EPR SPECTROSCOPY INVESTIGATION ON THE ANTIOXIDANT EFFICACY OF *SAMBUCUS NIGRA* L (DRY FLOWERS/BLOSSOM) EXTRACT

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ABSTRACT

The present study aims to evaluate the antioxidant efficacy and the lipid peroxidation degree of Sambucus nigra L. (S. nigra) blossom extract after induction of biotic stress. Dry stained extracts of S. nigra flowers were analyzed by direct and indirect in vitro EPR spectroscopy. The antioxidant activity of the extract was assessed by DPPH analysis, and the inhibition of lipid peroxidation - by PBN-spin adduction, after UV-B and thermal stress. The direct EPR stable radical structures presence was established either in non-irradiated and UV-B irradiated samples. Furthermore, as before and after UV-B and thermal biotic stress, S. nigra showed well-expressed scavenging abilities and protective properties against reactive oxygen and nitrogen species such as superoxide radicals (•O_2^-), hydroxyl radicals (•OH), and reduced lipid peroxidation.

Keywords: Sambucus nigra blossom, biotic stress, EPR spectroscopy.

INTRODUCTION

Bulgarian flora is a rich source of medicinal plants, which are the basis of traditional medicine [1]. Medicinal plants in Bulgaria have been used in healthcare since time immemorial. Numerous studies have shown the high content of phenolic compounds with antioxidant potential in some medicinal plants [2].

Sambucus nigra (*S. nigra*, black elderberry) belongs to the Adoxaceae family and is used to make tea, jams, syrups, infusions, juices, wines or medicines. It contains large amounts of useful compounds such as various sugars, organic acids, vitamins, polyphenols, etc. [3]. Moreover, high levels of polyphenolic compounds and anthocyanins in elderflowers inactivate harmful reactive oxygen species (ROS/RNS), directly protect cells from oxidative disturbances, stimulate vitamin function and directly inhibit lipid peroxidation and antioxidant activity. Elderberry flavonoids have antibacterial, antiallergic and antioxidant properties and are involved in the prevention of neurodegenerative, cardiovascular diseases, diabetes, cancer, etc. [4 - 6]. Młynarczyk et al. reported that the flowers and fruits of *S. nigra* are among the most widely used medicinal plants in the Bulgarian and Romanian phytopharmaceutical industry [7]. Aliakbarian et al. commented on the possible use of polyphenols from flowers and fruits of *S. nigra* due to their low bioavailability, solubility in water, rapid catabolism, low stability under biotic stress [8]. Polyphenols undergo oxidative degradation as a result of exposure to oxygen, ultraviolet (UV) light, enzymes, increased/decreased temperature and pH and metal ions, which quantitatively and qualitatively change their antioxidant properties [6]. Moreover, the polyphenolic content of *S. nigra* extract remains unchanged, thus protecting and neutralizing biomolecules from oxidative disturbances and preserving biochemical functionality [8]. In recent years, electron paramagnetic resonance (EPR) spectroscopy is increasingly used for this purpose as an adequate analytical technique that detects and

proves the structures of free radicals and specifically measures the levels of ROS/RNS [9, 10]. EPR spectroscopy methods are easily applicable to determine the ability of natural agents, their extracts, essential oils and bacterial metabolites *in vitro/in vivo* to inhibit specific radical species (peroxide, hydroxyl, organic or lipid-peroxide radicals) by their variable antioxidant activity [11 - 15]. Several reports have demonstrated the usefulness of *in vitro* EPR spectroscopy in determining radical capture, radioprotective (especially after UV/γ irradiation) and oxidative stress modulating activity of natural extracts, oils, etc. [16, 17]. EPR spectroscopy is an accurate method for studying and evaluating redox mechanisms, and antioxidant capacity, as well as for analyzing the quality, stability, and shelf life of natural plant extracts isolated from leaves, flowers, and fruits that are supposed to have antioxidant properties [18 - 20]. *In vitro* EPR spectroscopy directly proves the presence of stable radical structures, such as semiquinone, nitroxide, etc., in water-soluble or lipo-soluble samples [19, 20]. Most extracts of natural agents used in traditional medicine have stable radical structures and can be used strategically to develop protective creams, biomedicine, protection against UV-B or gamma radiation exposure, as cryoprotectants and as a sustainable resource for the biotechnology industry [21, 22].

Based on the following facts: 1) the application of abiotic and biotic stressers on natural extracts and compounds causes the formation of paramagnetic species; 2) EPR spectroscopy characterizes the radical structures and antioxidant capacity of various natural extracts and compounds, the purpose of the present study was justified. The aim is to study and compare the change in radical scavenging capacity, antioxidant, and radiomodulatory properties in dried *S. nigra* blossom extract before and after exposure to stressors, by using standard *in vitro* EPR methods.

EXPERIMENTAL

Plant material and extraction procedure

The row material, object of this study, was elderberry blossom (*S. nigra L*). Dry elderberry flowers (natural product without impurities) were purchased from a drug store, producer "Alin", Bulgaria. For each sample, 7 g of dry elderberry flowers were weighted by Sartorius Analytic balance (precision 0.1 mg) and put in a laboratory beaker (volume 250 mL). Distilled water was used as the extraction solvent, and the solid-liquid ratio was 1/30. The extraction process was performed in an ultrasonic bath (Elmasonic P 30 H, Elma, Germany), with a volume of 2.8 L. Its piezoceramic emitter has a frequency range of 37 - 80 kHz. The flask was fixed vertically in the center of the bath so that the solution was completely immersed in the liquid. The operating temperature (23ºC) was set in advance and kept constant by setting the thermostat built into the ultrasonic bath.

Thermal and ultraviolet-B oxidative stress induction

All samples were subjected to thermal stress in the range of 23ºC, 60ºC, and 90ºC with duration of 120 min. Also, all samples were irradiated from a distance of 20 cm from the light source with UV-B Transilluminator - 4000, with radiation 290 nm - 320 nm (peak 309 nm), purchased from Stratagene/USA. The experimental UV-B intensity was calibrated twice. For the maximum effect of UV-B radiation, the samples were irradiated in a wide range from 0 to 12 $kJ.m^{-1}$, without visible rays. UV-B irradiated energy was controlled with short-term exposure - 60 + 2 min; 120 + 2 min. To prevent the evaporation of the extract, the samples were kept in a horizontal position with a quartz coating to ensure maximum UV transparency [9, 10].

In vitro direct EPR study of elderberry extracts

The test samples (1 mL) of elderberry extracts were dissolved in deionized 50 vol.% water/ethanol, and were placed in standard quartz tubes. Measurements were started at first minute. The EPR parameters were: field center 3529.50 G, microwave power 20.61 MW, modulation amplitude - 10.00 G, field width 200.00 G, receiver bidding $2x10^3$, time constant 1310.72 ms; each sample was scanned three times. The g-factor and hyperfine characteristic constants aN and aH of the test samples were determined at room temperature (23°C) and a mean deviation of less than 2 %.

In vitro study of the formation of oxygen-centered radicals in extracts of elderberry flower

The formation of oxygen-centered radicals in elderflower extracts was determined by the method of Gao et al. with slight modifications [23]. The 1 mL of tested solutions (3.2 %) of elderberry extract was basified to pH = 8 with 5 mM NaOH. Measurements

were started at 1st minute, at room temperature (23°C), with parameters: field center 3513 G, microwave power 2.05 mW, modulation amplitude 10.00 G; each sample was scanned three times, and the mean deviation was less than 2 %.

In vitro indirect EPR study of antioxidant/ radical scavenging ability

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging capacity of the tested elderflower extract was determined by the method of Bernardo Dos Santos et al. [24], modified by Zheleva et al. [25]. To measure DPPH scavenging capacity, 98 % ethanolic DPPH solution (80 mM, stock) and test natural products (5 mM) were mixed and homogenized. After 1 minute of incubation in the dark, 0.20 μL of the mixture were transferred to non-heparinized capillary tubes (Micro-221), which were placed as sample tubes in standard EPR quartz tubes. Generation of the system (DPPH-H/R) was initiated after 1 minute and the DPPH solution was used as an internal standard. DPPH radical scavenging ability was calculatd relative to the equation:

$$Scavenged\ DPPH\ (\%) = [(I_O - I)/I_O] \times 100\%$$

where: I_o - the double-integrated intensity of the DPPH signal for the blank; I - the double-integrated DPPH signal intensity of the test sample.

In vitro indirect EPR study of alpha-phenyl-N-tetrabutylnitrone (PBN)

To measure the *in vitro* inhibitory ability, a PBN solution (5 mM, stock) dissolved in DMSO and 5 mM of the tested natural product was mixed and homogenized. After 30 minutes of incubation in the dark, 0.20 μL of the mixture were transferred to non heparinized capillary tubes. The PBN-generated adduct was a sextet. The amount of spin adducts formed between the PBN and the lipid radicals present was calculated after integrating the area under the EPR spectra recorded in the control sample and the samples containing the test product. The double-integrated PBN signal intensity from the test sample:

$$Scavenged\ PBN\ (\%) = [(I_O - I)/I_O] \times 100\%$$

Generation of the system was initiated after 30 minutes and PBN/DMSO solution was used as an internal standard at field center 3513 G, microwave power 2.05 mW, modulation amplitude 10.00 G, field width 200.00 G, 1 number of scans performed. The PBN inhibitory capacity of the test samples is calculated according to the given equation, where I_O is the double integrated intensity of the PBN/DMSO signal for the blank.

Statistical analysis

EPR spectral processing was performed using Bruker Win-EPR and Simfonia Software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., a one-way ANOVA, and Student-t-test to determine a significant difference between data sets. Results were expressed as mean ± standard error (SE). A value of $p < 0.05$ was considered statistically. Kinetic data were expressed as the average of two independent measurements, which were processed using the computer programs Origin 6.1 and Microsoft Excel 2010.

RESULTS AND DISCUSSION

EPR radical detection before and after oxidative stressors

Based on the fact that natural antioxidants contain stable radical structures [26, 27], we set out to study the presence of radical structures and their stability under various oxidative stressors.

Direct EPR spectroscopy recorded a stable singlet signal in *S. nigra* dry flower extract (Fig. 1(a)) with a g-factor 2.0052 ± 0.0002, and a peak-to-peak distance in the order of 9 G. The UV-B sample (Fig. 1(b)) showed an almost commensurate symmetric singlet intensity, but with a slight change in g value, characterized by $g\perp = 2.0049 \pm 0.0002$. Based on the data obtained, in identifying the registered free radical form, we hypothesized that the stable structure is due to the high content of rutin [28, 29]. Baycheva et al. [9] and Mendoza-Wilson [30] demonstrated that the presence of flavonoids and phenolic acids can form oxygen-centered free radicals in a strongly alkaline solution, stable enough to be detected by the EPR method. Alkalization of *S. nigra* flower extract resulted in resorption and abrupt disappearance of the EPR signal (Fig. 1(c)). From these results, it can be concluded that oxygen-centered stable structure is absent in *S. nigra* flower extract

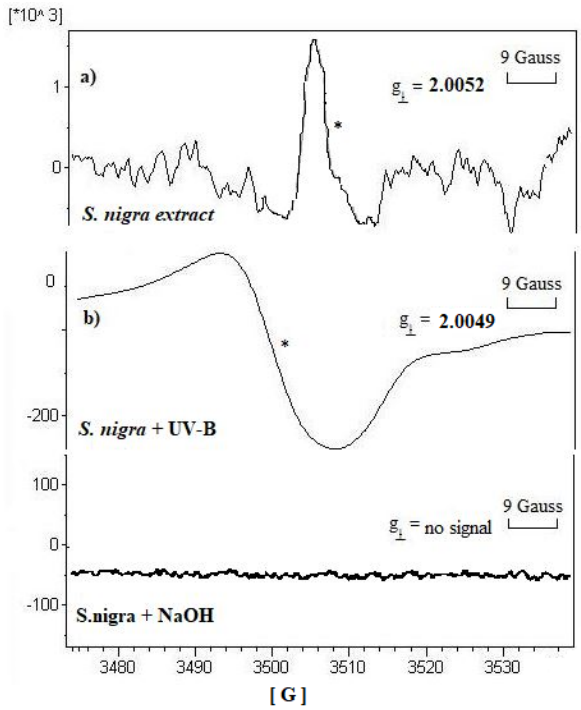

Fig. 1. Radical structures of *S. nigra* dry flower extract before radiation (1(a)), after UV-B radiation (1(b)) and (1(c)) after NaOH alkylation.

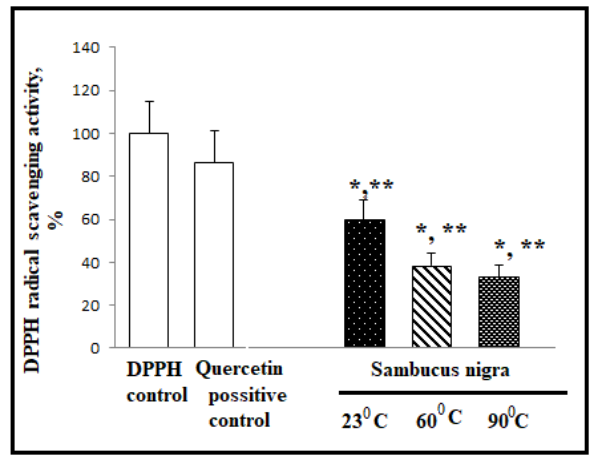

Fig. 2. DPPH radical - scavenging activity of *S. nigra* flower extract under different stressful environmental conditions (before/after the effect of UV-B radiation; before/after high-temperature heating) relative to DPPH ($p < 0.003$), and relative to Quercetin, as positive control ($p < 0.005$).

and the use of dry flower extract is inappropriate in production processes at pH > 7 due to possibly reduced antioxidant activity. In addition, Walz and Chrubasik commented that dilute *S. nigra* concentrate did not affect urine pH and did not affect both hydrogen and hydroxide ion concentrations, even with 24 hour excretion [31].

The biotic stress factors increase antioxidant activity

Over the last decade, the number of studies related to the protective ability of *S. nigra* blossom extracts to use as antioxidants in medicine and the pharmaceutical industry has increased. Data reported about UV-B radiomodulatory effects, immunostimulant, antiviral, and antibacterial activity of *S. nigra* that eliminate harmful ROS/RNS influences and counteract abiotic/biotic stressers that cause the development of many diseases in the human body [7, 29, 32]. Previous studies have commented on the strong free radical inhibitory ability and antioxidant/protective activity of elderberry extract due to the presence of large amounts of polyphenolic compounds, mainly flavonols, phenolic acids, and anthocyanins. Structural components are able to minimize the harmful effects of induced oxidative stress, subsequent redox changes in the studied system, as well as lipid peroxidation processes in cell constituents [4, 33, 34]. Michalak et al. found a difference in antioxidant activity in glycol extracts of rosemary and elderberry extract [29]. The presented results report a twofold decrease in the DPPH-scavenging ability of elderberry flower extract compared to rosemary and suggest that the inhibitory capacity is directly proportional to the content of polyphenols and flavonoids.

Based on the presented literature data, we investigated the 80 μM DPPH radical- scavenging activity of elderflower extract under different stressful environmental conditions (before/after the effect of UV-B radiation; before/after high-temperature heating). *S. nigra* flower extract at a concentration of 2 % tested under normal conditions (18 - 23ºC and humidity of 52 %) showed high inhibitory efficiency and antioxidant activity within 60.03 %, relative to DPPH ($p < 0.003$), and relative to quercetin ($p < 0.005$). The increase in temperature from 60°C to 90°C (Fig. 2) led to a twofold decrease in the scavenging values and neutralized ROS after induction of high-temperature biotic stress.

Plants photosynthesize and evolve under the

light environment, constantly exposed to UV (UV-C, UV-A, and UV-B regions) radiation. The UV-B spectral region (280 nm - 320 nm) is known to damage various physiological processes in plant cells, namely: photosynthetic damage, changes in membranes, biomass and epidermal deformation, proteins and DNA destruction, hormones inactivation, which influence the dry weight of the plant [9, 10, 35]. Many studies emphasize that fennel and elderberry extract alleviate UV-B-induced oxidative stress and subsequent inflammatory reactions by signaling the activation of the antioxidant system [36 - 38]. The plant „responses" to induced UV-B radiation vary depending on the variability and intensity of UV-B, as well as the degree of induction of antioxidant enzymes and protective molecules from the extract and activation of signaling pathways, preventing inflammation [39].

Baicheva et al. [9] and Yaneva et al. [10] note that cellular free radical damage caused by H_2O_2 and $\bullet O_2^-$ is the result of conversion to the more toxic •OH. In addition, the authors comment that UV-B causes constructive changes in plant cells and provokes the transformation of hydroxide (•OH) to superoxide ($\bullet O_2^-$) radical in the cell wall of the plant cell. Moreover, UV-B as a biotic stressers, changes the levels of gene expression and reduces the activity of endogenous enzymatic and non-enzymatic antioxidant systems of plant cells [40]. The results of the radical scavenging activity of elderflower extract after induction of UV-B stress relative to the stable DPPH radical are presented in Fig. 3.

S. nigra flower extract subjected to 2-hour UV-B induced biotic stress statistically significantly inhibited 51.03 % of the DPPH radical at a concentration of 2 %. It was found that the percentage of inhibited DPPH radicals in elderberry extract before irradiation did not differ statistically from the percentage of inhibited DPPH radicals at the same concentrations studied after UV-B induced biotic stress (51.33 %, $p < 0.05$). It is interesting to note that 60 min. of UV-B irradiation resulted in a decrease in antioxidant activity (51.33 % vs. 43.442 %, $p < 0.001$, Fig. 3.). A comparison of the results confirms that UV-B radiation as a stress factor does not affect the antioxidant activity of the extract. Moreover, elderberry extract, subjected to prolonged biotic stress, due to the presence of a stable radical structure inhibits ROS/RNS, probably by systemic activation of signaling

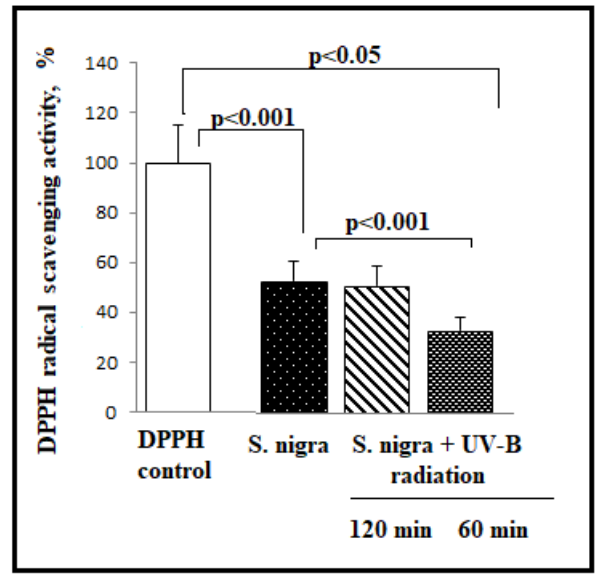

Fig. 3. DPPH scavenging activity of *S. nigra* flower extract in % before ($p < 0.001$) and after UV-B irradiation ($p < 0.001$).

pathways by antioxidant enzymes and non-enzymatic systems, inhibits residual toxicity and remodulates cellular signaling. In confirmation of our results on the antioxidant activity of aqueous extracts of elderberry blossom, Sidor and Gramza-Michałowska found 60.2 mg equivalents of ascorbic acid, relative to DPPH [41]. In addition, Yi Yang et al. [42] and Chen et al. [43] emphasize the positive effectiveness of UV-B radiation on various parts of medicinal plants and the significant increase in antioxidant activity and content of bioactive components.

The biotic stress factors increase lipid peroxidation

Inhibition of lipid peroxidation by natural agents is extremely important industrially. In addition, extracts with antioxidant properties are increasingly in demand in the emerging market for healthy ingredients. Peroxidation of cellular lipids is a characteristic reason for the deterioration of the lipid quality of extracts during processing or storage. Ongoing oxidative processes affect the lipid bilayer membrane and later determine the quality of the test substance, as well as determining the shelf life; loss of nutritional value; development of unpleasant sensory characteristics (tanning, darkening), and the appearance of toxic by-products [44, 45]. The

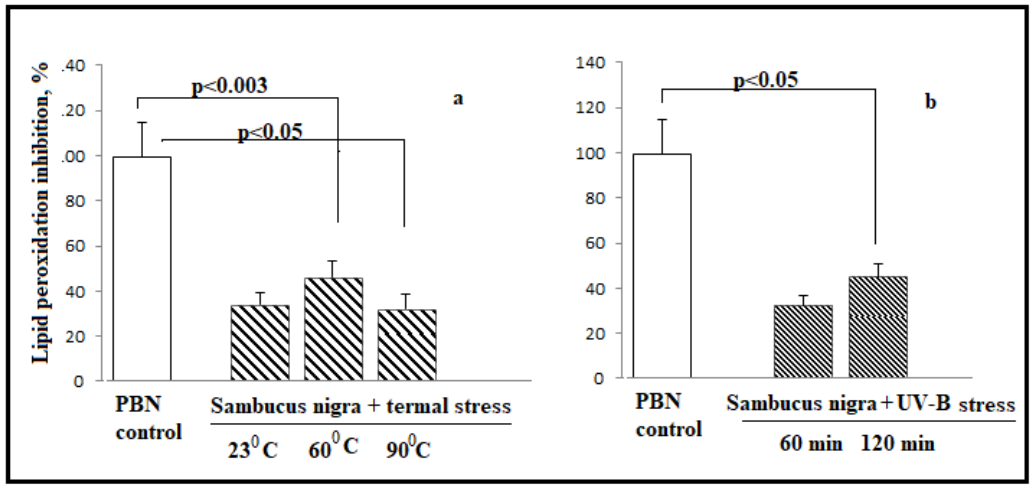

Fig. 4. Lipid peroxidation inhibition of *S. nigra* flower extract in % after: a) termal stress ($p < 0.003$) and b) UV-B irradiation ($p < 0.003$).

levels of reduction of lipid peroxidation from elderberry extract after adduction with PBN after artificially induced biotic stress - high temperature (60°C - 90ºC) and UV-B radiation are presented in Fig. 4. Maximum values of inhibition of lipid radicals from the extract of *S. nigra* flower after thermal stress were reported at 60 °C, within 41.7 ± 0.002 %, $p < 0.005$.

The levels of reduction of lipid peroxidation from elderberry extract after adduction with PBN after artificially induced biotic stress - high temperature (60°C - 90ºC) and UV-B radiation are presented in Fig. 4. Maximum values of inhibition of lipid radicals from the extract of *S. nigra* flower after thermal stress was reported at 60°C, within 41.7 ± 0.002 %, $p < 0.005$. In support of our results, elderberry extract statistically significantly reduced •OH, DPPH• radicals and inhibited lipid peroxidation in linoleic acid emulsion [41]. In contrast to our study, Araújo et al. reported that parts of plants or plant extracts exposed to biotic stress (temperature; UV-B) increase pigment content and malondialdehyde (MDA) concentrations [46]. Therefore, antioxidants, including *S. nigra* flower extract, act on the various stages of the radical formation process and this can be traced, taking into account their antioxidant action and lipid peroxidation in their cell membranes (*in vitro*), which implies successive steps of initiating, spreading and terminating the chain of radical formation and antioxidant inhibition [47, 48].

CONCLUSIONS

A direct EPR study of *S. nigra* dry flower extract was performed to determine antioxidant efficiency and lipid peroxidation.

- For the first time it was investigated and proved by direct EPR spectroscopy that a radical structure is present in the extract of dried elderflower;
- The results obtained for the dry flower extract of *S. nigra* under induced UV-B biotic stress prove the possible use of the obtained extract as a UV-B protector;
- Reduction of lipid radicals during artificially induced oxidative stress - high temperature (60°C - 90ºC), high frequency sonication (80 kHz), UV-B ionization in elderberry extract makes it a suitable tool for its inclusion in cosmetic emulsions.

These studies provide important information for the conduct of the production process using extracts of dried elderberry flower (*S. nigra*) in the pharmaceutical, cosmetic, and food industries and allow further research *in vivo*.

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