB-irradiated *S. virgaurea* L. ethanol extracts.

EXPERIMENTAL

The study was conducted at the Electron Paramagnetic Resonance (EPR) Centre of the Medical Faculty, at the Bioorganic Chemistry Laboratory of the Faculty of Veterinary Medicine, the Central Scientific Laboratory, Trakia University, Stara Zagora, Bulgaria and at the laboratories of the Chemical Engineering Department, University of Chemical Technology and Metallurgy, Sofia, Bulgaria in 2019.

Reagents and standards

The following standards were used for the experimental studies: (+)-catechin hydrate (≥ 96.0 %, HPLC), (−)-epigallocatechin (analytical standard), quercetin (≥95%, HPLC) (Sigma-Aldrich). The reagents: acetonitrile (AcCN, ≥ 99.8%), orthophosphoric acid (H_3PO_4, 85%), methanol (MeOH, ≥ 99.9%), ethanol (EtOH, p.a. ≥ 99.8%), L-ascorbic acid, CH_3COOH and NaOH (p.a., HPLC) were supplied by Sigma-Aldrich. Milli-Q water was used to prepare the mobile phase.

Extraction procedure

The periodic extraction of *Solidago virgaurea* L. was conducted in a stirred batch extraction reactor at solid/liquid ratio $\xi = 0.03$ m^3 kg^{-1}, temperature T = 30ºC and solvent - 70% EtOH [14, 15]. The test results were obtained at an agitation rate n = 4 s^{-1}, which proved that the process was limited by internal diffusion (the external diffusion resistance was eliminated). A weight analysis with accuracy of 1.10^{-4} g was used to determine the contents of the extracts. A specified amount of extract was taken periodically and dried to constant weight in an oven at T = 70^{0}C.

RP-HPLC analyses

The concentrations of catechins (catechin, epigallocatechin) and quercetin in the ethanol extracts tested were determined by newly developed liquid chromatographic techniques specific for each antioxidant. A reverse-phase RP-HPLC system consisting of a Hypersil BDS C18 column (5 μM, 4.6 × 150 mm), a Surveyor LC Pump Plus pump, a PDA detector, and an Autosampler Plus autosampler (Thermo Fisher Scientific) were used. The mobile phase consisted of MeOH:AcCN:H_2O = 40:15:45 (v/v) (+1% CH_3COOH). To achieve better separation of the components and purification of the extracts, all analyses were performed with a precolumn. The analyses of all the samples tested were performed in triplicate and the mean values of the reported concentrations were presented. The experimental HPLC chromatograms of the test sample extracts were analysed with ChromQuestTM chromatography workstation software system Version 4.2 (Thermo Electron Corporation).

UVB treatment

All ethanol samples were irradiated at a distance from the light source of 25 to 35 cm and a quartz cover was used to allow UV transparency and to prevent extract evaporation and keeping it in a horizontal position on UVB–vis Transilluminator-4000, Stratagene/USA (emitting between 290 nm and 320 nm (peak 309 nm)). The experimental UVB intensity was calibrated in each experiment. To obtain the value of dose response about the UVB radiation effect, the samples were irradiated over a wide UVB radiation range (0 to 12 kJ m^{-2}) without visible ray, at less than 291-293 K. The UVB irradiated energy was controlled with exposure time of 120 min. Dark, fresh air and 46 % relative humidity was circulated in the illuminator throughout the irradiation course.

In vitro antioxidant activity

FRAP assay: The ferric reducing antioxidant power/electron donation potential of untreated and UVB-treated *S. virgaurea L.* extract samples, was conducted according to the previously reported method with a slight modification [16, 17]. 50 μg mL^{-1} before was firstly tested to determine the exhibited maximal donation potential. Further, 50 μg mL^{-1} concentration was UVB-irradiated and the *FRAP* potential was determined immediately post irradiation. The reaction mixture was left for 10 min

at 293 K temperature and the absorbance was measured at 700 nm.

DPPH assay: The ability of the *S. virgaurea L.* extract before and after UVB-treatment to scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical was determined according to Cuendet *et al.* (1997) [18], with slight modifications [19]. Briefly, 1.0 ml of DPPH (100 μM) was added to 500 μl of 50 μg mL^{-1} volume concentration of the studied samples. Mixtures were incubated in the dark for 10 min and their absorbance at 517 nm was measured. Quercetin was used as a positive control.

$ABTS^{\bullet+}$ assay: The radical-scavenging activity of untreated and UVB-treated and *S. virgaurea* L. samples against 2,2-azinobis (3-ethylbenzothiazoline–6–sulfonic acid) was determined by Re *et al.,* (1999) [20] with modifications by Adhikari *et al*., (2012) [21]. The reaction mixtures were incubated at 297 K for 30 min and the chromogen intensity was measured at 734 nm. Antiradical activity of the examined samples was presented as the percentage of $ABTS^{\bullet+}$ radical-scavenging in μg/ml, conducted in triplicate and the results expressed as the mean ± standard deviation.

NO assay: The presence of nitrite potential, a stable oxidized product of nitric oxide ion scavenging of untreated and UVB-irradiated *S. virgaurea* L. samples (50 μg mL^{-1}) was determined according to the standard methodology described by Shirwaikar *et al.,* (2006) [22] with modification by Karamalakova *et al.,* (2017) [23]. The scavenging potential was evaluated as a decrease in % absorbance of the chromogen formed by diazotisation of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine recorded at λ = 546 nm.

SOD-like activity assay: The superoxide dismutase (SOD) assay of *S. virgaurea L.* extract, before and after UVB irradiation was carried out according to the method of Sun *et al*., (1988) [24] as follows: The xanthine/xanthine oxidase system (Sigma Chemicals, USA) was used to generate the superoxide anion ($\cdot O_2^-$). Following 20 min of incubation, the superoxide anion reduces nitroblue tetrazolium (NBT; Sigma Chemicals, USA) to formazan. Absorbance was measured at λ = 560 nm and L-ascorbic acid was used as a standard. One unit (U) of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the NBT reduction to formazan.

To study the effect of UVB radiation on the antioxidant activity and free radical scavenging, the samples were stored at a temperature of 293 K in the dry air/dark and re-examined for 24 hours at the 2nd and 6th month post irradiation.

In vitro Electron Paramagnetic Resonance spectral analysis

Direct EPR analysis: The *S. virgaurea* L. ethanol extract was divided into two parts: 1) before irradiation and 2) after UVB irradiation for 2 h. Immediately after irradiation storage, the two extract samples were examined in triplicate on an X-band-EMX^{micro} spectrometer at a temperature of 293 K. The sample tubes were always positioned exactly in the centre of the cavity and *g-value* was calculated as first derivative of the EPR signal [25]. Spectral settings were as following: centre field 3514.00 G, sweep width 200.00 G, microwave power 0.635 mW, modulation amplitude 10.00 G, gain 1×105, time constant 1310.72 ms, sweep time 133.12 s, 1 scan per sample; and for UVB-irradiated samples - 1.00 G modulation amplitude.

DPPH• radical-scavenging activity: The ability to scavenge DPPH• radical was studied according to Santos *et al.,* (2009) [26] with modifications [27]. Untreated and UVB-irradiated *S. virgaurea* L. extract (50 μg mL^{-1}) was added to 250 μl of EtOH solution of DPPH (80 μmol L^{-1}). Following 10 min incubation (293 K, 10 min/dark) the samples were transferred into the EPR cavity and the scavenging ability was calculated as follows:

$$Scavenged\ DPPH\ radials\ (\%) = [(I_0 - I)/I_0]x100 \quad (1)$$

where I_0 is the integral intensity of the DPPH signal of the control sample; I - the integral intensity of the DPPH signal after addition of the tested sample to the control.

A solution containing 250 μl of DPPH and 50 μl of EtOH was used as a positive control. The settings were as follows: microwave power 3.232 mW, modulation amplitude 5.00 G, gain 5.02×10^3, time constant 163.84 ms, 1 scan per sample.

To investigate the UVB effect on free radical stability and structural changes, samples were stored at a temperature of 293±2 K in the dry air/ dark and re-examined for 24 hours, on 2nd and 6th months post irradiation.

Statistical analysis

The data obtained from the *in vitro* antioxidant activity study were expressed as means ± standard error (SE) with Statistica 7.1, StaSoft Inc., one-way ANOVA. The statistical significance was determined by Student's *t*-test as the post-hoc test. A value of $p < 0.05$ was considered as statistically

significant. The kinetic data were expressed as the average of two independent measurements, which were processed using the computer programmes Origin 6.1 and Microsoft Excel 2010. EPR spectral processing was performed with Bruker WIN-EPR and Sim-Fonia software (Germany, IRCF 2003).

RESULTS AND DISCUSSION

RP-HPLC analysis of S. virgaurea L. ethanol extract

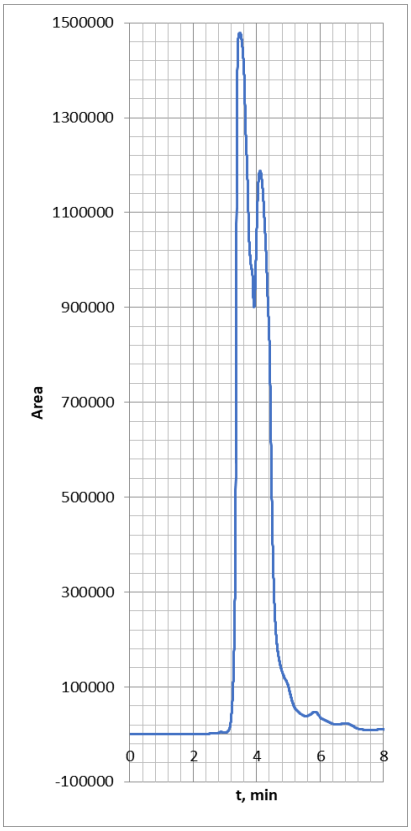
The concentrations of catechin, epigallocatechin and quercetin in the tested extract were determined by RP-HPLC on the basis of standard calibration curves of the corresponding three standards, which were characterized with high linearity ($R^2 > 0.998$).

The volume of all samples used in the RP-HPLC assays was 1.5 mL. The concentration of quercetin was determined at maximum wavelength λ = 360 nm, flow rate 0.5 ml min^{-1}, column temperature T = 30°C, detection time t = 5.8 min, volume of sample injected V = 20 µL. The catechin concentrations were determined at λ = 325 nm; flow rate: 0.5 mL/min, column temperature T = 30°C, detection time t = 3.5 min for epigallocatechin and t = 4.3 min for catechin, volume of sample injected V = 20 µL.

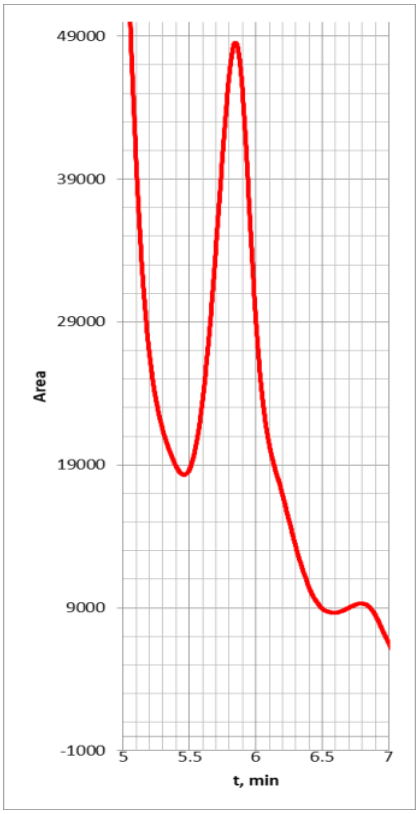
The concentrations of catechin, epigallocatechin and quercetin in the tested extract were 1384.23, 1427.04 and 18.9 µg mL^{-1}, respectively. The RP-HPLC chromatograms of the three biologically-active polyphenols are presented in Fig. 1.

In vitro antiradical activity promoted by UVB treatment

The photon energy of UVB radiation directly induces excessive production of ROS/RNS in medicinal plants, leading to the occurrence of oxidative stress damages. Plant are a potential source of natural antioxidants, which inhibit ROS/RNS activity by different mechanisms controlled by activation of the antioxidant enzymes system [28] and scavenging unstable free radicals such as superoxide, hydroxyl anion, nitric oxide and peroxynitrite. To identify whether free oxygen and nitrogen species function as signaling molecules in the UVB-provoked *S. virgaurea* L. extract response, different indirect methods were applied to evaluate the antioxidant activity. Previous studies have shown that the *S. virgaurea* L. extract possessed antioxidant activity [29]. In the present study, a maximal scavenging ability for untreated *S. virgaurea* L. extract and UVB-irradiated extract was observed at a concentration of 50 µg L^{-1}.



A.

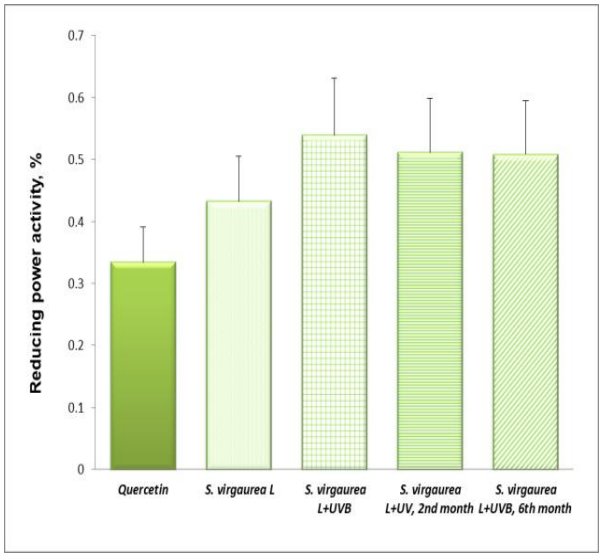


B.

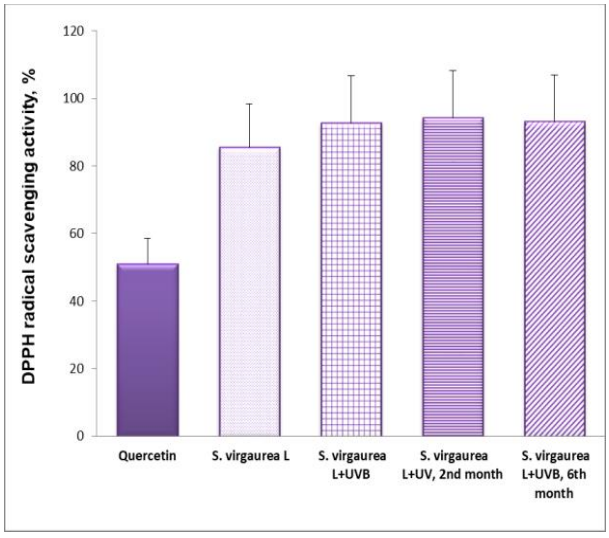
Fig. 1. RP-HPLC chromatograms of *S. virgaurea* L. extract of: **A.** catechin and epigallocatechin at λ = 325 nm; **B.** quercetin at λ = 360 nm.

FRAP assay (Fig. 2A) is generally associated with the presence of reductants in the untreated *S. virgaurea* L. extract and in the UVB-irradiated one, which exert antioxidant action by donating a hydrogen atom and reducing Fe^{3+} to Fe^{2+}. The ferric reducing activity for the UVB-irradaited *S. virgaurea L.* extract (0.539±0.01 %) was determined to be higher than that of untreated *S. virgaurea* L. (0.432±0.003 %) and the control sample (0.334±0.08 %, $p<0.005$). FRAP potential of the UVB-irradiated *S. virgaurea L.* was tested at different time intervals – on the 2nd and 6th months after treatment.

There were no significant differences ($p > 0.005$) in the FRAP activity between the post storage UVB-exposed samples compared to the immediate UVB-exposed ones, displaying a good restoring ability to the Fe^{3+} complex.



A.



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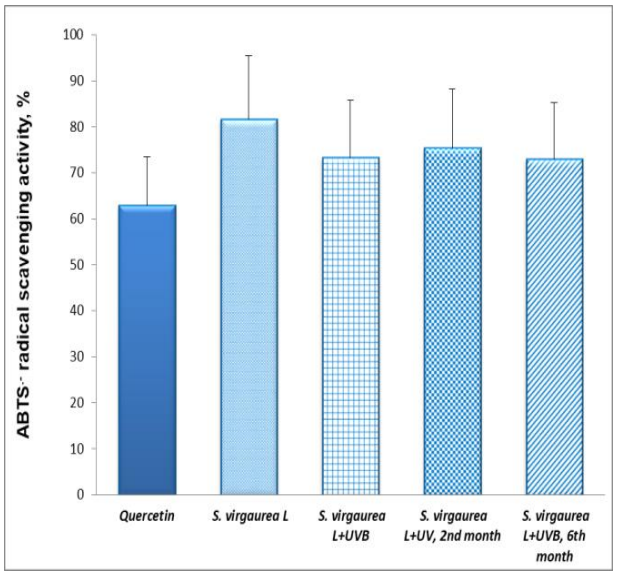
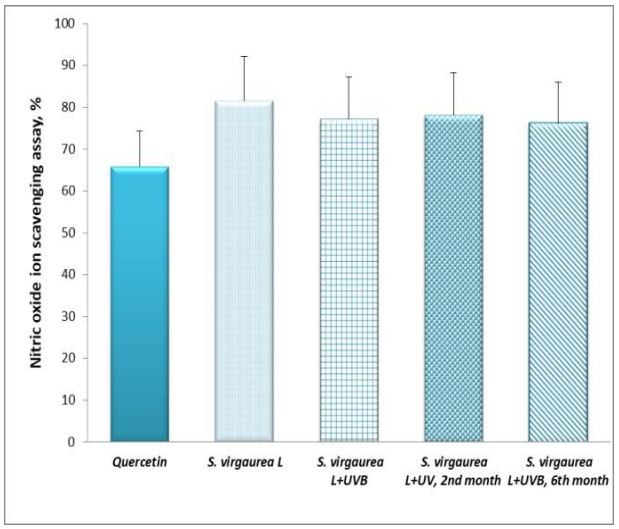


C.



D.

Fig. 2. Effect of UVB irradiation on the *in vitro* antiradical activity of *S. virgaurea L.* extract: **A**. FRAP assay, **B**. DPPH radical scavenging activity, **C**. $ABTS^{\bullet+}$ radical scavenging activity, **D**. Nitric oxide ion scavenging assay.

UVB-exposure increases the load of iron in the cellular environment and leads to hemolysis processes. The reduction of Fe^{3+} into the less harmful Fe^{2+} might be assumed as a possible mode of action by which *S. virgaurea* L. extract [29] would exhibit protective efficacy to oxidative damages caused by UVB-radiation *in vitro* [23, 30].

The DPPH as a stable lipophilic free radical with high sensitivity was used for evaluating the antioxidant activity of *S. virgaurea* L. extracts [31]. The $DPPH^{\bullet}$ scavenging activity of untreated *S. virgaurea* L. extract (85.56±0.88%, $p < 0.05$) and of the UVB-exposed sample (92.78±1.37%, $p<0.003$)

were statistically insignificantly increased, compared to the standard (50.99±0.19%) (Fig. 2B). It should be noted that UVB-radiation increased the DPPH$^{\bullet}$ ability to scavenge free radicals in the *S. virgaurea* L. extract, compared to quercetin. Meanwhile, UVB-radiation enhanced the DPPH$^{\bullet}$ radical scavenging after 2 and 6 months of storage. The considerable increase in the scavenging effects of the *S. virgaurea* L. extract after UVB radiation could be explained by the stable compounds as flavonoids, phenolic acids, glucosides, polysaccharides, anthocyanins, tannins, and vitamins present in the extract. These results prove the suitability of *S. virgaurea* L. extracts as a natural antioxidant for food applications [29-32].

The proton-radical analysis (ABTS$^{\bullet+}$ method*)* is excellent to define the total antioxidant activity of hydrophilic plant antioxidants [33]. The ABTS$^{\bullet+}$ proton scavenging abilities in both the UVB-irradiated *S. virgaurea L.* sample (73.221±1.16%) and the untreated extract (81.556±0.97%, 0.00) (Fig. 2C) increased as compared to quercetin (62.8± 0.58%) - the standard antioxidant used in this study. Untreated *S. virgaurea* L. extract was more effective in quenching proton radicals in the system. The present study shows that after 2 (75.3±1.00%) and 6 months (72.89± 0.47%) after UVB-irradiation the *S. virgaurea* L. extract has accumulated active components and maintains the ABTS$^{\bullet+}$ reduction levels. These results suggest that UVB-irradiation probably stimulates the production of secondary metabolites (flavonoids, phenolic acids, anthocyanins), the antioxidant activity and, thus, the extract is able to adapt and to protect itself from UVB-intensity irradiation [34].

Nitric oxide (NO$^{\bullet}$) is considered as one of the important molecules, which is the regulator of physiological functions. In addition to its physiological actions, the ROS can affect overload of NO$^{\bullet}$ radicals leading to oxidative cell damages and to compromised cell NO-functionality [23, 35]. The NO$^{\bullet}$ scavenging ability (Fig. 2D) of unirradiated *S. virgaurea* L. extract (81.65±1.75%, $p < 0.05$) and the UVB-irradiated sample (77.3±0.39%) increased as compared to the control sample (65.81±0.9%). Probably, *S. virgaurea* L. contains hydrophilic scavengers able to regulate the NO$^{\bullet}$ production even in the UVB-irradiated sample. UVB-irradiation incubation of *S. virgaurea L.* samples for 2 (78.13±0.63%) and 6 months (76.11±1.23%) recorded stable NO$^{\bullet}$ radicals scavenging in comparison to the untreated extract and the UVB-treated sample. Investigations demonstrated that endogenous NO/NO$^{\bullet}$ production is able to modulate primary metabolism and oxidative stress signalling responses in plant molecules [36]. The latter suggests that flavonoids and other *S. virgaurea* L. active components act as potential photoprotectors and ROS/NO scavengers formed as a result of long-term UVB-stress exposure [8, 36].

To identify whether ROS function as signalling molecules in the UVB-provoked oxidative stress in the *S. virgaurea* L. extract, SOD-like activity non-enzymatic method [37] was applied. The SOD-like activity of 1 mL untreated *S. virgaurea* L. extract was 9 times higher than that of the standard antioxidant L-ascorbic acid (1 mg) (Fig. 3).

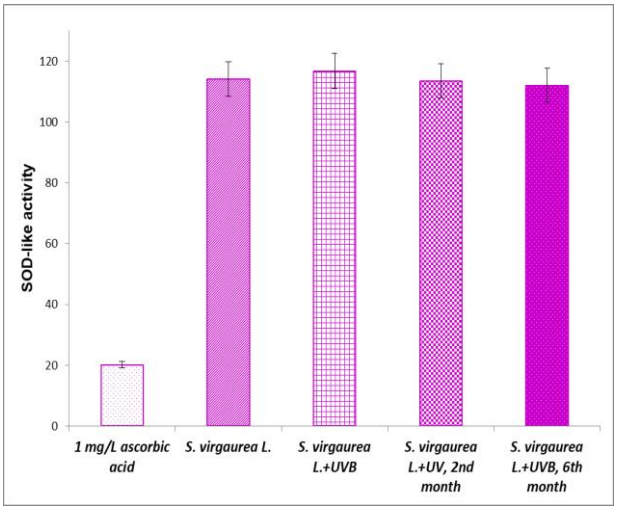


Fig. 3. Effect of UVB irradiation on the SOD-like activity of *S. virgaurea L.* extract.

Significantly slight increase in SOD-like activity in the UVB-treated extract in comparison with the non-treated sample was registered. In addition, the UVB-treated *S. virgaurea* L. registered 5 times higher SOD-like activity *vs* the standard, which was preserved for 2 and 6 months. The latter illustrates that abiotic stress of UVB-induced generation of superoxide radicals ($\bullet O_2^-$) was metabolically suppressed by the active compounds in the *S. virgaurea* L. extract into hydrogen peroxide (H_2O_2). Moreover, induced intracellular ROS were momentarily inhibited by the UVB-light-absorbing molecules of the *S. virgaurea* L. extract and converted into harmless molecules through SOD, H_2O_2, NAD (P) H oxidase and flavin oxidase [28].

EPR spectrum characterization before and after UVB-irradiation

An intensive symmetrical EPR singlet with g = 2.0043±0.0003 in the *S. virgaurea* L. sample (Fig. 4a) was recorded immediately after preparing the extract. A similar symmetrical signal with g-factor of 2.0054±0.0002 was registered in the UVB-irradiated extract (Fig. 4b) with almost commensurable singlet intensity. The g-factors of

the recorded spectra of *S. virgaurea* L. sample suggest the detection of *ortho*-semiquinone centred radical [38, 39] typical for the radical-scavenging of flavonoids, phenolic acids, glucosides, anthocyanins, tannins in the extract composition.

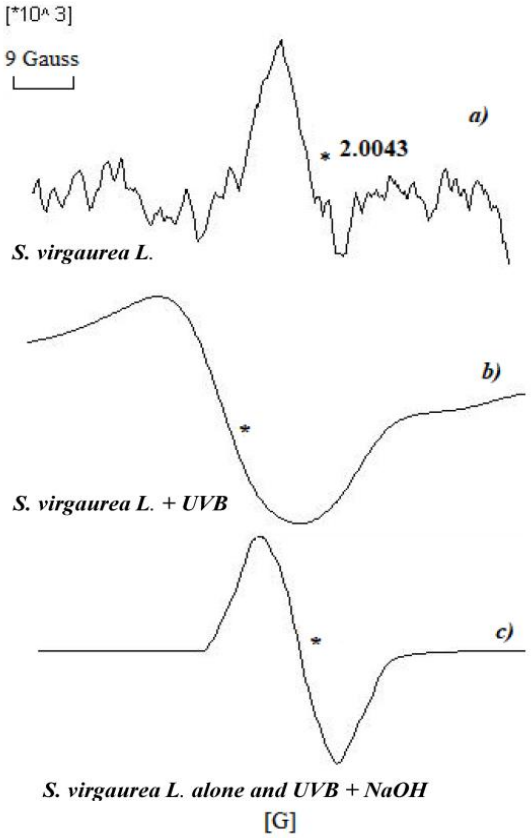


Fig. 4. EPR spectrum of *S. virgaurea* L. extract before and after UVB irradiation.

Considering that the EPR spectroscopy has the potential to verify the possibility of radical structures belonging to a semiquinone structure, we have studied the non-irradiated *S. virgaurea* L. extract and the UVB-irradiated sample after alkalization (Fig. 4c). Generating EPR radicals in both alkalized solutions resulted in intensive singlet signals of the *ortho*-semiquinone structures [40, 41] with equal g = 2.00456±0.0002 values.

Regarding the stable *ortho*-semiquinone structures in both samples for a more detailed analysis of the antioxidant capacity, we investigated the *in vitro* DPPH scavenging activity of the untreated *S. virgaurea L.* extract and of the UVB-irradiated sample. *S. virgaurea L.* extract (Fig. 5) displayed maximum radical-scavenging activity (89.11±7.45%). However, the UVB-treated sample was characterized with higher DPPH activity (91.37±9.71%), which was statistically insignificantly decreased on the 2nd (88.3 ± 5.71%) and 6th (85.2 ± 8.47%) months of UVB-endurance. The latter findings were in accordance with the results of Toiu *et al.*, (2019) [42]. The authors of the present study established that the flavonoids from *S. virgaurea* L. extract possess stronger antioxidant and DPPH radical-scavenging activity in comparison with vitamin C as a positive control.

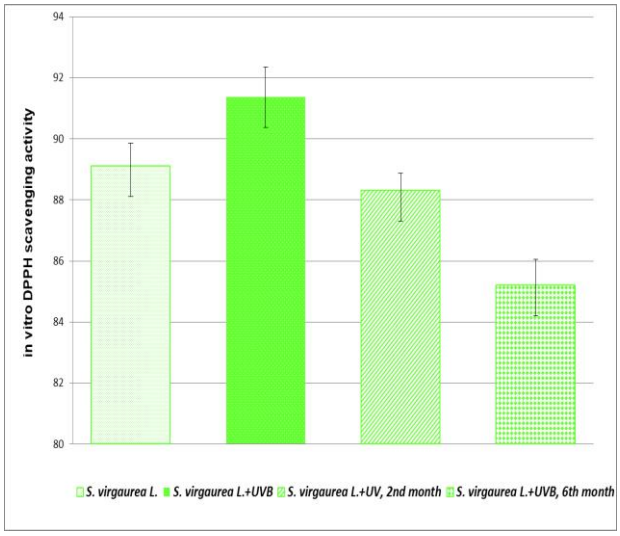


Fig. 5. *In vitro* DPPH scavenging activity of *S. virgaurea* L. extract before and after UVB irradiation.

Identical dependency was registered after ultra-sonication (150 gg cycle/20 sec) and dark incubation (at 297 K) of both extracts at different time intervals (the results are not presented).

CONCLUSIONS

In the present study the flavonoids content of *S. virgaurea* L. ethanol extract was determined by newly developed RP-HPLC methods. The concentrations of catechin, epigallocatechin and quercetin in the plant extract were 1384.23, 1427.04 and 18.9 μg mL^{-1}, respectively. It was established that the UVB-treated *S. virgaurea* L. extract demonstrated *in vitro* well-expressed DPPH radical-scavenging ability, SOD-like activity and antioxidant-protective capabilities due to the formation of stable *ortho*-semiquinone structures. The latter results prove that *S. virgaurea* L. extract is a promising ROS-scavenging antioxidant with UVB-protective properties and provoke future studies.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

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