Antioxidant and antigenotoxic potencies of *Sempervivum armenum* on human lymphocytes in vitro

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Abstract In this research, the genotoxic and antigenotoxic effects of methanol extract of *Sempervivum armenum* (MSA) were studied using micronucleus (MN) test and sister chromatid exchange (SCE) test systems in cultured human peripheral blood cells. According to the SCE and MN tests results, MSA reduced the genotoxic effects of aflatoxin B₁. In order to explain the reason for the antigenotoxic effects of MSA, antioxidants levels were determined. Cotreatments of 5, 10, 20 mg/mL concentrations of MSA with aflatoxin B₁ decreased the frequencies of SCE, MN and the malondialdehyde level and increased the amount of superoxide dismutase, glutathione and glutathione peroxidase which were decreased by aflatoxin. The results of this experiment showed that MSA has strong antioxidative and antigenotoxic effects and this antigenotoxic activities of MSA can be due to the antioxidant activities.

Keywords *Sempervivum armenum* · Antigenotoxicity · Antioxidant · Aflatoxin B₁

Introduction

Sempervivum L. (Crassulaceae) consists of approximately 50 species and 17 hybrids. It is mainly confined to the mountains of southern Europe, southwestern Asia, Mediterranean and the Caucasus (Topalov et al. 2006).

S. armenum, the first Turkish species, was found and published by Boissier and Huet du Pavillon (1856). The species is a yellow flowering and glabrous leaved plant from NE Turkey Collected by Huet du Pavillon in the Kassaklu valley in 1853 (Karaer and Celep 2010; Ozhatay et al. 2011). The first revision of *Sempervivum* L. in Turkey was completed by Muirhead (1972), who defined 11 species and 2 varieties. *Sempervivum* L. is represented with 14 species in Turkey (Muirhead 1972; Güner et al. 2000). Some species like *Sempervivum tectorum* L. and *Sempervivum armenum* are used in Anatolian folk remedy (Zeybek and Zeybek 1994; Baytop 1999).

The name “*Sempervivum*” has its basis in the Latin *Semper* (“always”) and *vivus* (“living”). Because this perennial plant keeps its leaves in winter and is very resistant to hard conditions of growth, *Sempervivum* is called “always living”. Their ability to store water in their thick leaves allows them to live on rocks and stony places in the mountain, alpine and subalpine belts.

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The earliest publications concerning the medicinal use of this plant deal with its influence on pharyngitis, tracheitis, thrush, combustion and otitis. Strawn over wounds, sores, burns, abscesses of areas, the fresh juice from squeezed leaves of *S. tectorum* is used as a refrigerant and astringent, a sure remedy for insect bites, as well as for ear pains and inflammations while the tea prepared from its leaves is recommended for ulcer treatment. *S. armenum* Boiss. et Huet subsp. *armenum* is a well known medicinal plant found in the Eastern Anatolia (Erzurum—Kars) region of Cat in Turkey (Sengul Koseoglu 2014).

Previous studies on the genus *Sempervivum* L. were directed to the biological activities of exchange isolated by different extraction methods (Stojicevic et al. 2008; Alberti et al. 2008; Kekesi et al. 2003; Abram and Donko 1999). The juice of *S. tectorum* was applied to herpetic eruptions of the skin, minor burns and wounds (Alberti et al. 2008). In addition to antinociceptive, liver-protecting, membrane stabilizing effect, antimicrobial, antiinflammatory and in vitro antioxidant features, *S. tectorum* extracts have been described in recent works (Stojicevic et al. 2008; Alberti et al. 2008; Kekesi et al. 2003; Abram and Donko 1999).

Therapies with preparations of *Sempervivum* species can be traced back to the origins of antiquity herbal medicine. However, extracts of *S. armenum* leaves have not been introduced into medical practice yet.

In this study, we examined the genotoxic and antigenotoxic effect of methanol extracts obtained from *S. armenum* (AFB₁) by using micronucleus (MN) and sister chromatid exchange (SCE) tests, in cultured human blood cells. Additionally, the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, glutathione (GSH) and malondialdehyde (MDA) levels in human blood culture were measured to determine the role of enzyme systems in the basis of antigenotoxic effects. This is the first report indicating the antioxidant and the antigenotoxic activity of methanol extract of the *S. armenum*.

Materials and methods

Plant material

The samples of *S. armenum* were collected from Erzurum region of Cat in Turkey, in 2012 (Altitude 2340 m). The samples were identified by Dr. Meryem

Sengul, using various flora books and papers (Davis et al. 1988). They were recorded in the herbarium of Faculty of Sciences, Ataturk University (ATA-9873).

Preparation of methanol extracts

Air-dried and powdered leaves of *S. armenum* (10 g) was extracted with 200 mL of methanol using the Soxhlet extractor (Isopad, Heidelberg, Germany) for 12 h at a temperature not exceeding the boiling point of the solvent (Lin et al. 1999). The extract was filtered using Whatman filter paper (No 1) then concentrated in vacuum at 40 °C using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) yielding to a waxy material. The extract was then lyophilized and kept in dark at +4 °C until tested.

Cytogenetic analysis

Peripheral blood lymphocytes were taken from four nonsmoking healthy donors between the ages of 22 and 26 years. Lymphocyte cultures were set up by adding 0.5 mL of heparinized whole blood from Roswell Park Memorial Institute. 1640 chromosome medium (Sigma) was supplemented by 15 % heat-inactivated fetal calf serum (Sigma), 100 IU/mL streptomycin (Sigma), 100 IU/mL penicillin (Sigma) and 2 % L-glutamine (Sigma). Lymphocytes were stimulated to divide by 2 % phytohemagglutinin (Sigma). AFB₁ (Sigma; 5 mM) and MSA (5, 10, 20 and 40 mg/mL) were added to the cultures just before incubation.

The experiments were conducted in seven groups as follows:

- Group 1: Control,
- Group 2: AFB₁ (5 µM),
- Group 3: MSA (10 mg/mL),
- Group 4: AFB₁ (5 µM) + MSA (5 mg/mL),
- Group 5: AFB₁ (5 µM) + MSA (10 mg/mL),
- Group 6: AFB₁ (5 µM) + MSA (20 mg/mL),
- Group 7: AFB₁ (5 µM) + MSA (40 mg/mL).

Sister chromatid exchanges

The cultures were incubated at 37 °C for 72 h and 5-bromo 2-deoxyuridine (Sigma) at 6 mg/mL concentration was added into the cultures. All cultures were maintained in dark. Then, 3 mg/mL of colcemide (Sigma) was added 1.5 h before harvesting to arrest the cells at metaphase. The cultures were centrifuged

at 1200 r/min for 10 min. The supernatants were used for enzyme analysis. Cells were harvested and treated for 25 min at 37 °C with hypotonic solution (0.05 M KCl), centrifuged for 10 min at 1200 r/min, and then fixed in a 1:3 mixture of glacial acetic acid/methanol (v/v). This fixative process was repeated three times. Then, the cells were spread on glass slides and air-dried in dark for 15 days. The staining of air dried slides was performed following the modified fluorescence 5 % Giemsa (Sigma) method for SCE (Speit and Haupter 1985; Anar et al. 2013). The slides were irradiated with 30 W, 254 nm ultraviolet lamp at 15 cm distance in Sørensen buffer 1 h, then incubated with standard saline citrate at 65 °C for 1 h and stained with 5 % Giemsa prepared with Sørensen buffer. In order to score SCE, 20 satisfactory metaphases were analyzed. For each treatment condition, well-spread second division metaphases containing 42–46 chromosomes in each cell were scored, and the values obtained were calculated as SCEs per cell.

Micronucleus assay

The cultures were incubated at 37 °C for 72 h then Cytochalasin B (Sigma) was added 44 h after PHA stimulation to a final concentration of 3 g/ml. Twenty-eight hours later (after 72-h cultivation), the cells were harvested by centrifugation (1200r x 10 min). The supernatant was removed. Cells were harvested and treated for 20 min at 37 °C with hypotonic solution (0.05 M KCl), centrifuged for 10 min at 1200 r/min, and then fixed in a 1:3 mixture of glacial acetic acid/methanol (v/v). This fixative process was repeated three times. The cell pellet was then resuspended in 1 mL of fresh fixative, dropped onto a clean microscope slide, incubated at 37 °C or at room temperature overnight, and stained with Giemsa dye. Encoded slides were scored blind by two independent individuals. Only binucleated cells were scored for MN analysis. For each subject, at least 1000 binucleated cells were analyzed for the presence of MN (Anar et al. 2013). For the MN scoring, the micronucleus criteria described by Countryman and Heddle (1976) were used: a diameter less than 1/3 of the main nucleus, non-refractility, not touching, and with the same colour as the nucleus or lighter. One hundred lymphocytes were scored to evaluate the percentage of cells with 1, 2, 3 or 4 nuclei. Nuclear Division Index (NDI) was calculated according to the following

formula: $[1X(1\text{ N}) + 2X(2\text{ N}) + 3X(3\text{ N} + 4\text{ N})]/n$ (n = total number of cells evaluated).

Biochemical analysis

The cell homogenates were prepared at 1:10 (w:v) dilution in 10 mM potassium phosphate buffer with pH 7.4. Samples were centrifuged at 3000 r/min for 10 min at +4 °C, and the supernatants were collected and immediately assayed for enzyme activities. All samples were measured in six replicates.

SOD assay

Copper (Cu)- and zinc (Zn)-SOD activities in the cell culture supernatant was detected by the method described by Sun et al. (1988). Assay reagent (2.45 mL; 0.3 mM xanthine, 0.6 mM ethylenediaminetetraacetic acid disodium salt (Na₂-EDTA), 0.15 mM nitroblue tetrazolium (NBT-Sigma), 0.4 M sodium carbonate, 1 g/L bovine serum albumin) was combined with 100 mL of the sample. Xanthine oxidase (50 mL, 167 IU/L) was added to initiate the reaction, and the reduction of NBT by superoxide anion radicals produced by the xanthine–xanthine oxidase system was determined by measuring the absorbance at 560 nm. Cu- and Zn-SOD activities were expressed in units (IU) of SOD per mg protein, where one IU is defined as that amount of enzyme causing half maximal inhibition of NBT reduction.

GSH assay

GSH levels in the cell culture supernatant were assessed according to the method described by Tietze (1969) and Andersen et al. (1996). Briefly, 100 µL of sample was placed on a 3-mL cuvette and then 750 µL of 10 mM 5-50-dithio-bis-2-nitrobenzoic acid (DTNB-Sigma) solution (100 mM monopotassium phosphate 5 mM Na₂-EDTA, pH 7.5 and GSH reductase (GSH-RD, 625 IU/L) was added and incubated for 3 min at room temperature. Then, 150 µL of 1.47 mM β-nicotinamide adenine dinucleotide phosphate (β-NADPH-Sigma) was added, mixed rapidly by inversion, and the rate of 5-thio-2-nitrobenzoic acid formation (proportional to the sum of reduced and oxidized GSH) was measured spectrophotometrically for 2 min at 412 nm. The reference cuvette contained equal concentrations of DTNB and NADPH but no

sample, and values were expressed in micromole (μmol) per gram protein.

GPx assay

GPx activity in the cell culture supernatant was measured by using the method described by Paglia and Valentine (1967). Briefly, 50 μL of sample was combined with 100 μL of 8 mM NADPH, 100 μL of 150 mM reduced GSH, 20 μL of GSH-RD (30 units/ μL), 20 μL of 0.12 M NaN_3 solution, and 2.65 μL of 50 mM potassium phosphate buffer (pH 7, 0.5 mM EDTA), and the tubes were incubated for 30 min at 37 °C. The reaction was initiated with addition of 100 μL of 2 mM hydrogen peroxide solution, mixed rapidly by inversion, and the conversion of NADPH to NADP oxidized was measured spectrophotometrically for 5 min at 340 nm. The enzyme activity was expressed in units per gram protein using an extinction coefficient of 6.22×10^{-6} for NADPH at 340 nm.

MDA assay

MDA levels in the cell culture supernatant were determined spectrophotometrically according to the method described by Ohkawa et al. (1979). A mixture of 8.1 % sodium dodecyl sulphate, 20 % acetic acid, and 0.9 % thiobarbituric acid (Sigma) were added to 0.2 mL of sample, and distilled water was added to this mixture to make the total volume 4 mL. This mixture was incubated at 95 °C for 1 h. After the incubation, the tubes were left to cool in cold water, and 1 mL distilled water with 5 mL n-butanol/pyridine (15:1 v/v) was added, followed by mixing. The samples were centrifuged at 4000g for 10 min. The supernatants were removed, and then, at 532 nm, the absorbance was measured with respect to a blank. 1, 1, 3, 3-tetraethoxypropane (Sigma) was used as the standard. Lipid peroxide levels were expressed in micromoles per liter MDA.

Statistical analysis

For the statistical analysis of biochemical parameters and analysis of SCE values, Mann–Whitney U test and Oneway ANOVA were used. The value of $p < 0.05$ was accepted as statistically significant. Results were expressed as mean + SD. For these procedures, Statistical Package for Social Sciences (SPSS version

16.0 for Windows; SPSS Inc., Chicago, IL, USA) was used.

Results

In the present study, our results showed that the use of AFB_1 alone caused significant SCE and MN formations on peripheral lymphocytes as shown in Table 1, Figs. 1 and 2. The formations of SCE and MN frequencies caused with the AFB_1 found to be statistically significant ($p < 0.05$). The simultaneous treatments of AFB_1 with different concentrations of MSA reduced the SCE and MN. It was found that statistically significant ($p < 0.05$). 5, 10 and 20 mg/mL concentrations of MSA reduced SCE and MN frequencies significantly. But 40 mg/mL concentration of MSA induced an increase of SCE and MN frequencies.

In the biochemical results, the activities of SOD, GPx and GSH, MDA levels of the control and experimental groups are presented in Table 2 and Fig. 2b. A decrease in the activities of SOD, GPx, GSH level and an increase in the MDA level were observed when the samples were treated with AFB_1 as shown in Table 2. Cotreatment of AFB_1 with MSA extract reduced the level MDA and increased the activities of SOD and GPx and GSH level. The statistical analysis showed a significant difference in SOD and GPX activities and GSH and MDA level between all treated samples with AFB_1 and simultaneous treatment of AFB_1 with MSA ($p < 0.01$ or $p < 0.05$).

Discussion

In this study, genotoxic, antigenotoxic and antioxidant activity of MSA were examined against AFB_1 using SCE and MN test systems. In the SCE and MN tests, AFB_1 was used as a positive control. AFB_1 is a well known mycotoxin and is known to stimulate the release of free radical, including reactive oxygen species, which lead to gene mutations, chromosomal aberrations, formation of MN and SCE (Alpsoy et al. 2009, 2011; Sedmikova et al. 2001; Anar et al. 2013).

In the SCE and MN tests, MSA showed a great antimutagenic potential against AFB_1 . The use of concentration of MSA (5, 10 and 20 mg/mL) showed

Table 1 Values for sister chromatid exchanges (SCEs)/cell and micronuclei (MN) in the cultures of human peripheral lymphocytes in the study groups

Culture types	Metaphase	Range of SCEs	Counted of MN	SCEs/Cell	MN/1000 cell
Control	20	3–8	1009	5.7 ± 0.23 ^a	3.4 ± 0.35 ^a
AFB1 (5 mM)	20	4–10	1004	8.68 ± 1.12 ^d	5.48 ± 0.96 ^c
MSA (10 mg/mL)	20	3–7	1010	6.3 ± 0.43 ^a	3.96 ± 0.43 ^{a,b}
AFB1 + MSA (5 mg/mL)	20	2–9	1007	7.9 ± 0.45 ^c	4.65 ± 0.24 ^c
AFB1 + MSA (10 mg/mL)	20	4–6	1004	7.01 ± 0.43 ^{b,c}	4.31 ± 0.43 ^{b,c}
AFB1 + MSA (20 mg/mL)	20	3–7	1003	6.5 ± 0.43 ^{a,b}	4.15 ± 0.23 ^{a,b}
AFB1 + MSA (40 mg/mL)	20	2–6	1003	8.01 ± 0.45 ^c	5.13 ± 0.32 ^d

^a $p < 0.001$ compared with control group^b $p < 0.001$ compared with AFB₁ group^c $p < 0.05$ compared with MSA (10 mg/mL) group^d $p < 0.05$ compared with MSA (40 mg/mL) group **Fig. 1** The effects of AFB₁ (5 mM), MSA (10 µg/mL) and different concentrations of MSA (5, 10, 20 and 40 mg/mL) together with AFB₁ on the number of SCEs and MN in human peripheral lymphocytes. All data are shown as mean ± SE.

Statistically significant differences (One-way ANOVA with Dunnett's multiple comparison test) are as follows; $p > 0.05$ (not significant, ns), $*p < 0.05$ (significant), $**p < 0.01$ (very significant), $***p < 0.001$ (extremely significant)

significant antimutagenic effects. However, 40 mg/mL concentration of MSA increased frequencies of SCE and MN. These results could be due to the chemical compounds (phenolic and flavonoids) and high doses of MSA. The antigenotoxic effect of MSA is correlated with its concentrations. In addition, some chemical compounds (phenolic compounds, organic acids and flavonoids) of MSA could have synergistic effects such as AFB₁.

Our results showed that MSA have antigenotoxic activity. This antimutagenic activity of MSA may be explained with inhibitor activities of the MSA on free radicals. Therefore, in the present study, SOD and GPx activities, GSH and MDA level were measured to determine the antioxidant capacity of MSA. The results

suggest that the activities of SOD and GPx and GSH level decreased, and the MDA level increased through AFB₁ application. On the other hand, with the application of increasing concentrations of MSA with AFB₁, the antioxidant enzyme activities (SOD and GPx) and GSH levels increased and the levels of MDA decreased.

According to the literature, the *S. tectorum* has important biological properties including antioxidant, antinociceptive, liver-protecting, membrane stabilizing effect, antimicrobial, antiinflammatory activity (Stojicevic et al. 2008; Alberti et al. 2008; Kekesi et al. 2003; Abram and Donko 1999). But, there is not enough information about *S. armenum*. Our study showed that *S. armenum* has biological activities like as antigenotoxic and antioxidant capacity. However,

Fig. 2 The effects of AFB₁ (5 mM), MSA (10 µg/mL) and different concentrations of MSA (5, 10, 20 and 40 mg/ml) together with AFB₁ on **a** SOD, GPx activities and **b** GSH and MDA activities. All data are shown as mean ± SE. Statistically significant differences (One-way ANOVA with Dunnett's multiple comparison test) are as follows; $p > 0.05$ (not significant, ns), $*p < 0.05$ (significant), $**p < 0.01$ (very significant), $***p < 0.001$ (extremely significant)

Table 2 Effects of AFB₁ and different concentrations of MSA on SOD and GPx activities, GSH and MDA levels

Groups	SOD (IU/mg protein)	GSH (IU/g protein)	GPx (IU/g protein)	MDA (nmol/mL)
Control	3.32 ± 0.06	3.66 ± 0.06	3.91 ± 0.06	4.21 ± 0.13
AFB ₁ (5 mM)	1.4 ± 0.09 ^a	2.15 ± 0.09 ^a	2.4 ± 0.16 ^a	5.92 ± 0.08 ^a
MSA (10 mg/mL)	3.02 ± 1.00 ^b	3.51 ± 0.09 ^b	3.93 ± 0.09 ^b	4.63 ± 0.23 ^b
AFB ₁ + MSA (5 mg/mL)	1.75 ± 0.12 ^c	2.39 ± 0.13 ^c	2.76 ± 0.13 ^c	5.25 ± 0.14 ^c
AFB ₁ + MSA (10 mg/mL)	2.32 ± 0.16 ^{b,c}	2.66 ± 0.11 ^{c,d}	3.07 ± 0.05 ^{b,c}	4.9 ± 0.07 ^{b,c}
AFB ₁ + MSA (20 mg/mL)	2.96 ± 0.20 ^b	3.18 ± 0.08 ^{b,d}	3.55 ± 0.17 ^{b,d}	4.54 ± 0.25 ^{b,c,d}
AFB ₁ + MSA (40 mg/mL)	2.29 ± 1.00 ^{c,d}	2.76 ± 0.06 ^{c,d,e}	3.06 ± 0.13 ^{b,c}	5.21 ± 0.17 ^{c,d}

^a $p < 0.001$ compared with control group

^b $p < 0.001$ compared with AFB₁ group

^c $p < 0.05$ compared with MSA (10 mg/mL) group

^d $p < 0.05$ compared with MSA (40 mg/mL) group

extracts of *S. armenum* leaves have not been introduced into medical practice yet. This is the first report indicating antioxidant and antigenotoxic activities of MSA against AFB₁. The antigenotoxic potency of MSA is important, especially against AFB₁ induced genotoxicity. (1) AFB₁ is an unavoidable food contaminant, (2) it can be found in many products such as wheat, barley and milk (Sedmikova et al. 2001; Signorini et al. 2012) and (3) it is the most potent, occurring and common mutagenic agent.

In conclusion, the results of our study indicated that MSA has strong antigenotoxic potential and antioxidant against AFB₁. Although, important data were obtained with the used test systems, further studies are needed. Further studies to fractionate the methanol extracts, to identify the active compounds, to adjust accurate dose and to determine all mechanisms of action should be carried out. The underlying mechanism of the antigenotoxic impact of MSA should be further investigated. The secondary metabolites of

MSA should be determined and the identified compounds should be assessed by new studies. This can enlarge the use of MSA in food and medical industries.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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