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Antigenotoxic and antioxidant activities of a polyphenolic extract from European *Dracocephalum moldavica* L.

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ABSTRACT

The aim of the present study was to investigate the antigenotoxic and antioxidant activities of the crude hydromethanolic extract from the aerial parts of European *Dracocephalum moldavica* L. (Moldavian dragonhead). The total phenolic content estimated by the Folin–Ciocalteu assay was 289.55 ± 2.63 mg of GAE/g of dry extract, and rosmarinic acid was the major polyphenol of *Dracocephalum moldavica* extract (107.11 ± 0.83 mg/g of dry extract). *In vitro* antioxidant assays revelead remarkable scavenging effects against DPPH (EC_{50} = 23.10 ± 0.10 μg/mL), ABTS (EC_{50} = 8.0 ± 0.10 μg/mL) and superoxide anion radicals (EC_{50} = 445.5 ± 2.3 μg/mL). The extract showed a high ferrous ion chelating activity (EC_{50} = 35.70 ± 0.40 μg/mL), a considerable reducing capacity, and good hydroxyl radical scavenging properties. *Dracocephalum moldavica* extract reduced, in a concentration-dependent manner, DNA damage induced by bleomycin in normal human dermal fibroblasts as measured by comet assay and micronucleus test. Exposure of dermal fibroblasts to *Dracocephalum moldavica* extract (100 μg/mL) after preincubation with bleomycin (10 μg/mL) resulted in the most significant antigenotoxic activity. The protective effects may be due to the free radical scavenging activity, iron-chelating properties and the possible intervention on DNA repair processes.

1. Introduction

Elevated levels of oxidative stress and redox imbalance induced by various exogenous agents such as ionizing radiations, UV light, chemicals, air pollutants, can alter the structure and functions of different biomolecules (DNA, proteins, lipoproteins, polyunsaturated fatty acids, amino acids). Continuous and cumulative DNA damage has serious consequences: cellular senescence, mutations, apoptosis, degenerative diseases, cancer (Aziz et al., 2012). Antioxidants can protect against genotoxic agents by decreasing oxidative stress and primary DNA damage (Caputo et al., 2012). An increasing number of studies have showed that different plant-derived extracts and phytochemicals possess significant antioxidant activity (Cao et al., 2015; Xia et al., 2014; Li et al., 2013) and display antigenotoxic effects against oxidative DNA-damage induced by various genotoxicants. Particularly, plant polyphenols have shown to be potent dietary antioxidants. In addition, they can modulate DNA-repair mechanisms being among the most promising phytochemicals that can be exploited as genoprotective agents (Ramos et al., 2011).

Dracocephalum moldavica L., Lamiaceae (Moldavian balm, Moldavian dragonhead) is a perennial herb native to Central Asia and naturalized in Central and Eastern Europe. It is traditionally used in gastric, liver and cardiovascular disorders, headache, but also as food additive (Dastmalchi et al., 2007b). The aerial parts of the plant are reported to contain various polyphenols, especially hydroxycinnamic acids (rosmarinic and caffeic acids) and flavonoids such as apigenin, luteolin and their glycosides, quercetin, diosmetin, kaempferol, acacetin, agastachioside and salvigenin (Yang et al., 2014). Recent investigations have revealed significant therapeu-

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tic effects of *D. moldavica* extracts: cardioprotective, antiplatelet (Jiang et al., 2014), neuroprotective (Sun et al., 2014), sedative (Martínez-Vásquez et al., 2012) and antiaging (Maimaitiyiming et al., 2014). The extracts have also the ability to atenuate the systemic hypoxia and cardiac pathological state in chronic mountain sickness (Maimaitiyiming et al., 2014). The polyphenols and their antioxidant properties are involved in most of the aforementioned activities. Although some studies have reported a high antioxidant activity of the polar extracts or flavonoid fraction obtained from *D. moldavica* (Krishnaiah et al., 2011), its protective capacity on oxidative DNA-damage has not been investigated, to the best of our knowledge. Also, the antioxidant studies on *D. moldavica* of European origin are limited (Povilaityté et al., 2001; Weremczuk-Ježyna et al., 2013).

In this respect, the present study reports the antigenotoxic effects of the crude hydromethanolic extract from the aerial parts of *D. moldavica* cultivated in Romania. Bleomycin (BLM), a glycopeptide-derived antitumor antibiotic and a well-known radiomimetic drug (Sidik and Smerden, 1990), was used as genotoxicant on normal human dermal fibroblasts. Similarly to low energy transfer radiation, BLM induces *single strand DNA breaks* (SSBs), *double strand DNA breaks* (DSBs) and even clustered DNA lesions (*locally multiple damaged sites*, LMDS) (Aziz et al., 2012). BLM-induced DNA breakage mechanism involves free radical attack on deoxyribose moieties in DNA nucleotides (Povirk, 1996; Regulus et al., 2007); the process is ferrous ion and oxygen-dependent (Kruszewski et al., 2001). The skin is one of the most susceptible organs to the BLM toxicity probably due to a poor inactivation of BLM (Sikic, 1986). BLM hydrolase, a specific protease which metabolizes BLM, is poorly expressed in dermal fibroblasts (Pratt et al., 1994). The antigenotoxic activity of *D. moldavica* extract was assessed using the comet assay and the micronucleus test. The polyphenolic profile of the extract was investigated by high performance liquid chromatography with diode-array detection (HPLC-DAD) and the antioxidant activity was determined by free radical scavenging, ferrous ion-chelating and reducing power assays.

2. Materials and methods

2.1. Chemicals

Gallic acid, (+) catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), thiobarbituric acid, potassium ferricyanide, iron (III) chloride were purchased from Sigma–Aldrich (Steinheim, Germany). Tris(hydroxymethyl) aminomethane (Tris), Folin–Ciocalteu's phenol reagent, hydrochloric acid, 3-(4,5-dimethyl-2-thiazolyl)-2-5-diphenyl-2H-tetrazolium bromide (MTT), Giemsa stain, dimethyl sulfoxide (DMSO) and ferrous chloride were from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), 2-deoxy-D-ribose, iron (II) sulfate heptahydrate and pyrogallol were supplied by Fluka (Steinheim, Germany). Trichloroacetic acid and potassium persulfate were from Riedel-de-Haën (Seelze, Germany). Ethidium bromide was purchased from Carl Roth (Karlsruhe, Germany). Normal and low melting point agarose, amifostin, bleomycin, sodium lauroyl sarcosinate, cytochalasin B, rosmarinic acid, apigenin, apigenin 7-glucoside, quercetin, caffeic and chlorogenic acids were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbeco's Modified Eagle's Medium (DMEM), 10% foetal bovine serum, phosphate buffered saline (PBS), streptomycin, penicillin were procured from Biochrom AG (Berlin, Germany). Ultrapure water was obtained using a SG Water Ultra Clear TWF water purification system (Siemens Water Technologies Corp., USA).

2.2. Plant material

Aerial parts of *D. moldavica* were collected from the experimental field of *Stejarul* Biological Research Centre (Piatra Neamt, Romania) in July 2012. The plant material was air dried in shade at room temperature for 7 days. A voucher specimen (DM1/Pharmacog/2012) was deposited in the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy *Grigore T. Popa*-Iasi (Romania).

2.3. Extraction

The powdered aerial parts (10 g) were extracted under reflux with 100 mL of 80% methanol for 1 h at 60 °C. The extract was filtered and the residue was re-extracted twice as described. The combined extracts were concentrated in a rotary evaporator under reduced pressure at 40 °C and finally freeze-dried (η = 47.74%). *D. moldavica* extract (DME) was stored in a freezer at −18 °C until use.

2.4. Quantification of total phenolics

Total phenolic content was determined by the Folin–Ciocalteu method (Wangensteen et al., 2004). 0.04 mL of DME (5 mg/mL in DMSO:water, 7:3, v/v), 3.16 mL of ultrapure water and 0.2 mL of Folin–Ciocalteu reagent were mixed and allowed to stand for 5 min followed by the addition of 0.6 mL of 20% sodium carbonate. After 2 h incubation at ambient temperature in dark, the absorbance was measured at 765 nm. The calibration curve was plotted using gallic acid (0.2–2 mg/mL) as standard. Total phenolic content was expressed as mg of gallic acid equivalents/g of dry extract (mg of GAE/g of extract).

2.5. RP-HPLC analysis of polyphenols

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed on an Agilent Technologies 1200 Series HPLC system with a diode array detector. The separation was carried out on a ODS Hypersil column (Thermo Scientific, 250 × 4.6 mm, 5 μ particle size) using the method of Kamden et al. (2013) with minor changes. The mobile phase consisted of (A) acetonitrile and (B) water and acetic acid (99:1, v/v). The elution profile was: 13% A in B (0–10 min, isocratic), 13–20% A in B (10–20 min, linear gradient), 20–27% A in B (20–35 min, linear gradient), 27–40% A in B (35–60 min, linear gradient), 40–70% A in B (60–70 min, linear gradient), 70–100% A in B (70–80 min, linear gradient), 100% A (80–90 min, isocratic), 100–13% A in B (90–100 min, linear gradient). For qualitative analysis, DME and the standards (rosmarinic acid, apigenin, apigenin 7-glucoside, quercetin, caffeic and chlorogenic acids) were dissolved in methanol and methanol-water mixtures at concentrations of 20 and 0.5 mg/mL, respectively. Prior to injection, both the extract and standards were filtered through MCE (Mixed Cellulose Ester) syringe filters (13 mm, 0.22 μm) (OlimPeak, Teknokroma, CEE). Solvents (A) and (B) were filtered through 0.45 μm Millipore membrane filters (Merck, Darmstadt, Germany). Volumes of 20 μL were injected. The flow rate was 0.5 mL/min. The detection wavelengths were set at 254, 280, 325 and 365 nm. The phenolic compounds were identified by comparing their UV spectra and retention times with those of commercial standards. Rosmarinic acid, the major polyphenol in DME, was quantified on the basis of a calibration curve (0.5–1.25 mg/mL); the result was expressed as mg/g of DME.

2.6. In vitro antioxidant activity

Stock solutions of DME in DMSO-ultrapure water (7:3, v/v) were prepared and succesive dilutions were obtained in a suitable concentration range for each antioxidant assay.

2.6.1. DPPH radical scavenging assay

DPPH radical scavenging activity was determined according to the method described by Malterud et al. (Malterud et al., 1993). Different dilutions of DME were prepared in concentrations ranging from 20 to 1.25 mg/mL. An aliquot of each dilution (0.05 mL) was mixed vigorously with 2.95 mL of DPPH in methanol (A_{517nm} = 1.00 ± 0.05). The absorbance of the mixture was measured at 517 nm before adding the extract (A_{start}) and after 5 min reaction time (A_{end}). Gallic acid (0.078–1.25 mg/mL) was used as positive control. The ability to scavenge the DPPH radical was calculated as follows:

$$\text{DPPHscavengingactivity}(\%) = 100 \times \left[\frac{A_{start} - A_{end}}{A_{start}}\right].$$

2.6.2. ABTS radical cation scavenging assay

The assay was carried out following the procedure described by Re et al. (1999). ABTS radical cation was generated by incubating ABTS stock solution (7 mM) with potassium persulfate (2.45 mM) at room temperature in dark for 16 h followed by dilution with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm (equilibration at 30 °C). An aliquot (0.02 mL) of different DME dilutions (0.625–5 mg/mL) was mixed with ABTS radical cation solution in a total volume of 2 mL. The absorbance at 734 nm was measured after 6 min reaction time. Gallic acid (0.0125–0.1 mg/mL) was the positive control. The ABTS radical cation scavenging capacity was calculated using the following formula:

$$\text{ABTSscavengingactivity}(\%) = 100 \times \left[\frac{A_{control} - A_{sample}}{A_{control}}\right],$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of DME/gallic acid.

The ABTS scavenging activity was also expressed as Trolox equivalent antioxidant capacity (TEAC). In this respect, the scavenging effects of different concentrations of Trolox (0.5–2 mM) against ABTS radical cation were evaluated. TEAC values were calculated by dividing the slope of dose-response curve of DME/gallic acid by the slope of dose-response curve of Trolox.

2.6.3. Superoxide anion radical scavenging assay

The assay was performed according to a slightly modified method of Marklund and Marklund based on pyrogallol autoxidation (Wang and Luo, 2007). In brief, 0.1 mL of each DME dilution (10–20 mg/mL), 2.8 mL of 50 mM Tris–HCl buffer (pH 8) containing 1 mM EDTA and 0.2 mL of 6 mM pyrogallol were mixed thoroughly. The absorbance of the mixture was recorded at 325 nm every 30 s for 4 min. (+)-Catechin hydrate (20–80 mg/mL) was used as positive control. The capacity to scavenge superoxide anion radical was calculated using the formula:

$$\text{Superoxide scavenging activity}(\%) = 100 \times \left[\frac{\text{slope}_{control} - \text{slope}_{sample}}{\text{slope}_{control}}\right],$$

where $\text{slope}_{control}$ and slope_{sample} are the slopes of the plots of absorbance vs. time for control and DME/gallic acid, respectively.

2.6.4. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging capacity was assayed by the deoxyribose method (Goel et al., 2002; Gutteridge, 1984) with minor changes. Briefly, DME dilutions (10–120 mg/mL; 63 μL) were mixed with 0.4 mL of 5 mM 2-deoxy-D-ribose and 0.1 M phosphate-saline buffer (pH 7.4) up to a final volume of 1.8 mL. After addition of 0.2 mL of 2 mM ferrous sulfate heptahydrate, the mixture was incubated at 37 °C for 1 h. Then, 1% thiobarbituric acid and 2.8% trichloroacetic acid (1 mL of each) were added followed by heating at 80 °C for 1 h. The mixture was cooled and the absorbance was measured at 532 nm. In this assay, (+)-catechin hydrate (0.625–10 mg/mL) was used as positive control. The ability to scavenge hydroxyl radical was calculated by the following formula:

$$\text{Hydroxylscavengingactivity}(\%) = 100 \times \left[\frac{A_{control} - A_{sample}}{A_{control}}\right].$$

2.6.5. Reducing power assay

The assay was assessed according to a previously described method (Berker et al., 2007) with minor changes. 0.05 mL of DME dilutions (0.89–4.42 mg/mL), 1.2 mL of 0.2 M phosphate buffer (pH 6.6) and 1.25 mL of 1% potassium ferricyanide were mixed and incubated at 50 °C for 20 min. A volume of 1.25 mL of 10% trichloroacetic acid was added to the mixture followed by centrifugation at 3000 rpm for 10 min. An aliquot of 1.25 mL of the upper layer was mixed with 1.25 mL of ultrapure water and 0.25 mL of 0.1% ferric chloride. After 90 s, the absorbance of the mixture was recorded at 700 nm. (+)-Catechin hydrate (0.16–0.82 mg/mL) was used as positive control. A high absorbance of the reaction mixture indicates a high reducing capacity.

2.6.6. Ferrous ion chelating assay

Ferrous ion chelating ability was evaluated by the method of Dinis et al. (1994) with minor changes. 0.05 mL of each DME dilution (1.25–10 mg/mL), 0.05 mL of 2 mM ferrous chloride and 2.7 mL of 50 mM Tris–HCl buffer (pH 7.4) were mixed thoroughly followed by the addition of 0.2 mL of 5 mM ferrozine. After incubation (10 min), the absorbance of the mixture was measured at 562 nm. Gallic acid (0.078–2.5 mg/mL) was used as positive control. The ferrous ion chelating activity was calculated as follows:

$$\text{Ferrous ion chelatingactivity}(\%) = 100 \times \left[\frac{A_{control} - A_{sample}}{A_{control}}\right].$$

2.7. Estimation of antigenotoxic potential

2.7.1. Cell culture

Normal Human Dermal Fibroblasts (PromoCell, Heidelberg, Germany) were cultivated in a DMEM medium supplemented with 10% foetal bovine serum, 100 μg/mL streptomycin and 100 IU/mL penicillin.

2.7.2. Preparation of moldavica extract, bleomycin and amifostin solutions

Stock solutions of DME and amifostin were prepared in DMSO-ultrapure water (5:5, v/v). Further dilutions were made in culture media (DMEM) to obtain the desired concentration. The final concentrations in the culture media did not exceed 0.1% DMSO (v/v). Bleomycin solution was prepared in the culture media.

2.7.3. Cell viability assay

Cytotoxicity assessment was performed using the MTT assay previously described (Mosmann, 1983). Dermal fibroblasts (1.5×10^4 cells) were seeded in 96-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated in the absence (control) or presence of different DME concentrations (25, 100 and 200 μg/mL), at 37 °C for 24 h.Then, 10 μL of MTT (5 mg/mL) were added to each well. The plates were further incubated for 3 h. The blue formazan precipitate was dissolved in 300 μL DMSO/well

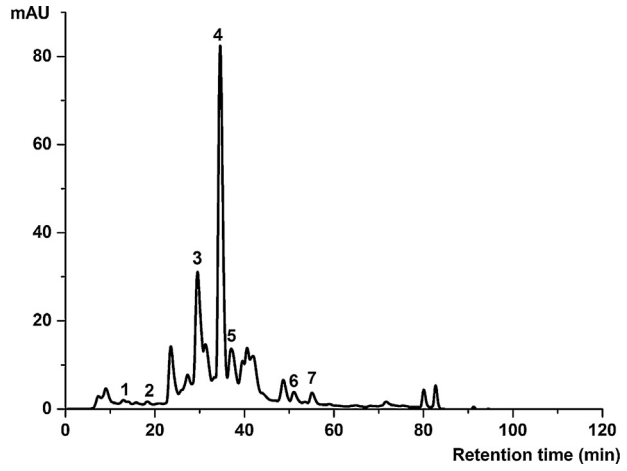

Fig. 1. High-performance liquid chromatography (HPLC) analysis of *Dracocephalum moldavica* extract. Chlorogenic acid (peak 1; retention time, R_t = 12.94 min); caffeic acid (peak 2; R_t = 18.29 min); ferulic acid (peak 3; R_t = 29.52 min); rosmarinic acid (peak 4; R_t = 34.58 min); apigenin 7-*O*-glucoside (peak 5; R_t = 37.06 min); quercetin (peak 6; R_t = 51.05 min); apigenin (peak 7; R_t = 55.10 min). Calibration curve for rosmarinic acid: $Y = 990.94x + 663.75$ ($r = 0.9998$). HPLC analysis was performed in triplicate.

and the absorbance was measured at 540 nm (PG Instruments T70, UK). The assay was carried out in fiveplicate. The results were expressed as percentages of control viability (100% viability).

2.7.4. Treatment of the cells

Cells were seeded at a density of 2×10^4 cells/well in 24-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and maintained in a 5% CO_2 atmosphere (Binder CB 150, Tuttlingen, Germany) at 37 °C. When the cells reached the confluence in the monolayer stage, they were incubated with BLM (10 μg/mL) for 6 h, followed by rinsing the cellular film with cold PBS; the rinse was repeated twice. After incubation with BLM, the cells were further incubated with DME (25 and 100 μg/mL) for 18 h. The same experimental protocol was applied in the case of the positive control, amifostin (AMI). It was used in a dose of 100 μg/mL. After 24 h incubation, the cells were detached by trypsinization and the cell suspension was obtained.

The groups of treated cells and controls were as follows:

- Sham control: normal dermal fibroblasts;
- BLM group: bleomycin (10 μg/mL) treated fibroblasts;
- DME 25 group: *D. moldavica* extract (25 μg/mL) treated fibroblasts;
- DME 100 group: *D. moldavica* extract (100 μg/mL) treated fibroblasts;
- AMI group: amifostin (100 μg/mL) treated fibroblasts;
- DME 25 + BLM treated group: *D. moldavica* extract (25 μg/mL) treated fibroblasts after the exposure to bleomycin (10 μg/mL);
- DME 100 + BLM treated group: *D. moldavica* extract (100 μg/mL) treated fibroblasts after the exposure to bleomycin (10 μg/mL);
- AMI + BLM treated group (positive control): amifostin (100 μg/mL) treated fibroblasts after the exposure to bleomycin (10 μg/mL).

2.7.5. Alkaline single-cell gel electrophoresis assay (Comet assay)

The degree of oxidative DNA damage was estimated by Comet assay (Olive and Banath, 2006). 200 μL of cell suspension in cold PBS were mixed with 1000 μL of low-gelling-temperature agarose at 40 °C. The agarose cell suspension was mixed and rapidly pipeted onto the agarose-covered surface of a precoated slide, avoiding to produce bubles. The agarose was allowed to gel for aprox. 5 min. After agarose has gelled, the slides were submerged in a covered dish containing lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH, pH > 13). The samples were maintained in the lysis solution overnight at 4 °C in the dark. After lysis, slides were submerged in the rinse solution (0.03 M NaOH, 2 mM Na_2EDTA, pH ≈ 12.3, room temperature) for three times. After three rinses, the slides were moved into the electrophoresis chamber filled with migration buffer (0.03 M NaOH, 2 mM Na_2EDTA, pH ≈ 12.3), the level of the migration buffer being 1–2 mm above the slides. The electrophoresis was conducted for 25 min at a voltage of 0.6 V/cm. After electrophoresis, the slides were removed from electrophoresis chamber, rinsed and neutralized with 400 mL of ultrapure water. 1% solution of ethidium bromide was spread on all the surface of slides and incubated for 20 min. The excess of stain was removed by rinsing the slides with 400 mL of ultrapure water. The images were captured using a Nikon Eclipse 600 epifluorescence microscope (Nikon, Japan). For each experimental variant, 600 cells were analyzed. Comet analysis was performed using AutoComet software (TriTek Comet Score, 1.5.2.6 version).

2.7.6. Micronucleus assay

Cytokinesis-blocked micronucleus (CBMN) assay was performed for assessing BLM-induced DNA damage in dermal fibroblasts (Fenech and Morley, 1986; Kirsch-Volders et al., 2000). After cells treatments, 3 μg/mL of cytochalasin B (stock solution: 1 mg cytochalasin B/mL DMSO) were added to the cultures. After 28 h incubation, the cells were treated with mild hypotonic solution of KCl (75 mM) for 3 min and fixed in 3:1 ethanol-acetic acid. After centrifugation (10 min, 4 °C) and removal of the supernatant (process repeated 3 times), the cells were gently dropped on a wet slide and stained with 5% Giemsa for 15 min. All slides were scored as the number of micronuclei per thousand binucleated cells. The analysis was done using a Nikon Eclipse 600 epifluorescence microscope (Nikon, Japan).

2.8. Statistical analysis

Results of chemical and antioxidant assays were expressed as mean ± standard deviation (SD). Results of genotoxicity tests were expressed as mean ± standard error of mean (SEM). In comet assay, Bonferroni multiple comparison test was used for the statistical evaluation and the results were considered statistically significant for $p < 0.001$. In MTT and micronucleus assays, the values were statistically analyzed using Student's t test; $p < 0.01$ and $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Phenolic content and profile

The total phenolic content of DME was 289.55 ± 2.63 mg of GAE/g; this value is lower than the one reported for the methanol extract from the aerial parts of Iranian *D. moldavica* (488.4 ± 1.8 mg/g) (Dastamalchi et al., 2007b). RP-HPLC analysis allowed the identification of chlorogenic and caffeic acids, apigenin 7-*O*-glucoside, rosmarinic acid, quercetin and apigenin (Fig. 1). Rosmarinic acid (107.11 ± 0.83 mg/g of DME) was found to be the major polyphenol representing 36.99% of the total phenolic content of DME. Rosmarinic acid was also found to be the most abundant polyphenol in Iranian *D. moldavica* (89.83 ± 1.38 mg/g dry extract) (Dastamalchi et al., 2007b).

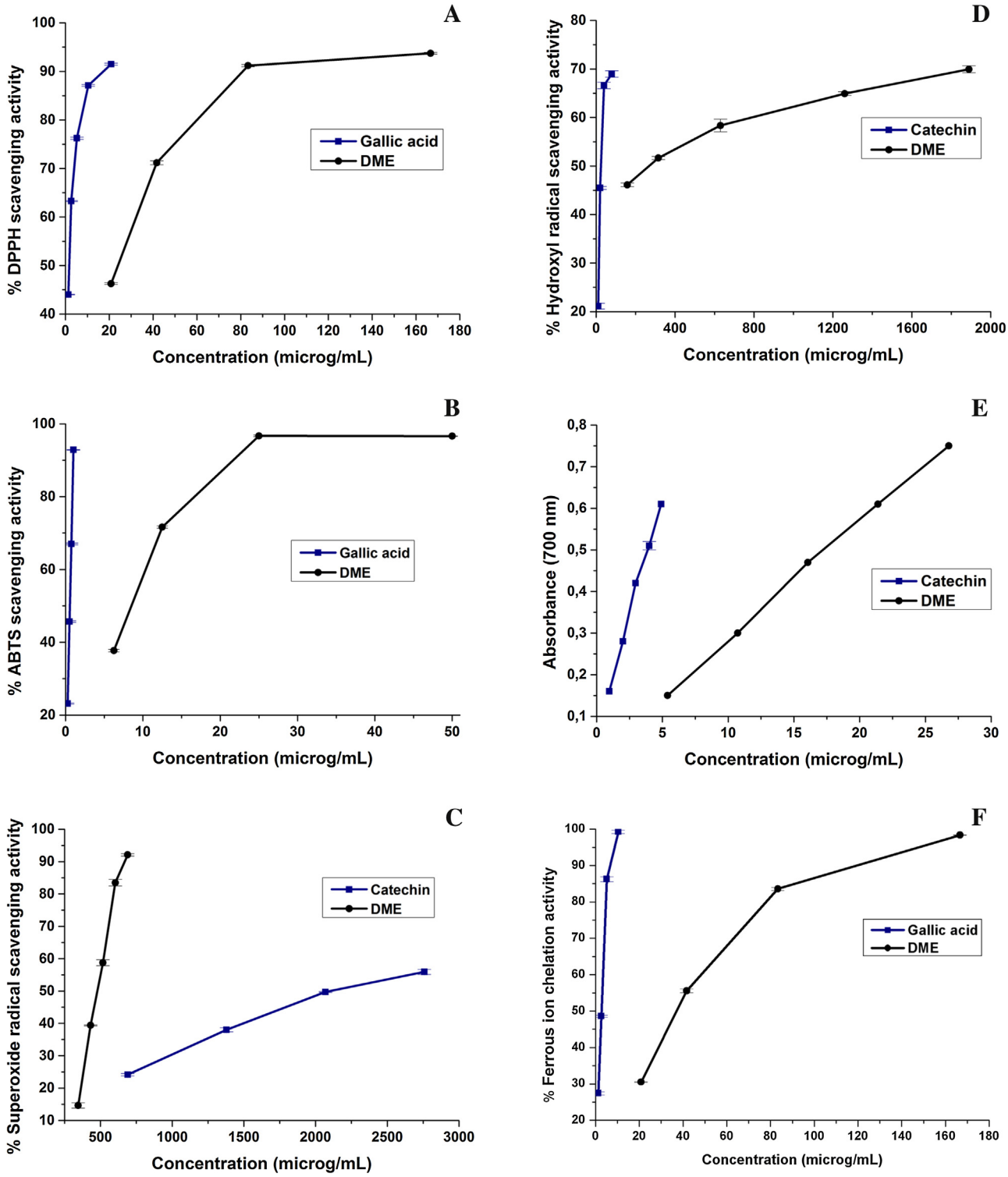

Fig. 2. Antioxidant activity of *Dracocephalum moldavica* extract (DME): (A) DPPH radical scavenging activity; (B) ABTS radical cation scavenging activity; (C) superoxide anion radical scavenging activity; (D) hydroxyl radical scavenging activity. (E) reducing capacity; (F) ferrous ion chelating ability. Values are given as mean ± SD of three replicates in each group.

3.2. *In vitro* antioxidant activity

Free radicals and oxidative damage are involved in the genotoxicity induced by BLM and other cytotoxic agents. In the presence of a redox-active metal such as Fe^{2+} or Cu^{+} and molecular oxygen, BLM is activated and generates superoxide and hydroxyl radicals which attack DNA, oxidize DNA bases, produce protein-damage and promote lipid peroxidation (Yen et al., 2005). Antioxidants can protect against oxidative injuries and can act as potential antigenotoxic agents. The capacity of DME to scavenge directly free radicals was investigated by DPPH and ABTS assays. Antioxidants having hydrogen atom- or electron-donating abilities convert DPPH radical (violet) into its non-radical form (yellow) with a consequent decrease in absorbance at 517 nm (Villaño et al., 2007). The

Table 1
In *vitro* antioxidant activity of DME.

Extract/positive control	DPPH radical scavenging assay	ABTS radical cation scavenging assay		Superoxide anion radical scavenging assay	Hydroxyl radical scavenging assay	Reducing power assay	Ferrous ion chelating assay
	EC_{50}	EC_{50}	TEAC	EC_{50}	EC_{50}	EC_{50}	EC_{50}
DME	23.1 ± 0.1	8.0 ± 0.1	0.79 ± 0.00	445.5 ± 2.3	269.3 ± 11.1	17.07 ± 0.21	35.7 ± 0.4
Gallic acid	1.6 ± 0.0	0.5 ± 0.1	18.61 ± 0.19	–	–	–	2.7 ± 0.0
(+)-catechin	–	–	–	1960.0 ± 13.7	24.0 ± 0.3	3.8 ± 0.0	–

The results are expressed as mean ± SD from three determinations. EC_{50} values (μg/mL) were calculated by linear interpolation between values above and below 50% activity (Apetrei et al., 2011). In reducing power assay, EC_{50} values were the concentrations giving an absorbance of 0.5 (Ferreira et al., 2007); TEAC, μM Trolox equivalent to 1 μg/mL of extract/positive control

DPPH scavenging activity of DME increased dose-dependently from 46.27 ± 0.18% at 20.83 μg/mL to 93.74 ± 0.18% at 166.66 μg/mL (Fig. 2). In this range of concentrations, the positive control, gallic acid, completely scavenged the radical. Dastmalchi et al. (2007b) evaluated the DPPH scavenging effects of different extracts from Iranian *D. moldavica* aerial parts using another experimental protocol based on a longer reaction period than the one used in our study (30 min vs. 5 min). Therefore, a direct comparison of our results with those reported by Dastmalchi et al. (2007b) is not possible. But it is worthy to note that Dastmalchi et al. (2007b) found the highest DPPH scavenging activity in the methanol extract; at 1 mg/mL (corresponding to 33.33 μg/mL in the reaction mixture), the methanol extract of Iranian *D. moldavica* aerial parts scavenged DPPH radical by 89.5 ± 0.2% after 30 min reaction time. In our study, a similar effect (91.19. ± 0.22%) was exhibited by DME at 83.33 μg/mL (in the reaction mixture) after 5 min of reaction with DPPH radical. Obviously, DME is an efficient free radical scavenger. According to the EC_{50} values (Table 1), DME was less active than gallic acid, a phytochemical with remarkable antioxidant activity due to its three phenolic hydroxyl groups in *ortho* position (Villaño et al., 2007).

The high free radical scavenging capacity of DME was confirmed by ABTS assay. Electron-donating antioxidants scavenge ABTS radical cation (blue-green) causing its decolorization and consequently, a decrease in absorbance at 734 nm (Re et al., 1999). ABTS scavenging activity of DME ranged from 37.70 ± 0.35% at 6.25 μg/mL to 96.63 ± 0.04% at 50 μg/mL after 6 min reaction time (Fig. 2). Gallic acid showed 100% scavenging effects in this concentration range. With regard to the EC_{50} and TEAC values calculated after 6 min reaction period (Table 1), DME was less active than gallic acid. Our results are consistent with those reported by Dastmalchi et al. (2007b) who recorded the ABTS scavenging effects of various extracts from Iranian *D. moldavica* aerial parts and gallic acid at different time points. After 5 min reaction time, Dastmalchi et al. (2007b) found TEAC values (mM Trolox) of 0.56 ± 0.02 and 17.56 ± 0.99 for the methanolic extract and gallic acid, respectively.

Superoxide anion radical plays an important role in BLM toxicity. Besides its involvement in the production of hydroxyl radicals, the superoxide anion radical can generate peroxynitrite ($ONOO^-$) by the reaction with endogenous NO leading to DNA adducts and denaturation of -SH-pool (Parihar et al., 2007). Also, it may reactivate BLM for damaging DNA (Yen et al., 2005). A pyrogallol autoxidation assay was used to evaluate the capacity of DME to scavenge the superoxide anion radical. In this assay, the superoxide anion radical, generated by the autoxidation of pyrogallol, converts pyrogallol to quinones which strongly absorb at 325 nm; superoxide scavengers significantly reduce the absorbance at 325 nm (Gao et al., 1998; Marklund and Marklund, 1974). As shown in Table 1 and Fig. 2, DME efficiently scavenged superoxide anion radical, being almost 4.5 times more active than the positive control, catechin. In a hypoxanthine/xanthine oxidase assay, a water extract from Iranian *D. moldavica* aerial showed a lower ability to scavenge superoxide anion radical in comparison with gallic acid and Pycnogenol (EC_{50} = 467.2 ± 73.6 μg/mL vs. 89.2 ± 9.7 and 163.5 ± 36.7 μg/mL, respectively) (Dastmalchi et al., 2007a). As the experimental protocols were different, a comparison of the superoxide scavenging effects of the extracts obtained from Romanian and Iranian *D. moldavica* aerial parts is not feasible.

Hydroxyl radical is a highly reactive oxygen species that attacks directly most biomolecules (DNA, carbohydrates, proteins, polyunsaturated acids). It causes more deleterious effects *in vivo* than any other reactive oxygen species. Hydroxyl radical can be generated in multiple pathways such as metal-mediated reduction of peroxides (Fenton reaction) or *via* superoxide anion radical (Perron and Brumaghin, 2009). The hydroxyl radical scavenging activity was investigated by 2-deoxy-D-ribose degradation assay (Goel et al., 2002; Gutteridge, 1984). Hydroxyl radicals, generated by ferrous sulfate heptahydrate in phosphate buffer, damage 2-deoxy-D-ribose with the release of thiobarbituric-acid reactive material which is spectrophotometrically quantified at 532 nm; a decrease of absorbance at 532 nm indicates hydroxyl radical scavenging activity. At 1890 μg/mL, DME exhibited 69.94 ± 0.72% hydroxyl scavenging activity (Fig. 2); a further increase in concentration did not result in an increase of activity. The extract was less active than the positive control, catechin (Table 1). However, DME (EC_{50} = 269.3 ± 11.1 μg/mL) protects 2-deoxy-D-ribose by scavenging hydroxyl radicals more pronounced than the water extract from Iranian *D. moldavica* aerial parts (EC_{50} = 19.4 ± 1.5 mg/mL) (Dastmalchi et al., 2007a).

Reducing power assay is also an electron transfer-based assay involving the reduction of potassium ferricyanide followed by the generation of Prussian blue (Berker et al., 2007). The reducing power of DME reached maximum value (absorbance 0.75 ± 0.00 at 700 nm) at concentration of 26.74 μg/mL (Fig. 2). According to the EC_{50} values it was 4.3 times less active than catechin (Table 1). Additionally, the water extract from Iranian *D. moldavica* had a moderate reducing capacity (0.51 ± 0.06 mmol ascorbic acid/g) being less active than well-known antioxidants such as ascorbic acid (5.58 ± 0.32 mmol ascorbic acid/g), butylated hydroxyanisole (3.14 ± 0.14 mmol ascorbic acid/g) or Pycnogenol (2.14 ± 0.13 mmol ascorbic acid) (Dastmalchi et al., 2007a).

Iron is the primary cause of peroxide and hydroxyl radicals generation and it is involved in many other oxidative stress related pathways that damage DNA and initiate lipid peroxidation (Perron and Brumaghin, 2009). Iron-binding activity is an important feature of antioxidant behaviour. In the ferrozine assay, chelating agents reduce the formation of ferrous ion-ferrozine complex leading to a decrease in absorbance at 562 nm (Dinis et al., 1994). In our study, DME chelated ferrous ions dose-dependently. At 166.66 μg/mL, the extract chelated ferrous ions by 98.36 ± 0.06%. Gallic acid exhibited a similar chelating activity (99.82 ± 0.10%) at 41.66 μg/mL (Fig. 2). Based on the EC_{50} values, DME showed a high ferrous ion chelating activity, but still lower than that of the positive control, gallic acid (Table 1). Using a slightly different experimental protocol, Dastmalchi et al. (2007a) found a lower activity for the water extract from Iranian *D. moldavica* aerial parts (EC_{50} = 4 mg/mL) than that of DME (EC_{50} = 35.7 ± 0.4 μg/mL).

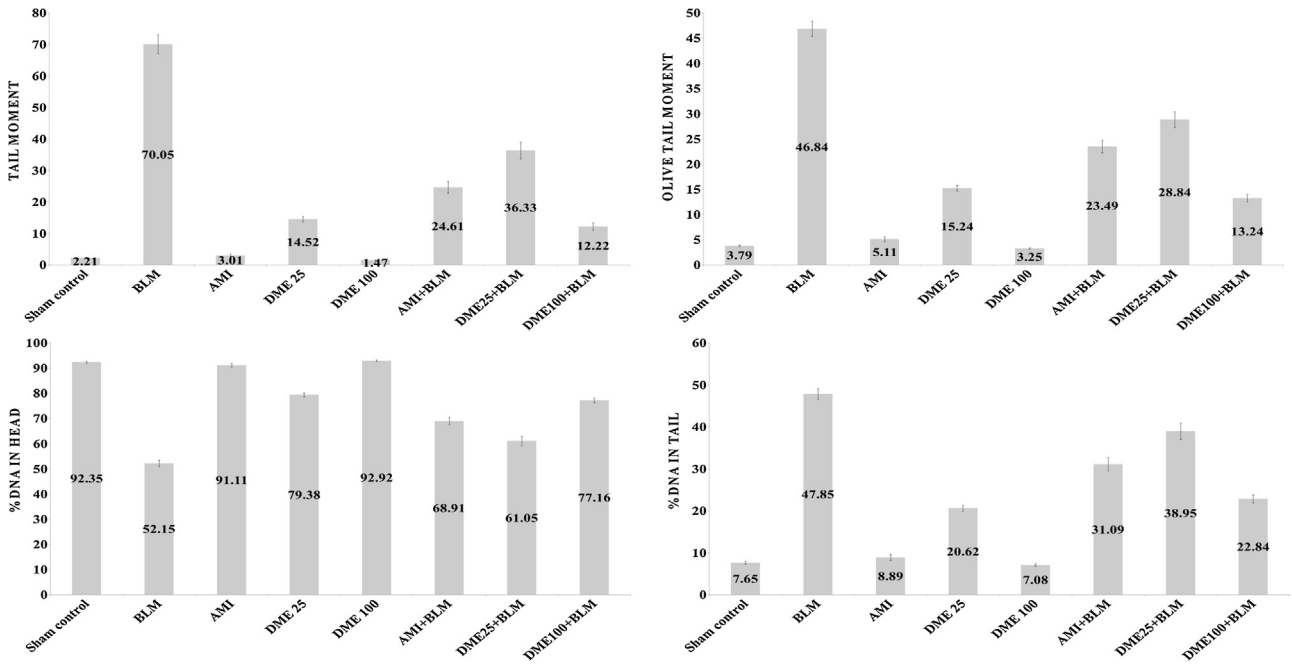

Fig. 3. Protective effect of *Dracocephalum moldavica* extract (DME) and amifostin (AMI) on bleomycin (BLM)-induced DNA damage in normal dermal fibroblasts. Results are given as mean ± SEM of three experiments in each group; Significant data at $p < 0.001$ compared to sham control (*) or BLM-alone group (**) (Bonferroni's Multiple Comparison Test).

Table 2
The effects of DME on human normal dermal fibroblasts viability upon 24 h incubation.

Group	(%) Cell viability
Normal dermal fibroblasts	100.0 ± 0.0
Fibroblasts + DME 25	92.3 ± 2.2*
Fibroblasts + DME 100	96.1 ± 1.6*
Fibroblasts + DME 200	83.3 ± 6.7**

The values are expressed as mean ± SEM from five replicates. Significant data at *$p < 0.01$ and **$p < 0.05$ compared to control (*t* Test).

3.3. Effect on cell viability

Normal human dermal fibroblasts incubated with DME (25–200 mg/mL) showed no important decrease in cell viability up to a concentration of 200 mg/mL (Table 2). Hence, 25 and 100 mg/mL were selected as non-toxic concentrations in the subsequent experiments.

3.4. Effect on bleomycin-induced DNA damage

Similar to ionizing radiation, BLM can induce different types of DNA damage. The genotoxicity of BLM depends on its ability to bind a transition metal (iron) followed by the activation of BLM-iron complex in the presence of oxygen and a reducing agent (Aruoma et al., 1999; Hoffmann et al., 2007; Pratt et al.,1994). The redox cycling of BLM-iron is accompanied by an increase in DNA damage (Pratt et al., 1994). The activated BLM is able to oxidize the 4-position of 2-deoxyribose leading to DNA strand breakage: SSBs, DSBs and DNA interstrand cross-link (Regulus et al., 2007). In addition, BLM also induces cytosine damage in cellular DNA, apurinic/apyrimidinic sites (Gruber and Anuszewska, 2000), release of free bases or generation of base propenals (Sidik and Smerden, 1990). It is appreciated that the DNA damage pattern in HeLa cells caused by either a treatment with 12 μg/mL BLM for 30 min or exposure to 40 Gy ^{137}Cs are astonishingly similar to each other (Grigaravičius et al., 2009). In our study, the effects of DME have been assessed by the comet assay. This is a highly sensitive technique that determines the extent of DNA damage (SSBs, DSBs, alkali labile sites, interstrand cross-links). The migration of DNA fragments out of the cell nucleus and the characteristic appearance of a *comet* are quantified by some parameters: tail moment (measures both the amount of DNA and DNA distribution in the tail), olive tail moment (product of the distance between the centre of gravity of the head and the centre of gravity of the tail and%DNA in tail) (Nair and Salvi, 2008), %DNA in head and%DNA in tail (Liao et al., 2009). Amifostin (WR-2721), an aminothiol with cytoprotective and radioprotectant effects (Nici et al., 1998), was used as positive control. Its active metabolite, 2-[(aminopropyl)-amino] ethanethiol (WR-1065) has also been reported to protect against BLM-induced cytotoxicity in V79 cells (Jagetia et al., 2007). In this assay, the dose of BLM (10 μg/mL) and time de incubation (6 h) were selected according to previous studies. 10 μg/mL of BLM dose induces a post-mitotic differentiation in fibroblasts and transcriptional changes of genes related to the expression of the senescent phenotype relevant to late normal tissue radiation injury (Westbury et al., 2011). Also, Jagetia et al. (2007) determined the highest DNA damage in V79 cells (%DNA in tail = 40.64 ± 1.03) after 6 h treatment with BLM. Fig. 3 and Fig. 4 show changes in the values of comet attributes (tail moment, olive tail moment, %DNA in tail and%DNA in head) in BLM and DME treated groups and also in BLM + DME treated groups comparative to sham control. Exposure of fibroblasts to BLM caused an increase in DNA damage evidenced by a greater migration of DNA into the comet tail. In a concentration-dependent manner, DME posttreatment significantly decreased BLM-induced DNA damage when compared to BLM alone-treated cells. The most important decrease in DNA damage was determined in DME 100 + BLM group. Exposure of dermal fibroblasts to *D. moldavica* extract 100 μg/mL after preincubation with BLM (10 μg/mL) resulted in a most significant decrease of comet attributes compared to BLM-alone treated fibroblasts: tail moment decreases from 70.05 ± 3.01 to 12.22 ± 1.19, olive tail moment from 46.84 ± 1.51 to 13.24 ± 0.77 and%DNA in tail from 47.85 ± 1.27 to 22.84 ± 0.93. In the posttreated groups, DME

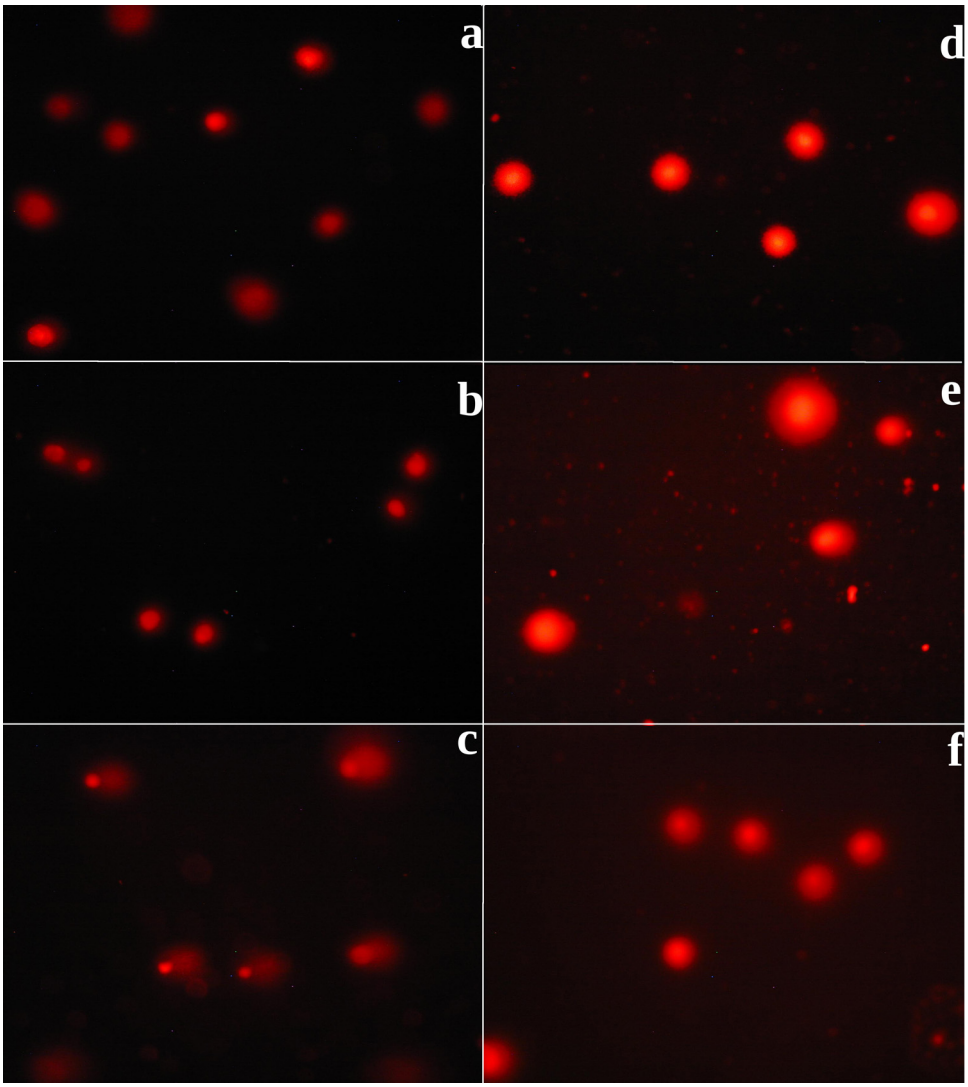

Fig. 4. Photomicrographs of comet length in normal and treated fibroblasts. (a) normal fibroblasts; (b) amifostin (AMI)-treated fibroblasts; (c) bleomycin (BLM)-treated fibroblasts; (d) *Dracocephalum moldavica* extract (DME) 100-treated fibroblasts; (e) AMI + BLM treated fibroblasts; (f) DME 100 + BLM treated fibroblasts.

100 induced a more pronounced protective effect than amifostin (100 μg/mL) (Fig. 3). The incubation with DME 100 did not result in any DNA damage while DME 25 alone induced some increases of the comet parameters (%DNA in tail, olive tail moment, tail moment) in comparison to sham control.

Extracts from different plant species (*Camellia sinensis*, *Origanum vulgare*, *Curcuma longa*, *Podophyllum hexandrum*) or polyphenols (naringin, genistein, epigallocatechin gallate, rutin, quercetin, silymarin, curcumin, resveratrol, rosmarinic acid, ellagic acid, ferulic acid) showed the capacity to provide protection against DNA-damage induced by BLM or other genotoxicants (radiation, H_2O_2, doxorubicin, mitomycin) in various mammalian cells (Aherne and O'Brien, 1999; Friedmann Angeli et al., 2010; Jagetia et al., 2007; Silva et al., 2008). The main mechanisms involved in their antigenotoxic activity are related to free radical scavenging properties, iron chelation, inhibition of lipid peroxidation or stimulation of endogenous antioxidant response. In our study, the experimental protocol involved an initial exposure of dermal fibroblasts to BLM followed by incubation with DME or amifostin. In this case, it is more likely that the protective effect occured by the intervention of DME on physiological mechanisms of DNA repair (Friedmann Angeli et al., 2010). For RA, the major component of DME, there has been reported a high protective activity against DNA-damage induced by *tert*-butyl hydroperoxide in PC12 cells. RA acts on specific intracellular mechanisms of DNA repair; it increases DNA repair enzymes activity, namely OGG1 (8-oxoguanine DNA glycosylase 1). This is an important component of base excision repair (BER) pathway that is involved mainly in the correction of oxidative DNA-damage, including BLM-induced DNA lesions (Silva et al., 2008). The decrease of comet tails also highlights that the protective effect of DME could also be mediated by BER pathways (Friedmann Angeli et al., 2010). In case of treatment with DME 100 μg/mL, the cells were exposed to a lower concentration of RA than the dose used in other studies (29.73 μM vs. 50 μM) (Silva et al., 2008). A possible explanation of the higher activity of DME 100 μg/mL is the fact that the other polyphenols in DME potentiated the activity of RA. Quercetin showed a high capacity to protect against the formation of DNA strand breaks (Silva et al., 2008). In addition, ferulic acid enhanced DNA repair in animals exposed to whole body gamma-radiation (Maurya et al., 2005) while chlorogenic acid increased the expression of DNA repair enzymes in HCT-116 cells (Bernstein et al., 2007). Another explanation might be related to the fact that the cells type, genotoxic agent and time of incubation are different. Besides the possible activity on DNA repair machinery, the free radical scavenging effects of DME also contribute to its genoprotective activity. DME has a

Table 3
Effect of DME on BLM-induced micronuclei in normal dermal fibroblasts.

Group	Mean total micronuclei ± SEM	Decrease (%)
Sham control	0.89 ± 0.12	–
BLM	4.33 ± 0.33	–
AMI	3.00 ± 0.58	–
DME 25	2.33 ± 0.33*	–
DME 100	1.93 ± 0.88*	–
AMI + BLM	3.33 ± 0.67	23.10
DME 25 + BLM	3.00 ± 0.58	30.72
DME 100 + BLM	1.33 ± 0.33**	69.28

Values are expressed as mean ± SEM ($n = 3$). Significant data at $^{*}p < 0.05$, $^{**}p < 0.01$ compared to BLM-alone group (*t* Test).

pronounced scavenging activity against superoxide anion radical. In BLM-system, superoxide anion radical, not hydroxyl radical, is considered the major DNA-damaging species (Aruoma et al., 1999). Iron chelating properties and mild reducing abilities of DME can also interfere the redox recycling of BLM.

3.5. Effect on bleomycin-induced micronuclei frequency

Micronuclei are small membrane-bound DNA fragments originating from acentric fragments or whole chromosomes that do not attach to the mitotic spindle during mitosis. They are effective biomarkers that measure the cytogenetic damage (Klein et al., 2000; Slowinski et al., 2004). Oxidative DNA lesions induced by BLM may cause micronuclei formation, chromosomal abberations and gene mutation (Cooke et al., 2003; Khouri et al., 2007). In our study, BLM increased the total number of micronuclei but subsequent treatment of fibroblasts with DME in concentrations of 25 and 100 μg/mL resulted in a decrease in micronuclei induction (Table 3). Posttreatment with DME 100 μg/mL showed the highest reduction (69.28%) in the total micronuclei; the effect was significant when compared to BLM-alone group. Therefore, DME posttreatment was found to be more effective than amifostin (Table 3). DME itself had no mutagenic effect.

Some polyphenols identified in DME showed a noticeable protective activity against clastogenic effects of certain anticancer drugs or ionizing radiation. RA significantly reduced the frequency of micronuclei and DNA injuries induced by doxorubicin in V79 cells (Furtado et al., 2010) and gamma irradiation in human lymphocytes (Sánchez-Campillo et al., 2009; Furtado et al., 2010). Apigenin protected from micronuclei induction in irradiated human peripheral blood lymphocytes (Begum and Prasad, 2012). Also, ferulic acid decreased the micronuclei frequency in human peripheral blood lymphocytes exposed to gamma-radiation (Prasad et al., 2006). It is considered that the antioxidant effects are mainly involved in the anticlastogenic potential of plant polyphenols (Furtado et al., 2010; Furtado et al., 2008).

4. Conclusion

The polyphenolic extract from European *Dracocephalum moldavica* protected against bleomycin-induced genotoxicity in normal human adult dermal fibroblasts as it was confirmed by the comet assay at molecular level and micronucleus test at genomic level. The extract itself had no mutagenic effect. The protective effects might be due to the free radical scavenging activity, iron-chelating properties and the possible intervention on DNA repair processes. *Dracocephalum moldavica* extract showed a high antioxidant activity mediated *via* direct free radical scavenging and metal-chelating properties. It appeared to be a more potent antioxidant than polar extracts from Iranian *Dracocephalum moldavica*. The major polyphenol in the extract was rosmarinic acid, also identified in plants of Russian and Asian origin (Martínez-Vásquez et al., 2012). Further investigations are necessary for a better understanding of mechanisms underlying antigenotoxic effects of *Dracocephalum moldavica* of European origin. The European species might be an important source of rosmarinic acid, both the drug and the phytochemical being of interest as adjuvants in chemo- or radiotherapy. At the same time, it raises the question if the association of *Dracocephalum moldavica* with bleomycin could diminish the cytotoxic effect of this radiomimetic agent in patients with tumors. Also, European *D. moldavica* may be a promising radioprotective candidate.

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