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An ethanol extract ability of cultivated white oregano (*Origanum heracleoticum* L.) of Bulgarian flora to attenuate oxidative stress effects formed under short-term UV-B radiation

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Ultraviolet (UV) radiation is a spectral part of the sun's rays, and plants have developed different mechanisms to cope with this potential oxidative stress factor. Due to their high sensitivity to UV-B exposure, the plant leaves need to react rapidly to minimize reactive oxygen species (ROS) generation. White oregano (*Origanum heracleoticum* L.) is an aromatic plant used as a spice and cultivated in the last years in Bulgaria, and its tolerance to short-term UV-B radiation remains unclear.

The aim of the present study was to determine for the first time the strong antioxidant activity towards DPPH stable spin-trap, *in vitro* direct and indirect EPR spectral characteristics and superoxide dismutase (SOD) content in Bulgarian *O. heracleoticum* leaves extract after UV-B exposure for 2 h (0 to 12 kJ/m). All measurements were performed with electron paramagnetic resonance (EPR) spectroscopy, twice: immediately and 6 months after the end of UV-B stress. Our data suggested that radical-scavenging and antioxidant abilities of the Bulgarian *O. heracleoticum* extract were not affected by short–term UV-B stress. Statistically significantly higher superoxide ($\bullet O_2^-$) scavenging activity (127.54±10.91U) (almost 6 times) after short-term UV-B exposure also supported the antioxidant properties of *O. heracleoticum* extract. Based on the EPR singlet signals with equal values of $g_\perp$=2.00456±0.0002 after alkalization we assumed that the *o*-semiquinone radical originates from the polyphenol substances present in high concentrations in Bulgarian white oregano extract. Our results indicated that Bulgarian *O. heracleoticum* extract has developed several antioxidant defense mechanisms and plasticity to dissipate excess UV-B energy and has the ability to remove free-radicals' stress.

Keywords: Bulgarian white oregano, EPR spectroscopy, antioxidant activity.

INTRODUCTION

Plants are a source of compounds with antioxidant activity; they use sunlight throughout their lifecycle for photosynthesis, in the processes of regulation and development. As a consequence, all plant species have evolved under the light environment, exposed to ultraviolet (UV) (UV-C, UV-A, and UV-B regions) radiation. UV radiation in the UV-B spectral region (280 - 320 nm) has received much attention in the last 20 years because rays from this region are known to damage various physiological plant processes including: DNA damage, direct photosynthetic damage, membrane changes, protein destruction, hormone inactivation, biomass reductions, and epidermal deformation [1, 2]. UV-B radiation exposure decreases plant height, leaf area and plant dry weight, increases auxiliary branching and leaf curling [3]. As reported by Jansen *et al.*, an excessive amount of UV-B radiation damages the plant cells [4]. Furthermore, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anion ($\bullet O_2^-$) play a prominent role in different stages of plant pathogenesis. Free radicals directly damage plant cells by disrupting membrane phospholipids, proteins and nucleic acids [4, 5]. Allan and Fluhr [6] noted that cellular damage caused by H_2O_2 and $\bullet O_2^-$ free radicals is the result of conversion to even more reactive species as highly toxic hydroxyl radical (•OH). In addition, Pristov and co-workers [7], by using spin- trapping electron paramagnetic resonance (EPR) spectroscopy, have found that UV irradiation sets off constructive changes in plant cells and provokes the transformation of hydroxyl radical (•OH) into $\bullet O_2^-$ radicals on the cell wall polygalactic acid. There is evidence that *M. oleifera* plants, exposed to UV-B irradiation increase the content of pigments and malondialdehyde (MDA) concentration [8]. Moreover, the plant cells irradiated with UV-B rays induce ROS-overproduction by altering gene expression levels and reduction of the activity of endogenous enzymatic and non-enzymatic antioxidant systems [9]. Furthermore, Yokawa *et al.* commented that free radicals participate as signaling molecules in the effective regulation of cellular redox homeostasis [10].

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In contrast, many researchers have demonstrated that short-term UV-B rates stimulate protective and photomorphogenic responses [11, 12] that affect the resistance of medicinal plants and prevent plant cells from UV-B oxidative stress and other biotic stress types [13]. Several studies report that the effects of UV-B radiation on different parts of medicinal plants significantly increase the antioxidant activity and content of bioactive components [14, 15].

White oregano *(Origanum vulgare* subsp. *hirtum* (Link) Ietswaart), (Syn. *O. heracleoticum* L., *O. hirtum* L.) is an herbaceous, perennial plant with white flowers and pinnate leaves, belonging to the Lamiaceae family. It originates from Europe, the Caucasus, Southwest and Central Asia, Mediterranean regions and was cultivated in Greece, Italy, Spain and Bulgaria [16-18]. The flowers, stems, leaves and other parts of *O. heracleoticum* contain essential oils (carvacrol, thymol, *p*-cymene), phenolic acids (rosmarinic and caffeic acids), flavonoids (apigenin, luteolin, salvigenin, cirsimaritin, diosmetin), anthocyanins [16, 19]. The highest antioxidant activity of *O. heracleoticum*, comparable to the synthetic antioxidants, was associated with compounds as phenols, tannins, rosmarinic acid and carvacrol [16, 19]. Oregano essential oils containing carvacrol and phenolic compounds have been shown to possess antioxidant, antibacterial, antifungal, diaphoretic, antispasmodic, anticancer and analgesic properties [20, 21]. Many investigations have determined EPR antioxidant activity and free radical formation of teas, extracts and oils of cultivated oregano after gamma-radiation [22, 23] estimations. The scientific reports on the photo-related, UV-protecting activities of natural products and raw materials and the importance of prevention UV-exposure-connected damages have increased over the last decade.

Therefore, the aim of the study for the first time was focused on the effects of short-term UV-B radiation (2 h) on the changes in the character of the free radical species of the cultivated Bulgarian *O. heracleoticum* ethanol samples, using direct and indirect electron paramagnetic resonance (EPR) spectroscopy. Additionally, the antioxidant activity and radiomodulatory properties *in vitro* of irradiated oregano samples were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide dismutase (SOD) scavenging assays.

EXPERIMENTAL

Plant material and alcoholic extraction procedure

The object of this study was cultivated Bulgarian white oregano (*O. heracleoticum*). The raw material (dried leaves) was purchased from "BulgarLuk" OOD– Katunica (Parvomai region, Bulgaria), in 2018 and deposited in the Faculty of Technic and Technologies, Department of Food Technologies, Yambol, Bulgaria. Identification of the spice material was performed according to the requirements of the European Pharmacopoeia, *EC 1441/2007* [24], and the chemical composition was determined (Table 1).

Table 1. Chemical composition of Bulgarian white oregano

Components	Content, %
Moisture	5.98±0.03
Protein	12.56±0.07
Fat	3.96±0.08
Fibers	10.36 0.09
Ash	8.90±0.04
Essential oil, (v/w)	5.74±0.05
Tannins	13.84±0,09

In total, 100 g ofleaves material was weighed and extracted with 200 ml of 70% ethanol (Sigma-Aldrich Co.) on an ultrasonic bath at 60^0C and followed sonication. The *O. heracleoticum* raw material was separated from the resulting mycelium after filtration by vacuum filter and the residue was washed twice with ethanol. A rotary evaporator (Witeg Labortechnik GmbH) at a water temperature of 60-65^0C was used to separate the solvent. The sample was collected at 4^0C/dark in plastic bags till further analyses. The resulting ethanol extract was analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/ MS) [25, 26]. Carvacrol (60,47%), thymol (8,55%), cymen-7-ol acetate, thymolhydroquinone, caryophyllene oxide, benzophenone, ethyl linoleate and other compounds were identified in the composition of *O. heracleoticum* extract according to ISO standards (ISO 11024-1:1998, ISO 11024-2:1998) [25, 26].

UV-B treatment

UV-B–vis Transilluminator-4000 capable of emitting between 290 nm and 320 nm (peak 309 nm) were purchased from *Stratagene/ USA*. The experimental UV-B intensity was calibrated in each experiment. To obtain the value of dose response about the UV-B radiation effect, the samples were irradiated over UV-B radiation wide range (0 to 12 kJ/m) without visible ray. The UV-B irradiated energy was controlled with short-time exposure - 120+2 min. Dark, fresh air and 40-41% relative humidity was circulated in the illuminator throughout the irradiation course. All samples were irradiated from 20 to 30 cm distance of the light source and a quartz cover was used to allow UV

transparency and to prevent the extract of evaporation and kept in a horizontal position.

Antioxidant activity on O. heracleoticum extracts by spectrophotometricall study

The superoxide dismutase (SOD) content of *O. vulgare* extracts, before and after UV-B exposure was determined according to Sun *et al.* [27] method. The xanthine/xanthine oxidase system (Sigma Chemicals, USA) was used to generate the superoxide anion ($\bullet O_2^-$). Superoxide anion reduces nitro blue tetrazolium (NBT; (Sigma Chemicals, USA)) to formazan after 20 min of incubation in dark. 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.

Antioxidant activity on O. heracleoticum extracts by EPR spectroscopy analysis

The scavenging ability of DPPH• (Sigma Chemicals, USA) was studied according to Santos *et al.* [28]. Thus, 30 µl of standard or 30 µl of oregano samples (30 µg/ ml) before and after UV-B-radiation were mixed with 250 µl DPPH• ethanol (96%) solution (80 µmol/l) and incubated in dark (3 min). After incubation at 20-23^{0}C room temperature the samples were transferred into the EPR cavity (6 mm thin wall, Wilmad Lab Glass), to obtain the EPR spectra. The scavenging ability was calculated according to the following formula:

$$\text{Scavenged DPPH radicals (\%)} = [(I_0 - I)/I_0] \times 100,$$

where I_0 is the integral intensity of the DPPH• signal of the control sample and I is the integral intensity of the DPPH• signal after addition of the test sample to the control sample. The settings were as follows: center field 3516 00 G, sweep width 200.00 G, modulation amplitude 5.00 G. The percent obtained DPPH• scavenging is proportional to the antioxidant activity of *O. heracleoticum* extracts. IC_{50} values denote the concentration of the sample that is required to scavenge 50% of DPPH• free radicals.

Direct EPR characterization of O. heracleoticum extracts

The 70% *O. heracleoticum* ethanol extract was divided into two parts. First part of the extract was not irradiated while the second was short-term UV-B irradiated (2 h^{-1}). Immediately after irradiation storage, the two oregano extracts were examined by direct EPR spectroscopy. The same measurement was done after their 6-month storage. EPR study was made on a X-band- EMX ^{micro} spectrometer (Bruker, Germany) equipped with a standard resonator. Spectral processing (g-value calculation) was performed with Bruker WIN-EPR and Sim-Fonia software. The following EPR settings were used: center field 3513.50 G, microwave power 20.03 mW, modulation amplitude 10.00 G; gain 2×10^2; time constant 327.68 ms; sweep time 61.44 s.

EPR characterization of O. heracleoticum extracts after system alkalization

A 10 mM sodium hydroxide (NaOH (*Sigma Chemicals, USA*)) water solution was added to the 70% *O. heracleoticum* ethanol extract and to the UV-B irradiated (2 h^{-1}) oregano extract in a ratio of 1:1. The signal was detected after 5 min of incubation, and settings were the same as above. Deionized and distilled water was used for all experiments. Other chemicals used were of analytical or HPLC grade.

Statistical Analysis

The EPR spectral processing was performed using Bruker Win-EPR and Sim-fonia software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student-t-test to determine significant difference among data groups. The results were expressed as means ± standard error (SE). A value of $p<0.05$ was considered 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.

RESULTS AND DISCUSSION

Short-term UV-B radiation increase in antioxidant activity

Herbal antioxidants are molecules that mitigate free radicals generation, biotic and abiotic oxidative stress and protect oxidation-chain reactions from terminating *in vitro* [15]. Leyva-López *et al*. [29] reported that phenolic compounds and flavonoids from oregano species possess a high antioxidant activity. Phenolic compounds support plants to survive and to adapt to irregularities in the environment through physiological functions. On the other hand, flavonoids, and in particular dihydroxy B ring in the structure, have the ability to absorb UV-irradiation and to stimulate pigmentation, growth regulation, feeding insects, and oxidative disease resistance [30].

The most commonly used to determine the antioxidant activity of *O. heracleoticum* extracts *in vitro* towards DPPH stable radical, was EPR spectroscopy.

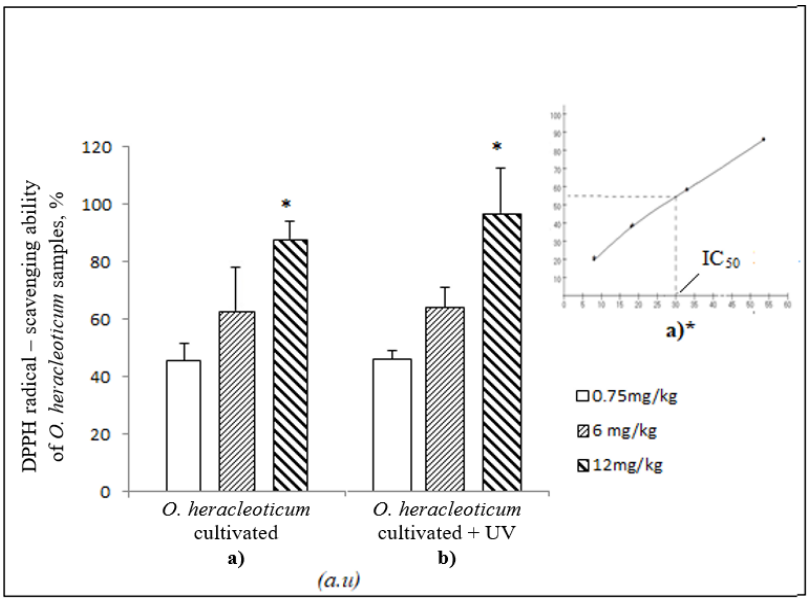

Fig. 1.

The untreated *O. heracleoticum* extract acted as a radical acceptor and significantly increased DPPH scavenging activity in a concentration-dependent manner in the EPR system. The maximum inhibition (88.755± 8.18%. $p<0.004$) (Fig. 1a) was obtained at a concentration of 12 mg/ml. Moreover, linear dependence of *O. heracleoticum* extracts concentration of 30.5±0.61 mM was defined as IC_{50} (Fig. 1a).

Our finding was in support of Amarowich *et al.* [31] and Shokrzadeh *et al.* [32]. The authors reported that ethanol *O. heracleoticum* extract possessed high scavenging capacity towards DPPH in a dose-depend manner, as opposed to thyme and marjoram extracts or to commercial antioxidant butylated hydroxytoluene (BHT). The effect of UV-B radiation on the oregano extract antioxidant activity is presented on Fig 1b. The radiation dose significantly enhanced the DPPH radical-scavenging abilities of the oregano extract (95.31±3.77 %, $p<0,005$) in a dose-dependent manner, compared to the DPPH standard.

The same dependency was established after ultra-sonication (150 gg cycle/ 10 sec) and incubation in the dark (at 25^0C) of both, untreated and irradiated samples at different time intervals (5 - 60 min). The 30-min incubation recorded a statistically significant maximum of EPR signal intensity (the results are not presented). UV-B high energy ROS mediation is particularly harmful to the DNA molecules whose nucleotides have absorption maximum near the border of the UV-B region waves [33, 10]. The well expressed DPPH-scavenging activity by the oregano extract is probably due to the development of effective mechanisms for UV-B radiation protection. Our results were sustained by investigations of Baranauskaite *et al.* who reported that the *O. heracleoticum* antioxidant activity is due to a variety of phenolic compounds and *carvacrol* potency [33].

Short-term UV-B radiation activate SOD-stress tolerance

UV radiation exposure accelerates the ROS overproduction across that of the normal oxygen metabolism, which leads to excessive oxidative stress effects, in relation to the different parts of plants. The superoxide radical generates a number of reactive and harmful secondary metabolites, such as alkoxyl radicals, H_2O_2, •OOH, ONOO−, •OH, lipid peroxyl that lead to damage to plant cells [34]. The adverse effects of $•O_2^-$ production could be reduced *in vivo* by the enzymatic activation of SOD, the levels of ascorbic acid, or anti-oxidant components in the plant cells, that increase the endogenous protection levels [35]. The reactivity of the 1 mg *O. heracleoticum* extract towards $•O_2^-$ radicals is shown on Fig. 2. It is seen that the SOD-like activity of *oregano* extract (111.93±11.34 U; $p < 0.003$) was 5 times higher than that of the used standard antioxidant L-ascorbic acid (1 mg).

Statistically significantly higher $•O_2^-$ scavenging activity (127.54±10.91U; $p<0.05$) (almost 6 times) after short-term UV-B radiation exposure also supported the antioxidant properties of the *O. heracleoticum* ethanolic extract. UV-B treated *O. heracleoticum* extract showed significant decrease and control of oxidation stress damages, plasticity and rapid response to environmental conditions. Our results indicate that the oregano plant has developed several antioxidant defense mechanisms to dissipate excess UV-B energy and free radical elimination capabilities [36].

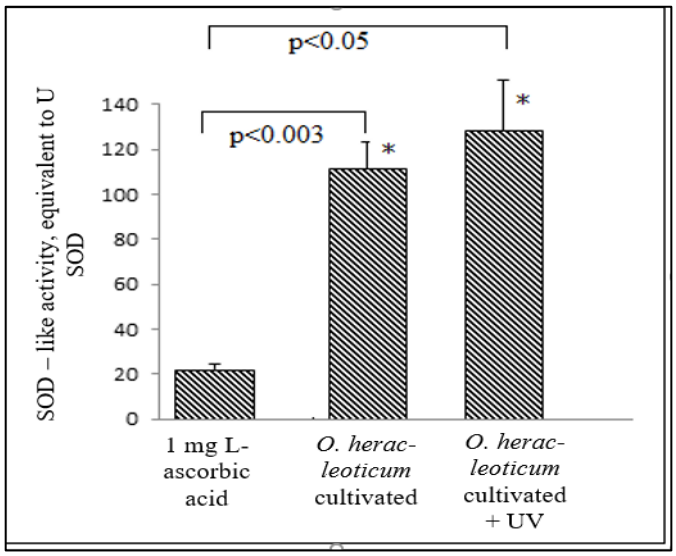

Fig. 2.

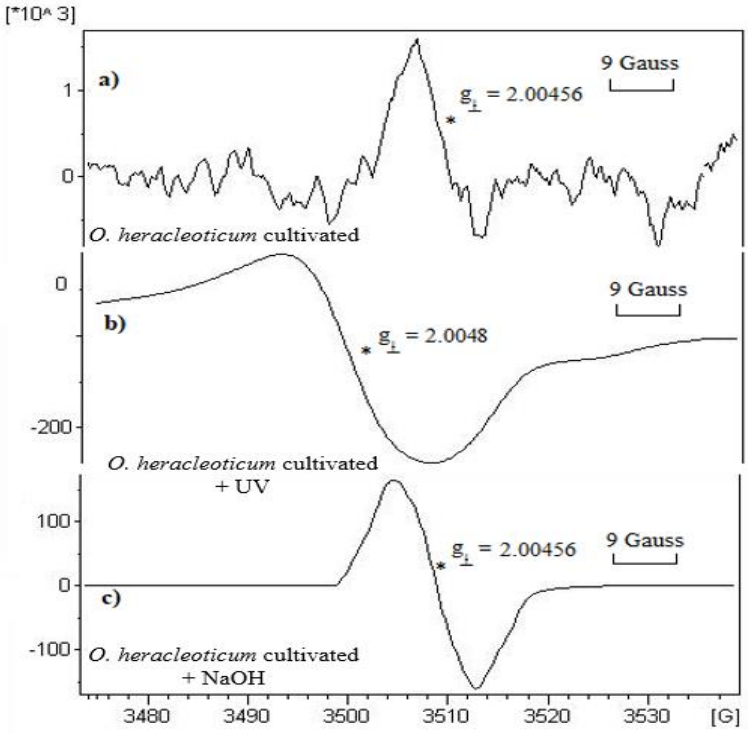

Fig. 3.

The acclimation to oxidative disorders is accomplished by initiating a series of enzymatic and non-enzymatic processes to prevent significant damage reaching the plasma membranes, chloroplasts or other plant organelles [37].

EPR spectral detection before and after short-term UV-B exposure

Moreover, gene expression could increase *oregano* plant tolerance to different stresses (UV-B irradiation), and many researchers invert attention to the potency of SOD-overexpression to increase oxidative stress tolerance [37, 38]. Importantly, the high concentration and synergism between carvacrol and thymol available in our oregano sample and the presence of other phenolic and no-phenolic components, also working as oxidation inhibitors, is positive for the high antioxidant activity [39, 40].

The detailed EPR spectral analysis (3513.50 G magnetic field) of the *O. heracleoticum* reference samples and of the short-term UV-B irradiated sample is depicted in Figure 3a. The *O. heracleoticum* non-irradiated spectrum was detected as a singlet almost symmetrical signal line characterized with a $g_{\perp}$=2.00456±0.0002 factor. The UV-B exposed (Fig. 3b) sample shows almost

commensurate singlet intensity, but with a slight change in the g value, characterized with $g_{\perp}$=2.0048±0.0002. The scavenged signal in both samples could be attributed to *o*-semiquinone radical structures produced by the oxidation of polyphenolic compounds present in plants, in accordance in previous investigations [41, 42].

Various EPR studies comment that flavonoids and phenolic compounds scavenging radicals effectively could form stable *o*-semiquinone structures in alkaline solution [43, 44]. To verify the possibility that the radicals registered in *O. heracleoticum* non-irradiated and UV-B treated extract belong to *o*-semiquinone class, their EPR spectra were evaluated in alkaline solution (1:1) (Fig. 3c). The application of alkalization on oregano samples led to EPR singlet signals with equal values of $g_{\perp}$=2.00456±0.0002. Based on earlier data [42-44] we assume that an *o*-semiquinone radical is practically not affected by short–term UV-B stress and originates from the polyphenol substances presented in high concentration in cultivated *O. heracleoticum* extract. Our EPR results complete the understanding that oregano extract acts as a strong reducing agent against ROS and in particular superoxide radical anion ($\bullet O_2^-$), and activates signaling pathways that prevent structural changes provoked by UV-B irradiation [45].

CONCLUSION

The current study for the first time demonstrates that the ROS inhibition and the negative oxidative stress effects caused by short-term UV-B exposure in cultivated Bulgarian *O. heracleoticum* extract lead to an increase in antioxidant activity. In conclusion, low doses of UV-B radiation induce protective effects based on signaling responses in plant cells and modulation in biological processes by activating enzyme activity tolerance and protecting constructive changes in plants compounds.

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

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