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Original Contribution

INVESTIGATION OF THE MODULATORY ROLE OF BULGARIAN *MENTHA PIPERITA L.* OIL AGAINST RADIATION-INDUCED OXIDATIVE DISORDERS AND ANTIOXIDANT STATUS *IN VITRO*

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ABSTRACT

This investigation report on the free radical-scavenging and radio-modulatory properties of Bulgarian *M. piperita* L. oil. The chemically pure oil (97 %) was tested alone and after expositer to ultraviolet radiation (290-320 nm) and gamma radiation at doses of 5, 10, 20 and 30 Gy. The *in vitro* antioxidant status studied by different spectrophotometrically methods of *M. piperita* alone was found to be lower compared to UVB and 10 Gy radiated oil. By direct EPR spectroscopy, single almost symmetrical EPR signals were registered in oil alone (g= 2.00492 ± 0.0003), UVB (g= 2.00586 ± 0.0002) and 10 Gy irradiated (g= 2.0019 ± 0.0002) samples. Antioxidant activity and radical scavenging capacity of *M. piperita* oil alone and after radiation was also evaluated by the DPPH test. The intensities of the EPR signals of *M. piperita* oil alone, and irradiated samples demonstrated the possible formation of stable radical structures. The EPR spectra considerably higher after radiation, and excellent DPPH scavenging capacity exhibited by *M. piperita* indicate good antioxidant potential, radio-modulatory activity and prevent effectively radiation–induced oxidative disorders.

Key words: *M. piperita*, EPR, Radiation, Oxidative disorders

INTRODUCTION

Widespread evidences indicate that exposure to ionizing and non-ionizing radiation results in significant changes in biological systems, viz., DNA, carbohydrates, polyunsaturated membrane and proteins, and caused increase in free radicals levels [1]. Ionizing radiation-induced reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide anion radicals ($^{\cdot}O_2^-$), hydroxyl radicals ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($RCOO^{\cdot}$) and $NO^{\cdot}$ radicals are active oxygen/ nitrogen components that are detoxified by endogenous antioxidant system [2]. After radiation exposure it is possible to generate excessive amounts of ROS and RNS, where the detoxifying cellular enzymatic and non-enzymatic mechanisms [3] in the body are insufficient. This unleashes pro-oxidant processes leading to oxidative stress (OS), irreversible cell dysfunction and cell death [4]. Over the last 20 years, antioxidants have been subjected to numerous studies related to their inhibition of the oxidation reaction effect, reducing the frequency of oxidative stress-disorders, associated with exposure to ionizing radiation (IR). Therefore, attention has been focused on the use of essential oils and their phytochemicals, for radical-scavenging activity and reduction in the radiation-induced ROS/ RNS and improvement of health [5, 6].

Peppermint in East Europe commercially grown for production of essential oil for pharmaceutical properties, body lotions, lip balms and dry leaves were used for herbal tea. Bulgarian *Mentha piperita* L. (Peppermint, *M. piperita*, *Lamiaceae family*), is a cultivated natural hybrid of *Mentha sivestris* L.,*Mentha longifolia* L., *Mentha viridis* L., *Mentha aquatica* L., also known as a hybrid mint or ‘’Bulgaro- Michum’’. It is a unique plant species with high quality [7, 8, 9]. The main constituents in extracted oil was monoterpene - menthol (66.4 - 68%) (*represented in free form menthol, isomenthol, and neomenthol*), and carboxyl esters, particularly- menthyl acetate [9]. The oil also contains small amounts of many additional compounds including menthone, *a*-pinene, *b*-pinene, sabinene, limonene, myrcene, cineole, *a*- and

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g- terpinene, hexanol, caryophyllene, pulegone and ets [9, 10].

Many investigators reported that *M. piperita* extract have several important properties including antioxidant, radical-scavenging, antibacterial [6, 11, 12], intestinal, neuronal, and cardiac preparations [13] vasodilatation, irritation, hyper secretion, influenced drug effects [14, 15]. Oral administration of *M. piperita* extract protected mice against the radiation-induced sickness [14, 16]. For the first time observed that the chloroform/ mint extract protected against the γ-radiation-induced oxidative stress and mortality [7, 16]. Also, was shown that the *M. piperita* extract possesses significant radioprotective properties [16, 17] in patients undergoing cancer treatment [7], and for reduction in irritable bowel syndrome (IBS) symptoms [18, 19] and anticancer properties [20].

IR is recognized method for maintaining the quality of aromatic herbs, oils, spices and vegetable, for a long time (Directive 1999/3/EC) [21]. Increased antioxidant activity was observed in essential oils derived from pre-irradiated leaves and fruits [22, 23]. Statistical analyses of antioxidant effects of UVB and gamma (γ) radiation on the content of volatile oils in spices showed that there were significant differences between γ-radiated and samples alone [22-24].

The usage of *in vitro* and *in vivo* model systems, serve as models for preliminary observations in the evaluation of pharmacological activities, radiation-induced oxidative changes in chemical composition and different EPR spectrum shapes as a function of temperature and time [23, 25, 26].

Keeping in view the multifarious properties of *M. piperita*, the present study was focused to find out the radioprotective properties of essential oil with a view to elucidating the mechanism of action, using a standard spectrophotometrically and Electron Paramagnetic Resonance technique (EPR) *in vitro* models.

MATERIALS AND METHODS

Isolation and characterization of *M. piperita* oil

The plant mass was collected early in the morning from experimental plots of 25 m² in 4 replicates. *M. piperita* plants were irrigated during a vegetative period to maintain 70-80% of the FWC. *M. piperita* was collected in full flowering form [8, 9, 10, 12]. The essential oil content in the biomass for the analyses was measured using hydrodilation in Clevenger Apparatus. *M. piperita* oil (purity 97%) was provided by "Institute for roses and aromatic plants'' Kazanlak, Bulgaria [8, 9, 10, 12].

Chemicals

2,2-dipheniyl-1-picrylhydrazyl (DPPH), Quercetin, 2,2-azinobis (3-ethylbenzothiazoline–6–sulfonic acid) sulfanilamide, Naphthylethylenediamine, Cholesterol (1:1 molar ratio), Soyalecithin were purchased from Sigma Chemicals, USA. Deionized and distillated water was used for all experiments. Other chemicals used were analytical or HPLC grade.

Irradiation

M. piperita oil samples (*in de-aerated capillary*) were exposed to UVB (*UV-vis Transilluminator- 4000, Bulgaria; 290-320 nm; two lamps; 220V ~ 50 Hz; microwave power 7.70VA; for 2 hrs rate; humidity- 40 %*) and to ^{60}Co radiation at dose 5, 10, 20, 30 Gy using γ- chamber Gamma Cell 5000 (*dose rate of 1.37 Gy/h, Board of Radiation and Isotope Technology, India*). Dosimetry was carried out using Baldwin Farmer's secondary dosimeter and Fricke's Chemical Method and all the radiation safety measures were strictly followed during experimentation. Fresh air was circulated in the chamber throughout the course of irradiation.

ANTIOXIDANT ACTIVITY

Reducing Power Assay

The reducing power potential of UVB and γ-irradiated samples and oil alone, was determined by the method of Oyaizu, 1986 [27]. A 50 μg/ml concentration of *M. piperita* oil alone, after UVB, and 5, 10, 20, 30 Gy irradiation was firstly tested to determine the concentration at which oil exhibited maximal reducing potential both, immediately and 24 hours post irradiation. The reaction mixture was left for 10 minutes at room temperature (27^0C) and the absorbance was measured at 700 nm. An increased absorbance of the reaction mixture indicates increased reducing power:

$$\%Inhibition=[(OD_{control} - OD_{test})/OD_{control}]x\ 100$$

DPPH radical- scavenging activity

Radical scavenging activity of UVB and γ-irradiated (5, 10, 20, 30 Gy) samples and *M. piperita* oil alone against the stable DPPH radical was determined according to Cuendet at all, 1997 [28], with modifications. Briefly, 1.0 ml of DPPH (100μM) was added to 50 μg/ml 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. The percent of DPPH radicals scavenged was calculated according to the equation:

$$\% \text{ Inhibition} = (A_{control} - A_{test}) / A_{control} \times 100$$

Non-enzymatic antioxidant ($ABTS^{\bullet+}$) radical-scavenging assay

The $ABTS^{\bullet+}$ diammonium salt radical cation decolourization test is also a radical-scavenging test. $ABTS^{\bullet+}$ assay of UVB and 5, 10, 20, 30 Gy γ- irradiated samples and *M. piperita* alone (50 μg/ml volume) was performed by Re et al., 1999 [29] with slight modifications. The reaction mixtures were incubated at 24^0C for 30 min and the intensity of chromogen was measured at 734 nm against PBS as control. Antiradical activity of examined sample was presented as the percentage of $ABTS^{\bullet+}$ radical scavenging and calculated according to the equation:

$$\% \text{ Inhibition} = (A_{control} - A_{test}) / A_{control} \times 100$$

RADIATION PROTECTION ACTIVITY IN LIPID PHASE

Nitric oxide (NO^-) ion scavenging assay

The presence of nitrite potential, a stable oxidized product of nitric oxide (NO^-) of UVB and 5, 10, 20, 30 Gy γ- irradiated samples and *M. piperita* alone (50 μg/ml) was determined according to the standard methodology described by Shirwaikar et al., 2006 [30]. The scavenging potential was evaluated as decrease in percent absorbance of the chromogen formed by diazotisation of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine recorded at 546 nm.

Membrane protection activity against radiation- induced damage

Cholesterol and Soyalecithin (1:1 molar ratio) were suspended in an appropriate amount of chloroform. A thin film was developed by complete evaporation of chloroform in a rotary evaporator (Buchi, New castle, USA) at 40^oC. The film was subjected to hydration in (0.1 M, pH 7.4) PBS and were incubated in (40^oC) water bath for 4 hr. The stock solution was diluted with PBS to the final concentration in terms of phospholipid content [31]. Different treated oil samples, liposome only (untreated), radiation only (2.5Gy), liposome + *M. piperita* oil and liposome + *M. piperita* oil+ 2.5 Gy were evaluated for the levels of malondialdehyde, the final product of membrane degeneration. A radiation dose of 2.5 Gy at a dose rate 1.37 kGy/h was used and after exposure the samples immediately were incubated for 1h at 37^oC. 10 % TCA and 0.5% thiobarbituric acid, 1:1 ratio and 0.025 M NaOH were added. The mixture was heated in water bath (80ºC) for 1 h and absorbance was measured at 535 nm [32].

ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY (EPR) ANALYSIS

***In vitro* direct EPR studies on *M. piperita* oil alone and after UV or γ-irradiation**

EPR experiments were carried out an X-band EMX^{micro} spectrometer (Bruker, Germany) equipped with standard resonator. Spectral processing (*g*-value calculation) was performed with Bruker WIN-EPR and Sim-Fonia software. Spectra of *M. piperita* oil alone were at following settings: center field 3514.00- 3516.00 G, sweep width 200.00 G, microwave power 0.634-3.323 mW, modulation amplitude 1.00- 5.00 G, gain 1×10^5, time constant 1310.72 ms, sweep time 5.24 s, 1 scan per sample. The EPR settings for the UVB and γ-irradiated samples were the same, only the modulation amplitude was reduced to 1.00- 5.00 G.

EPR determination of DPPH radical scavenging activity

By studies according to Santos et al., 2009 [33] with slight modifications Zheleva et al., 2012 [34] was determine the ability to scavenge stable DPPH radical. *M. piperita* oil (50 μg/ ml) alone, UVB and γ-irradiated samples was added to 250 μl ethanol solution of DPPH (80 μmol/ l). After 10 min/ dark incubation at 25^oC samples were transferred into the EPR cavity, and the scavenging ability was calculated as follows:

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

where $\mathbf{I_0}$ 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 tested oil sample to the control sample.

The control samples contained 250 μl of DPPH/ ethanol solution + 50 μl ethanol. The settings were as follows: center field 3516.00 G, sweep width 200.00 G, microwave power 3.232 mW, modulation amplitude 5.00 G, receiver gain 5.02×10^3, time constant 163.84 ms, 1 scan per sample.

STATISTICAL ANALYSIS

Statistical analysis was performed with Statistica 6.1, StaSoft, Inc. and results were expressed as means ± standard error (SE). Statistical significance was determined by the Student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS and DISCUSSION

Plethora of essential oils and herbal extracts was screened to minimize UVB and γ radiation induced oxidative changes and degradation of macromolecules and membrane-lipids peroxidation, after radiotherapy or unplanned radiation exposures [1, 35, 36]. The oxidation of membrane macromolecules leads to persistent oxidative disturbances, obstruction of intracellular processes, may result in tumor formation and oxidative stress [36]. Therefore, it is necessary to observe an effective radiation-protected natural antioxidant, supporting delaying or inhibiting cellular damages, mainly through the properties of free radical extraction [36]. Thus, *M. piperita* L. essential oil was evaluated for its *in vitro* stable antioxidant activity, scavenging of ROS/RNS [37] and radiation- modulative properties.

The reducing power potential is generally associated with the presence of reductants, which exert antioxidant activity by breaking the radical chains by donating an electron [38]. Maximal reducing power for *M. piperita* alone at 50 µg/ml concentration was 0.245 ± 0.036 %. After exposure of UVB, oil exhibited higher reducing power (0.346 ± 0.006 %), compared to oil alone (0.245 ± 0.036 %). Maximum reducing ability was found for 10 Gy sample (0.474 ± 0.021%), compared to *M. piperita* alone (0.245 ± 0.036, $p<0.05$). *M. piperita* possess good reducing potential towards Fe^{3+} complex as after UVB up to 10 Gy **(Figure 1).**

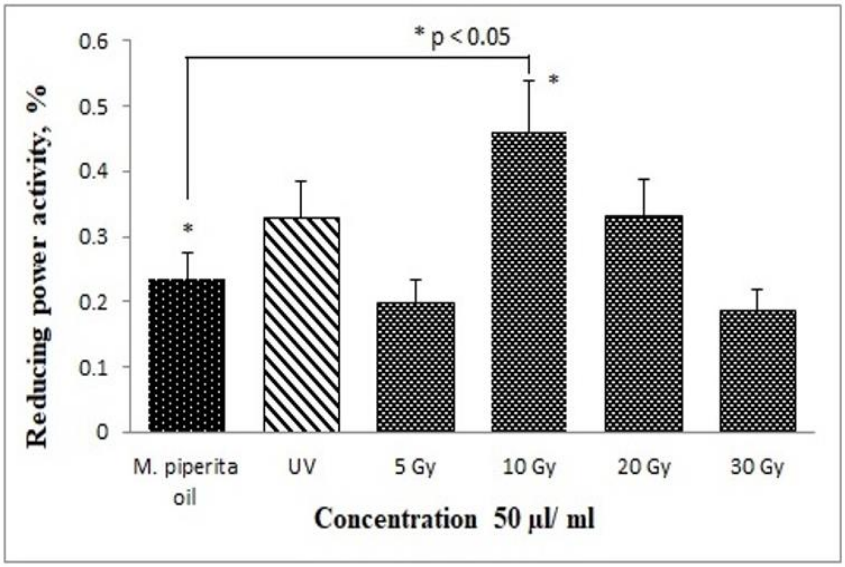

Figure 1. The percent reducing power potential (%) of *M. piperita* oil alone and after UV and 5-30 Gy γ–radiation. Results are expressed as mean of replicates (in thrice) from three independent experiments. (*) Significants ($p < 0.05$) as compared to *M. piperita* oil alone.

Results of 24h post-irradiation treatment of *M. piperita* samples showed significantly decrease in percent reducing power potential compared to oil alone and to samples measured immediately following irradiation (*results not shown*). IR increases the iron loading in the cellular environment resulting in hemolysis [38]. Probably, *M. piperita* exhibits antioxidant protection to overcome radiation- oxidative disorders *in vivo*, by reducing Fe^{3+} to less harmful Fe^{2+} cations.

Figure 2 indicate *in vitro* DPPH radical inhibitory effect was found to be strongest in 10 Gy irradiated *M. piperita* samples (83.52% DPPH, $p<0.05$) with slight difference in case of UVB irradiated *M. piperita* samples (79.4% DPPH, $p<0.04$) and *M. piperita* alone (73.7% DPPH, $p<0.05$) at concentration 50µg/ml, compared to positive control. Previous studies showed that menthol is the main component of *M. piperita* oil [12, 40, 41] and menthone, limonene and methyl acetate as second components, were responsible for scavenging effectiveness towards DPPH radicals and good antioxidant activity of oil [12, 42]. Considerable increase in DPPH- radical scavenging of the *M. piperita* oil at UVB and 10 Gy radiations could be explained with unchanged stable structures in compound as menthol, menthone, limonene and methyl acetate which probably largely determine its antioxidant activity [43].

The total antioxidant activity of oil-hydrogen-releasing antioxidants and those derived from lipid-peroxyl radicals are readily stained by the ABTS free radical scavenging models method [42].

At 50 µg/ml, both UV (43.1 ± 1.41%) and 10 Gy irradiated (58.44 ± 1.33 %) samples **(Figure 3)** exhibited increase in scavenging abilities towards ABTS•+, compared to *M.*

piperita alone (41.87± 0.91%). Only at 10 Gy irradiated (58.44 ± 1.33 %; p<0.05) samples were established significant increases in $ABTS^{\bullet+}$ scavenging, compared to standard. According to Singh, Shushni and Belkheir [44], *M. piperita* has high ABTS inhibitory potential, due to the content of menthol, methyl acetate and etc.

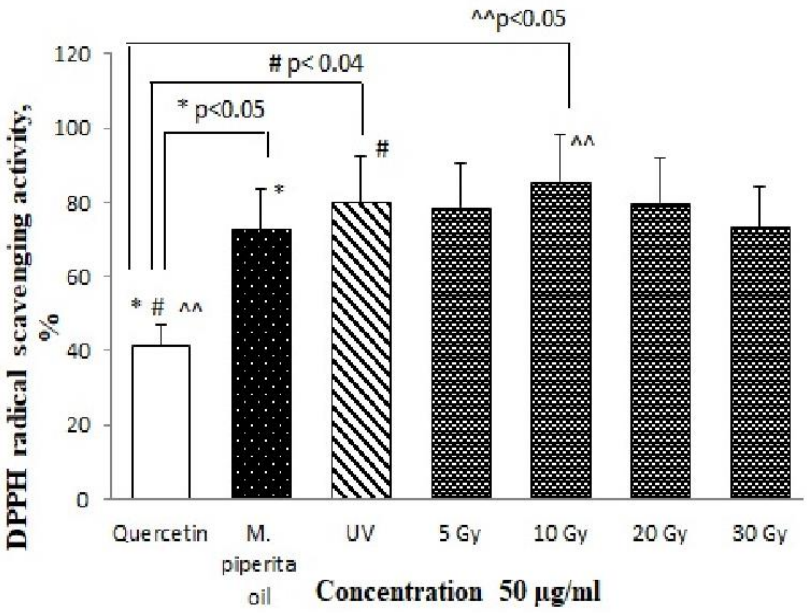

Figure 2. The DPPH- radical scavenging activity (%) of *M. piperita* oil alone and after UV and 5-30 Gy γ–radiation. Results are expressed as mean of replicates (in thrice) from three independent experiments. Significants (*p < 0.05; #p<0.04; ^^p<0.05) as compared to Quercetin, as positive control.

Essential oils were widely used as oxidation-food protectors and can serve as radical-scavengers in the pharmaceutical and cosmetic industries, especially neutralizing radiation. On the other hand, Jordan et al. [45] reported that essential oils containing α- and γ-terpene, terpinolein, etc., were capable of donating hydrogen atoms to stabilize $ABTS^{\bullet+}$. These components predominate in the *M. piperita* in this study at very low concentrations. Probably, this fact explains the low observed inhibitory activity of the oil alone, compared to the quercetin, as positive control.

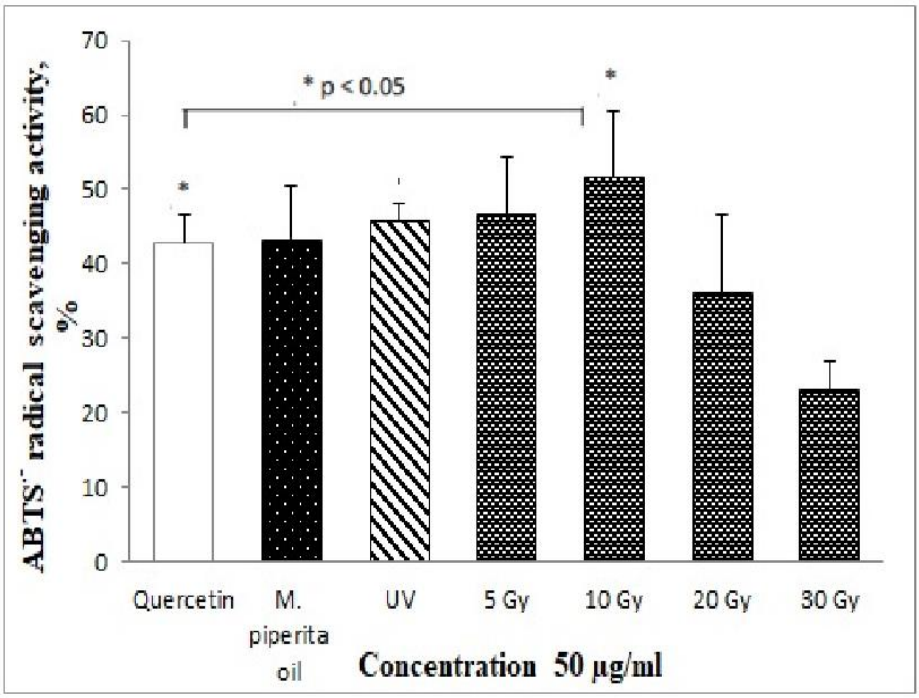

Figure 3. The $ABTS^{\bullet+}$ radical scavenging activity (%) of *M. piperita* oil alone and after UV and 5-30 Gy γ–radiation. Results are expressed as mean of replicates (in thrice) from three independent experiments. (*) Significants (p < 0.05) as compared to Quercetin as positive control.

The past decades have identified nitrogen oxide ($NO^{\bullet}$) as reactive nitrogen species (RNS), important molecule messenger/ plant growth regulator, whose concentration increases during oxidative cellular damages, but also during specific plant development processes [46, 47]. $NO^{\bullet}$ scavenging ability against **(Figure 4)** increases with increase the radiation of 10 Gy. At 50µg/ml maximum NO• scavenging activity were 79.11 ± 3.1%, p< 0.05 for UVB samples and 89.92 ± 7.01%, p<0.05 for 10 Gy irradiated samples, compared to *M. piperita* alone (59.92 ± 5.61%). *In vitro* tested samples of *M. piperita* alone and after raditaion reduced $NO^{\bullet}$ production indicating that oil contains hydrophilic antioxidant substances possessing $NO^{\bullet}$ scavenging ability. Furthermore, for tested samples of UVB and 10 Gy oil, showed higher scavenging activity compared to *M. piperita* alone, suggesting that after radiation a new additional structures were involved in reducing of $NO^{\bullet}$ radicals levels.

Pathologically, IR induced oxidative disorders generates $NO^{\bullet}$, and play a critical role in initiation and progression of pro-oxidative damages and dysfunction of the nervous system, as well [48].

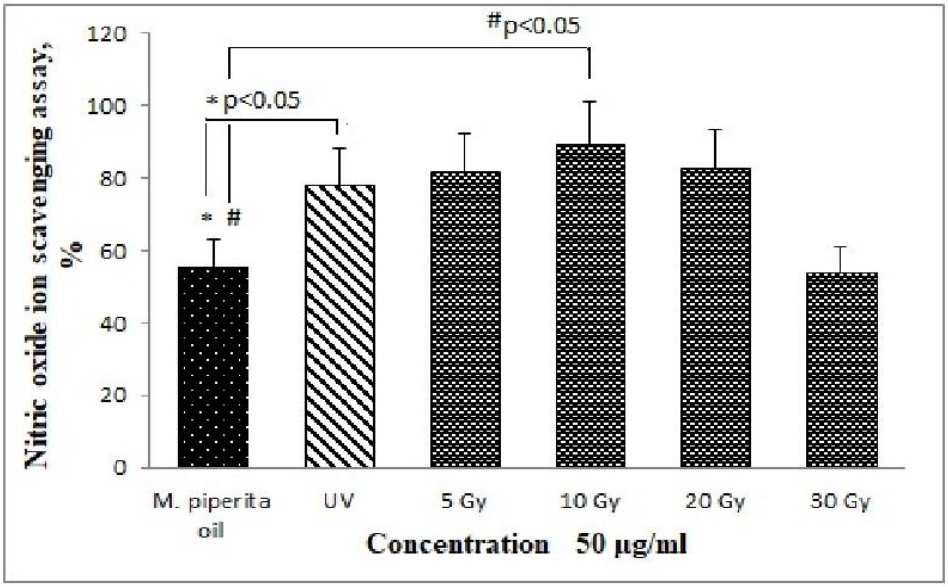

Figure 4. The Nitric oxide radical scavenging activity (%) of *M. piperita* oil alone and after UV and 5-30 Gy γ–radiation. Results are expressed as mean of replicates (in thrice) from three independent experiments. (*, #) Significants ($p < 0.05$), as compared to oil alone.

Samarth and Kumar, 2003 and Samarth et al., 2004 [14, 49] comment that *M. piperita* extract has been radioprotective in whole-body of animals when administered prior to 8-10 Gy of γ-radiation exposure. Also, $O_2^{\bullet-}$ anion reacts with $NO^{\bullet}$ radicals to serious toxic products affecting protein, lipids and neurons [50]. Bearing in mind, *M. piperita* extracts exhibits antioxidant- scavenging activity, Kma L, 2014 [51] show that *M. piperita* could be used as neuron-protective agent against radiation-induced apoptosis in the central nervous system.

In fact, natural flavonoids and polyphenols have potential to inhibit lipid peroxidation by suppression of highly-reactive lipid-peroxyl radicals and reduce iron in lipooxygenase enzyme and thus prevent initiation of lipid peroxidation reaction [52]. The membrane system (artificial liposomes) was utilized to estimate the possibility of *M. piperita* oil to protect the membrane lipids of liposomes against 2.5 Gy peroxidation (dose rate =1.26 KGy/hr). The most effective dose for inhibition of peroxidation in the tested liposome system was 100µg/ml. *M. piperita* oil exhibited significantly higher membrane protection at 150µg/ml (0.0696 ± 0.34%; **p<0.04). The test region at 100-150µl/ml of 2.5 Gy was registered maximal lipid-peroxidation protection **(Figure 5).** 2.5 Gy *M. piperita* samples indicated two times higher anti-lipid peroxydation, compared to *M. piperita* alone. We assume, that the highest membrane protection after IR, due to the chelation of transition metal ions [53]. Good ability of leaf extract of *M. piperita* to significant decrease in malondialdehyde inhibition in liver homogenates after radiation is in agreement with our reported results [54].

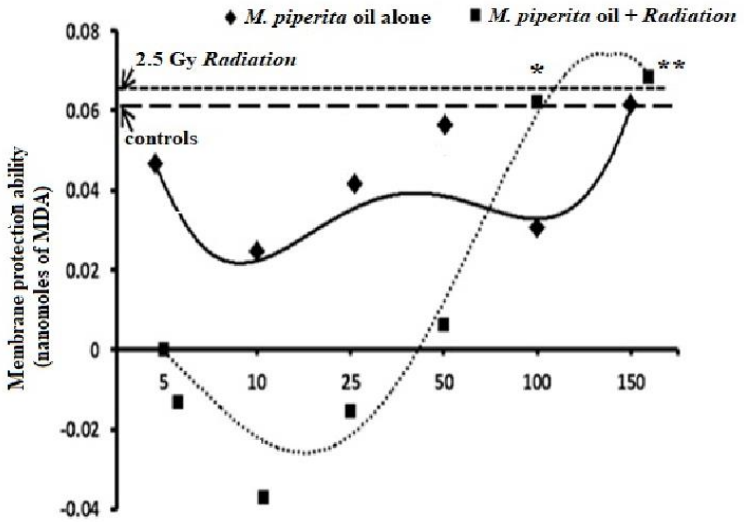

Figure 5. Analysis of the membrane protection ability (%) of *M. piperita* oil utilizing an artificial membrane system (liposome). The artificial system were irradiated with dose rate =1.26 KGy/hr, for 3.1 min. A significant (*p< 0.04) decrease in the formation of malondialdehyde (MDA) was recorded at 150 µg/ml of 2.5 Gy irradiated *M. piperita* samples. The lipid peroxidation activity is expressed as nanomoles of MDA formed. Each experiment was performed in triplicate and was repeated three times.

IR degrades the membranes and walls of the cell and so facilities a more efficient release of the extractable substances [55]. EPR spectra registered in the *M. piperita* oil alone and UVB and 10 Gy irradiation are presented on **Figure 6.** The spectra registered in *M. piperita* oil alone exhibited almost a symmetrical single EPR spectral line with g= 2.00492± 0.0003 at 3514 G magnetic field, and EPR signal intensity DI/N- 1.826. The EPR signals registered after UVB (g= 2.00586 ± 0.0002 with a small characteristic splitting) and 10 Gy (g= 2.0019 ± 0.0002) (DI/N- 1.831, arbit. units) radiation were different, compared to signal in *M. piperita* alone. It is suspected, that the radical structure presenting in *M. piperita* alone was affected by both types radiation, without affecting ($p > 0.05$) the spectra intensity. The emergence of a new radical structure in 10 Gy irradiated *M. piperita oil* is due the lower g-value. Similar structural alterations after radiation exposure were recorded in *Lavandula Angustifolia M*., *Rosa Damascena L.* and *Silybum Marianum* [56, 57, 58].

DPPH stable radical easily react with *in vitro* antioxidants and free radicals that possess functional groups donating $H^{\cdot}$ and these interactions written as:

$$\mathbf{DPPH^{\cdot} + R^{\cdot} = DPPH - R} \qquad (1)$$

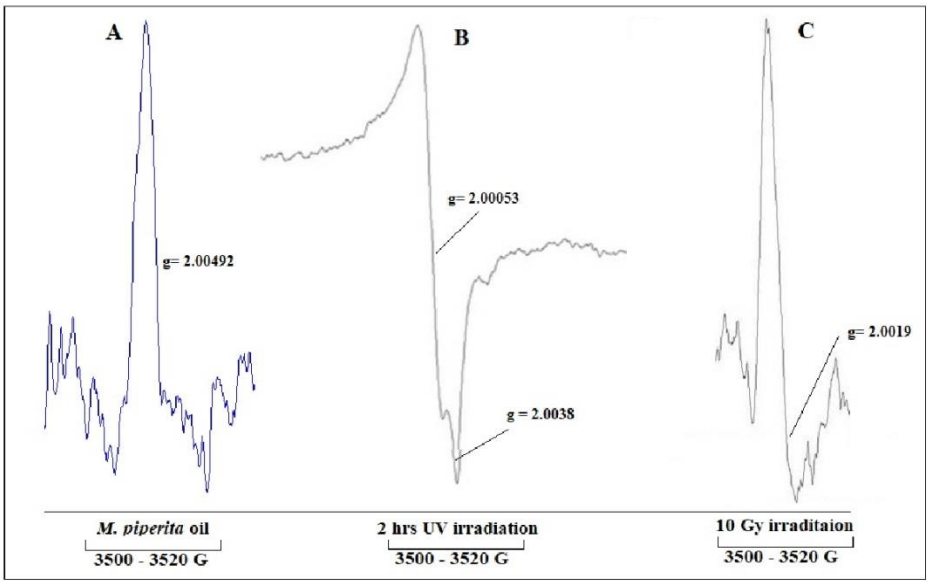

Figure 6. First derivative of the absorption curve (arbit. units) of *M. piperita* oil registered before radiation (**A**) after 2h UVB (**B**) and 10 Gy irradiation (**C**) with respect to the applied magnetic field (G).

Using the DPPH we have applied a direct EPR method [59] to evaluate and compare the *M. piperita* radical- scavenging capacity alone and after radiation **(Figure 7).** UVB (89.97 ± 0,037 %, p<0.003) and 10 Gy- irradiated (94.57 ± 0,09 %, p<0.05) samples, exhibited 2 times higher scavenging capacity, compared to *M. piperita* alone (48.81 ± 0,099%). Amplification in scavenging ability, present at oil alone and irradiated *M. piperita* samples, might be explained by strong antioxidant activity and the formation of stable radical in the chemical composition, resistant to IR- oxidation**.** Samartha et al., 2017 [60] consider that pretreatment *of M. piperita* extract/ oil prior exposure to γ-radiation in animals has been shown to provide protection against hematopoietic injury in bone marrow, intestine and testis in bone marrow cells and significantly reduced the number of chromosomal aberrations.

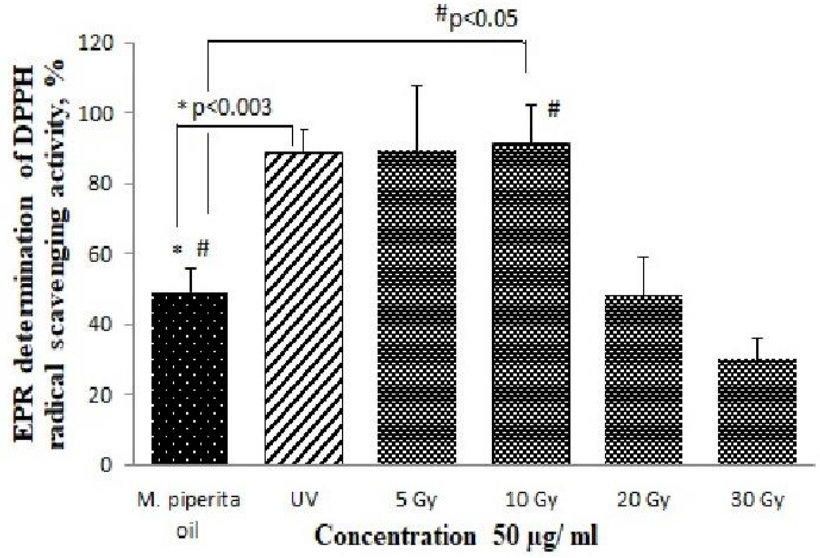

Figure 7. The percent scavenged DPPH radicals of *M. piperita* oil alone, after UVB and 10 Gy irradiation. Results are expressed as mean of replicates (in thrice) from three independent experiments. (*, #) Significants (p < 0.05), as compared to oil alone.

CONCLUSION

For the first time modulatory role of Bulgarian *M. piperita* oil against radiation-induced oxidative disorders was tested by different in vitro methods. Bearing in mind both, the presence and stability of the new radical structures formed after irradiation and higher protective effect (in the respect of excellent DPPH radical scavenging capacity) we consider oil might be suitable for medicinal treatment, preparation of cosmetics, and as *in vitro* good antioxidant/ radioprotector, as well.

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