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Protective effects of *Salvia officinalis* extract against cyclophosphamide-induced genotoxicity and oxidative stress in rats

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Abstract: Medicinal plants, especially rich in polyphenolic compounds, have been suggested to be chemopreventive on account of antioxidative properties. *Salvia officinalis* L., an aromatic and medicinal plant, is widely used in folk medicine and is well known for its antioxidant properties. Therefore, the present study was designed to investigate the antioxidative, possible genotoxic, and antigenotoxic potency of *S. officinalis* extract against cyclophosphamide (CYP)-induced oxidative stress and genotoxicity in Wistar albino rats. Animals were orally dosed with *S. officinalis* extract (50, 100, and 150 mg/kg body weight) for 7 days before the administration of a single intraperitoneal dose of CYP (40 mg/kg body weight). The biochemical and cytogenetic determinations were carried out 24 h after CYP injection. The activities of malondialdehyde, superoxide dismutase, and catalase were determined in liver, kidney, and heart tissues. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) and the ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE/NCE) were also evaluated. Additionally, the antioxidant capacity of the extract was analyzed. The micronucleus assay revealed that all doses of the extract tested presented no genotoxic activity; in addition, the 2 highest doses reduced the MNPCEs and increased the PCE/NCE ratio in the bone marrow and restored the oxidative stress markers in CYP-treated rats. In correlation with these findings, *S. officinalis* extract exhibited high antioxidant capacity. The results of the present study suggest that the methanolic extract of *S. officinalis* has a protective effect against CYP-induced oxidative stress and genotoxicity through its antioxidant property.

Key words: Cyclophosphamide, *Salvia officinalis*, micronucleus, oxidative stress, antioxidant capacity

Introduction

Cyclophosphamide (CYP) is a cytotoxic alkylating agent that has been used extensively in veterinary medicine as an antineoplastic agent for the treatment of several tumors such as sarcomas and carcinomas of the lung and mammary gland (1). However, this drug has serious side effects such as inducing genotoxic effects and renal (2) and hepatic damage (3), thereby limiting its therapeutic use. Its cytotoxic effects result from the reactive metabolites that alkylate DNA and form a variety of DNA adducts that sufficiently alter DNA structure or function (4), leading to formation

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of chromosome aberrations and micronuclei (5,6). This antitumoral is also able to generate active oxygen species such as superoxide anions and hydroxy radicals that induce oxidative stress and inhibit the activity of antioxidant enzymes in several tissues. The use of antioxidants mitigates the side effects associated with CYP treatment and more efficient and comfortable therapy can be achieved (7–10).

In recent years, considerable attention has been devoted to medicinal plants particularly rich in polyphenols, mainly flavonoids and phenolic acids, which exhibit antioxidant properties due to their hydrogen-donating and metal-chelating capacities as potential chemopreventive agents (11). The phenolic compounds have demonstrated protective effects against deleterious effects of genotoxic carcinogens by scavenging reactive oxygen species (ROS) and enhancing host antioxidant defense systems (12,13). It is known that many plant infusions have a large number of these molecules (14) and, hence, it is reasonable to investigate whether plants have the capacity to prevent the genotoxic potency of specific mutagens or carcinogens that are known to generate free radicals in nontumor cells both in vivo and in vitro.

Salvia officinalis L., one of the widest-spread members of the family Lamiaceae, has been used as a traditional herbal medicine against a variety of diseases in Turkey (15). The plant is reported to have multiple pharmacological effects, including antibacterial (16,17), antiviral (18), antiinflammatory (19), hypoglycemic (20), fungistatic (21), antimutagenic (22), anticancer (23), and antioxidative (24) effects. The leaves of *S. officinalis* possess some therapeutic effects due to the presence of mainly flavonoids; phenolic compounds such as carnosic, rosmarinic, caffeic, and salvianolic acids; and other phenolic structure-based compounds (25–27) especially found in alcohol-soluble fraction (28).

Several experimental studies have demonstrated the antioxidant properties of *S. officinalis* extract and some of its constituents (9,29,30), but no detailed study has been carried out on the chemoprotective effect of *S. officinalis* leaf extract against genotoxins. Therefore, the present study was undertaken to evaluate the antimutagenic and antioxidant potential of the methanolic extract of *S. officinalis* on CYP-induced genotoxicity and oxidative stress in rats and to investigate the possible mechanism(s) underlying these effects. Genotoxicity was induced by administrating a single CYP dose in the presence or absence of *S. officinalis* extract. Herbal protection was assessed by monitoring micronucleus frequency in bone marrow and endogenous antioxidants superoxide dismutase (SOD) and catalase (CAT) and lipid peroxidation in liver, kidney, and heart tissues, as well as by determining the in vitro antioxidant capacity of the plant extract.

Materials and methods

Cyclophosphamide monohydrate with a technical purity of 97% was obtained from Acros Organics (Belgium). All chemicals were of analytical reagent grade, and the chemicals required for all biochemical assays were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA).

All of the experiments were carried out with male Wistar rats, aged 8–10 weeks, weighing 220–280 g. The male rats were chosen according to previous studies that found that the clastogenic effects of CYP were more pronounced in males than females (31). The animals were housed in polypropylene cages at room temperature (22-24 °C) with a 12-h light/dark cycle and were acclimatized for 2 weeks prior to the experimental use. Food and water were provided ad libitum. The experimental protocol was approved by İstanbul University's Veterinary Faculty Ethic Committee.

The aerial parts (leaves and flowers) of *Salvia officinalis* L. were collected from the Gülpınar village of Kumbağlar district, Ayancık, Çanakkale, Turkey, during its flowering season between the months of May and July in 2008. The voucher specimen of this plant was deposited at the herbarium of the Department of Biology, Cumhuriyet University, Sivas, Turkey (CUFH), with voucher number AA 4332.

The plant was dried under shade at 25 °C and ground in a blender. A 2-g dry plant sample was soaked in 80% methanol overnight and homogenized in an Ultra-Turrax apparatus by gradually increasing the number of cycles per unit time. The obtained

extract was transferred to centrifuge tubes, centrifuged for 10 min (5000 rpm), and subsequently filtered through a filter paper into a 100-mL flask. The same procedure was repeated 3 times with 25-mL portions of 80% methanol for the remaining part of the plant. All filtered extracts were combined and evaporated to dryness using a rotary evaporator; the residue was dried under vacuum (yield: 20.1%) and stored at 4 °C until use (32). The methanolic extraction method was chosen according to studies that reported the alcoholic extraction as the best method for the extraction of polyphenol compounds, and it was found that the alcoholic extract includes a higher content of phenolic compounds than the water extract (28,30).

The animals were divided into 8 groups of 6 animals each. Animals in group 1 received only the standard diet and served as the control. Animals in groups 2, 3, and 4 received 50 mg/kg, 100 mg/kg, and 150 mg/kg body weight (bw) *S. officinalis* extract alone by gavage for 7 days, respectively. Group 5 was treated with a single dose of CYP (40 mg/kg bw) by intraperitoneal route. Groups 6, 7, and 8 received methanolic *S. officinalis* extract at concentrations of 50 mg/kg, 100 mg/kg, and 150 mg/kg bw by gavage for 7 days, respectively, followed by CYP 1.5 h after the final feeding. The doses of *S. officinalis* extracts were based on the common dosage for humans, such that the intake of leaf at a dose of 4–6 g/day and of alcoholic extract at 2.5–7.5 g/day for a 70-kg person is recommended (33). The animals were sacrificed 24 h after CYP treatment. Both femurs were removed and used for analysis of micronucleated polychromatic erythrocytes (MNPCEs). The liver, kidney, and heart tissues were surgically removed and kept at –80 °C until the biochemical analyses.

Bone marrow cells for MNPCE assessment were isolated and slides were prepared as described previously (31,34) with slight modifications. Briefly, rats were killed and the femurs were excised and trimmed immediately. Bone marrow cells of both femurs were flushed from the channel into a centrifuge tube containing fetal calf serum. The cell suspension was centrifuged at 2200 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in a drop of serum and smears were prepared on glass slides. Four slides were prepared for each rat. The air-dried slides were fixed in methanol and stained with May-Grünwald and Giemsa. A total of 2000 polychromatic erythrocytes (PCEs) were screened per animal for scoring the frequency of PCEs containing micronuclei and the ratio of PCEs to normochromatic erythrocytes (NCEs). The criteria for scoring and identification of micronuclei were similar to those of earlier studies (35).

The effects of treatments on lipid peroxidation and antioxidant enzymes were determined by corresponding assays in every group. Tissue samples were weighed and homogenized in appropriate buffer (1.15% KCl) using a Teflon homogenizer (Miccra D-1, ART Prozess & Labortechnik GmbH, Germany). The homogenate was centrifuged at 4000 rpm for 5 min at 4 °C. Products of lipid peroxidation were estimated by measuring the concentration of malondialdehyde (MDA) expressed as thiobarbituric acid-reactive substances according to the method of Yoshoiko (36). Total SOD activity was analyzed according to the method of Sun et al. (37) with a slight modification by Durak et al. (38) based on the inhibition of nitroblue tetrazolium reduction by the xanthine/xanthine oxidase system as a superoxide generator. CAT activity was determined by measuring the decomposition of hydrogen peroxide according to the method of Aebi (39) and was expressed as k/g protein, where k is the first-order rate constant. Contents of protein were measured in the homogenates according to the method of Lowry et al. (40) using bovine serum albumin as the standard.

The Trolox equivalent antioxidant capacity (TEAC) values of plant extract and tissue homogenates were determined by the cupric ion reducing antioxidant capacity (CUPRAC) method (41) based on the reduction of bis(neocuproine) copper(II) as the chromogenic oxidizing agent by antioxidants. A standard curve was prepared using different concentrations of Trolox (TR). The results were expressed as mmol TR per gram weight.

The statistical analyses were performed using SPSS 11.5. Statistical comparisons among several groups were made by one-way ANOVA. In any case, a difference was considered as statistically significant at $P < 0.05$.

Results

The anticlastogenic effects of pretreatment with *S. officinalis* extract on the frequency of MNPCEs and the PCE/NCE ratio are shown in the Figure. There was a significantly greater frequency of MNPCEs in CYP-treated rats (group 5) compared with the control (group 1) and the groups given only extract (groups 2, 3, 4). Pretreatment with 100 and 150 mg/kg bw resulted in a MNPCE frequency of 27.62 and 20.38 in 2000 PCEs, respectively, which was significantly ($P < 0.05$) less than the rate of 52.25 observed with CYP treatment. The number of MNPCEs was significantly different between the groups treated with CYP (groups 5, 6, 7, 8) and the control (group 1) ($P < 0.05$). Furthermore, the ratio of PCEs to NCEs, as one index of cytotoxicity, was decreased in animals treated with CYP. However, in animals pretreated with *S. officinalis* extract, the PCE/NCE ratio was increased significantly ($P < 0.05$) compared to the CYP group (group 5).

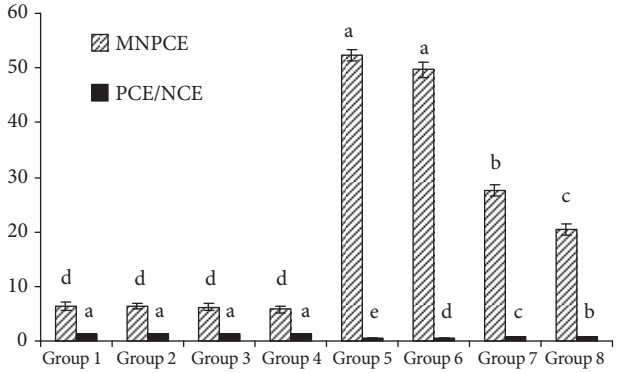

Figure. Effect of *Salvia officinalis* extract on the frequencies of MNPCEs and the PCE/NCE ratio on cyclophosphamide-treated rats. Values are expressed as mean ± SE; n = 6 for each treatment group. Values with different letters are significantly different ($P < 0.05$).

The effects of *S. officinalis* and CYP on the levels of lipid peroxidation in rat liver, kidney, and heart tissues are shown in the Table. CYP treatment led to a significant increase in MDA levels in all groups as compared to the control group. Despite this, significant restoration in the MDA profile was observed in animals that received *S. officinalis* pretreatment (100 and 150 mg/kg bw) as compared to animals that received only CYP.

As shown in the Table, the activity of SOD in liver, kidney, and heart tissue homogenates was significantly decreased in CYP-treated rats when compared with the control. Oral administration of methanolic extract at dosages of 100 and 150 mg/kg bw showed a significant increase in SOD activity in all tissues of CYP-treated rats as compared to animals that received only CYP.

Table. Effect of pretreatment of *Salvia officinalis* extract on the levels of lipid peroxidation expressed as MDA (μM/g protein), CAT (k/g protein), and SOD (U/g protein) activities in rat liver, kidney, and heart tissues (n = 6 per group). Values are expressed as mean ± standard error. In each row, values with different superscripts indicate a significant difference ($P < 0.05$).

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Liver	**MDA**	19.08 ± 0.87^{c}	18.04 ± 0.67^{c}	17.56 ± 0.79^{c}	16.28 ± 0.61^{c}	35.06 ± 1.33^{a}	33.45 ± 0.73^{a}	28.22 ± 0.81^{b}	25.97 ± 1.44^{b}
	CAT	93.89 ± 3.15^{b}	95.90 ± 2.81^{b}	97.81 ± 1.39^{b}	106.40 ± 2.05^{a}	24.10 ± 0.87^{f}	40.57 ± 1.73^{e}	50.76 ± 1.58^{d}	67.00 ± 1.84^{c}
	SOD	126.17 ± 1.86^{bc}	130.16 ± 2.45^{b}	137.80 ± 3.63^{ab}	146.75 ± 3.54^{a}	85.52 ± 1.67^{e}	91.29 ± 1.96^{de}	101.36 ± 1.81^{d}	112.62 ± 2.42^{d}
Kidney	**MDA**	18.15 ± 1.14^{c}	18.29 ± 0.96^{c}	17.34 ± 0.95^{c}	12.45 ± 0.63^{d}	28.79 ± 1.31^{a}	27.13 ± 0.70^{a}	23.77 ± 0.56^{b}	21.49 ± 1.21^{b}
	CAT	74.93 ± 1.61^{b}	75.33 ± 1.48^{b}	76.56 ± 1.79^{b}	84.17 ± 1.88^{a}	18.71 ± 0.60^{f}	42.43 ± 1.49^{e}	48.23 ± 1.28^{d}	54.93 ± 1.80^{c}
	SOD	148.17 ± 2.75^{b}	154.21 ± 1.79^{b}	162.38 ± 3.24^{a}	166.38 ± 3.05^{a}	95.30 ± 1.79^{e}	100.70 ± 1.93^{e}	113.95 ± 4.75^{d}	125.49 ± 2.07^{c}
Heart	**MDA**	17.97 ± 0.82^{d}	17.57 ± 0.87^{d}	16.76 ± 0.73^{d}	15.82 ± 0.83^{d}	30.77 ± 0.91^{a}	30.23 ± 0.76^{ab}	28.16 ± 0.88^{b}	22.83 ± 0.84^{c}
	CAT	54.64 ± 1.36^{b}	56.80 ± 1.52^{b}	57.09 ± 1.43^{b}	63.42 ± 1.49^{a}	14.73 ± 0.93^{e}	16.55 ± 1.29^{e}	25.53 ± 1.53^{d}	36.75 ± 1.02^{c}
	SOD	96.94 ± 1.64^{b}	97.81 ± 1.74^{b}	99.45 ± 2.13^{ab}	105.01 ± 2.71^{a}	59.88 ± 2.15^{e}	60.03 ± 2.22^{e}	66.07 ± 1.62^{d}	78.90 ± 1.30^{c}

In CYP-treated groups, CAT levels were significantly decreased in all tissues as compared to the control animals. In the pretreatment groups receiving the methanolic extract of *S. officinalis* prior to CYP, CAT activity was significantly increased in liver and kidney tissues in a dose-dependent manner. In heart tissue, only doses of 100 and 150 mg/kg bw restored CAT activity as compared to animals that received only CYP.

Cupric ion reducing capacity of the methanolic *S. officinalis* extract was investigated for the first time in this study. The TEAC value of the methanolic extract of *S. officinalis* was 0.56 ± 0.03 mmol TR/g weight.

Discussion

CYP is an effective anticancer drug that belongs to the class of nitrogen mustards. Its usage is severely limited by its physiological side effects, and its possible induction of genotoxicity in nontumor cells has been documented in humans as well as in a variety of animal species (42,43). The cytotoxic effect of CYP is directly connected with free radical-mediated metabolism. It is rapidly metabolized in the liver by cytochrome P-450 enzymes and generates active alkylating metabolites such as 4-hydroxycyclophosphamide, aldophosphamide, and acrolein, which interfere with cellular DNA synthesis in dividing cells and induce DNA single-strand breaks (44) that may result in micronucleus formation and cell death (45,46). Acrolein also produces oxidative stress resulting in a decrease in the activities of antioxidant enzymes and in an increase in lipid peroxidation and the production of intracellular ROS such as superoxide anion radicals, hydroxyl radicals, and singlet oxygen. These reactive oxygen and nitrogen species damage cellular lipid, proteins, and DNA (47). It was reported that acrolein and lipid peroxidation product MDA belongs to the carbonyl compounds, which are very reactive and can interact with amino acids of protein, causing structural and functional changes in the enzymes (46,48). In our study, we observed that intraperitoneal administration of CYP led to oxidative stress as evident from significant increases in MDA level and decreases in antioxidant enzymes such as SOD and CAT. Despite this, in animals pretreated with *S. officinalis* extract, the extent of oxidative stress was significantly decreased, especially with the 2 highest doses of the extract.

The micronucleus test is one of the simplest short-term assays for biomonitoring of the genotoxicity of chemical carcinogens and the effect of putative chemopreventive agents (12). The micronucleus in young erythrocytes arises primarily from chromosomal fragments or lagging chromosomes that are not incorporated into the daughter nucleus at the time of cell division in the erythropoietic blast cells, and changes in the incidence of MNPCEs are considered to reflect chromosomal damage (49,50). Evaluation of erythropoietic cytotoxicity is a convenient method of monitoring erythropoiesis. The occurrence of fewer PCEs relative to NCEs is considered to be an indicator of mutagen-induced cytotoxicity (51).

In our study, administration of *S. officinalis* extract for 7 days resulted in suppression of the mitotic activity of the erythropoietic system and inhibition of the micronucleus formation induced by CYP in rat bone marrow cells. *S. officinalis* extract significantly reduced the frequency of MNPCEs, increased the PCE/NCE ratio, and showed a protective and anticlastogenic effect against the side effects of CYP wherein the protection was more pronounced at a dose of 150 mg/kg bw.

A relationship between genotoxicity and oxidative stress has been well demonstrated in many experimental animal models (12,52,53). Many authors found that genotoxicity and chromosomal instability induced by many agents are directly correlated with the parameters of oxidative stress (54,55). Numerous studies have demonstrated that ROS such as superoxide, hydroxyl radical anion, and hydrogen peroxide are important mediators of DNA damage and tissue injury (56). SOD and CAT are the important antioxidant enzymes of cell defense against free radical damage. SOD constitutes an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 and molecular oxygen, which are deleterious to polyunsaturated fatty acids and proteins (57). CAT is a hemoprotein, which catalyzes the reduction of hydrogen peroxides and is known to be involved in the detoxification of H_2O_2 concentrations (58). In the present study,

the activities of SOD and CAT decreased in CYP-treated rats as reported earlier (57,59), which could be due to inactivation of cellular antioxidants by the lipid peroxides and ROS that are produced due to CYP intoxication. However, pretreatment with the *S. officinalis* extract restored the enzyme levels and decreased the formation of lipid peroxidation byproduct MDA. The restoration of oxidative stress by improving the antioxidant defense system might be ascribed to the free radical scavenging/antioxidant properties of the phytochemical constituents present in *S. officinalis*.

There is increasing evidence that a plethora of plant components such as vitamins, flavonoids, terpenoids, polyphenolics, carotenoids, catechins, and plant steroids can act as inhibitors of mutagenesis (13,22). It is known that chemoprotective agents are capable of exerting their antigenotoxic effects by a single mechanism or a combination of mechanisms. These mechanisms include the scavenging of ROS, the inhibiting of formation of reactive carcinogenic metabolites, the inducing of carcinogen-detoxifying enzymes, and the influencing of apoptosis and inhibiting of cell proliferation (52,60).

Several reports indicated that the compounds responsible for antioxidative activity of *S. officinalis* are mainly phenolic acids and flavonoids, namely rosmarinic acid, caffeic acid, carnosol, and carnosic acid (11,61). Through their free radical scavenging capacity, *S. officinalis* and its phenolic compounds have been shown to have protective effects against oxidative stress (30). Therefore, the antioxidant capacity of the plant extract was determined by the CUPRAC method, which is capable of assaying both hydrophilic and lipophilic antioxidants. The CUPRAC method, using a cupric-neocuproine (2,9-dimethyl-1,10-phenanthroline) chelate as the chromogenic oxidant, is based on the redox reaction with antioxidants producing the cuprous-neocuproine chelate showing maximum light absorption at 450 nm (41). The TEAC value of methanolic extract was found to be 0.56 ± 0.03 mmol TR/g weight. Since there are no data on the TEAC values of *S. officinalis* methanolic extract before measurement by the CUPRAC method, the result was only compared to the data on the TEAC value of *S. officinalis* measured by modified CUPRAC assays in acetone aqueous solution (n = 3). The TEAC value of the herbal tea extract of *S. officinalis* in acetone aqueous solution was reported as 0.356 ± 0.005 mmol TR/g weight (32). The TEAC value of the hydroalcoholic concentrated extracts of *S. officinalis* measured by ABTS method was reported in the range of 351.87–479.04 μmol TR/mL extract (62). Since TEAC is a quantification of the effective antioxidant activity of the extract, a higher TEAC value would involve greater antioxidant activity. Our finding was higher than the reported results that the methanolic extraction of *S. officinalis* has a higher antioxidative activity, which could be correlated to the total content of polyphenols.

The pharmacological properties of *S. officinalis* extract, which are mostly described as antioxidants, might explain the way in which it protects the tissues and bone marrow chromosomes against CYP-induced genotoxicity and oxidative stress. It was reported that essential oil of *S. officinalis* L. and *S. officinalis* (sage) in the form of tea infusion did not show any mutagenic effect and showed antimutagenic effect against UV-induced mutations in *Escherichia coli* and *Saccharomyces cerevisiae* in the *Salmonella*/microsome mutagenicity assay Ames test (22) and in the wing spot test of *Drosophila melanogaster*, respectively (63). The latter authors concluded that antioxidant activity and suppression of metabolic activation could be mechanisms through which sage or some of its components act as desmutagens (63). In our study, the inhibition of micronucleus formation by the extract suggests that the antimutagenic potential of the extract is mostly probably due to antioxidant and anticarcinogenic activity related to the high antioxidant capacity of the extract. The results of the in vitro antioxidant studies carried out, as evidenced by measuring the antioxidant capacity of the extract, also revealed that *S. officinalis* extract may contain powerful inhibitor compounds that might act as primary antioxidants and react with free radicals.

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