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ORIGINAL ARTICLE

Assessment of Protective Effects of Methanolic Extract of *Salvia verbenaca* Roots Against Oxidative Damage Induced by Hydrogen Peroxide

Hidrojen Peroksitin Neden Olduğu Oksidatif Hasara Karşı *Salvia verbenaca* Köklerinin Metanol Ekstresinin Koruyucu Etkilerinin Değerlendirilmesi

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ABSTRACT

Objectives: *Salvia verbenaca* is a medicinal plant that has been traditionally used in Algeria for the treatment of wounds and emptied abscesses. The present study aimed to evaluate the cytotoxicity of methanolic extract of *S. verbenaca* roots and explore its ability to bestow protection against oxidative damage induced by H_2O_2 (200 µM).

Materials and Methods: The cytotoxic effects and protective properties of *S. verbenaca* on human monocytic leukemia cells (THP-1) was studied using thiazolyl blue tetrazolium bromide assay. The protective effects of the extract against H_2O_2-induced oxidative damage was evaluated using single cell gel electrophoresis (comet) assay and 2,7-dichlorodihydrofluorescien diacetate (H2DCFDA) assay.

Results: *S. verbenaca* extract was found to be non-cytotoxic at concentrations <500 µg/mL. However, the use of 500 and 1000 µg/mL of the extract decreasedcell viability. H2DCFDA assay provided evidence for anti-oxidative properties of *S. verbenaca*. Addition of *S. verbenaca* (1 and 10 µg/mL) resulted in significant reductionin H_2O_2-induced reactive oxygen species (ROS) production. Further, comet assay showed that addition of the extract resulted in a significant reductionin the length and % DNA content of comet tail. Additionally,nuclei in the cells also appeared to be devoid ofdegradation.

Conclusion: The use of *S. verbenaca* root extract conferred protection against H_2O_2-induced ROS production and DNA breakage *in vitro*.

Key words: *Salvia verbenaca* root, THP-1 cells, reactive oxygen specices production, comet assay

ÖZ

Amaç: *Salvia verbenaca*, Cezayir'de geleneksel olarak yaraların ve boşalan apselerin tedavisinde kullanılan tıbbi bir bitkidir. Bu çalışma, *S. verbenaca* köklerinin metanol ekstresinin sitotoksisitesini değerlendirmeyi ve H_2O_2 (200 µM) ile indüklenen oksidatif hasara karşı koruyucu etkisini araştırmayı amaçlamaktadır.

Gereç ve Yöntemler: *S. verbenaca*'nın insan monositik lösemi hücrelerindeki (THP-1) sitotoksik etkileri ve koruyucu özellikleri tiyazolil mavi tetrazolyum bromür testi ile belirlendi. Ekstrenin H_2O_2 kaynaklı oksidatif hasara karşı koruyucu etkileri, tek hücre jel elektroforezi (comet) testi ve 2,7-diklorodihidroflorescien diasetat (H2DCFDA testi) kullanılarak değerlendirildi.

Bulgular: *S. verbenaca* ekstresinin <500 µg/mL konsantrasyonlarında sitotoksik olmadığı bulundu. Bununla birlikte, 500 ve 1000 µg/mL ekstrenin kullanılması hücre canlılığını azaltmıştır. H2DCFDA testi, *S. verbenaca*'nın antioksidan özellikleri için kanıt sağlamıştır. *S. verbenaca* (1 ve 10 µg/mL) ilavesi, H_2O_2 ile indüklenen reaktif oksijen türlerinin (ROS) üretiminde önemli derecede azalma ile sonuçlandı. Ayrıca, comet analizi, ekstrenin eklenmesinin comet kuyruğunun uzunluğunda ve % DNA içeriğinde önemli bir azalmaya neden olduğunu gösterdi. Ek olarak, hücredeki çekirdeklerde degradasyon olmadığı görüldü.

Sonuç: *S. verbenaca* kökü ekstresinin kullanılması, *in vitro* olarak H_2O_2 kaynaklı ROS üretimine ve DNA kırılmasına karşı koruma sağladı.

Anahtar kelimeler: *Salvia verbenaca* kökü, THP-1 hücreleri, reaktif oksijen türlerinin üretimi, comet testi

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INTRODUCTION

Plants of the *Salvia* genus are widely known for their applications in food, cosmetics, and pharmaceutical industries.[1] *Salvia* is the largest genus of the Lamiaceae family with ~1000 species distributed all across the globe, particularly in the Mediterranean basin, South-East Asia, and Central and South America.[2] Essential oils and concentrates from *Salvia* species are endowed with numerous therapeutic properties, including antimicrobial, hypoglycemic, antiphlogistic, antituberculous, and anti-inflammatory properties. The use of these oils also aids in cancer prevention.[3-5] In Algeria, 18 species of *Salvia* genus are known. Among these, *Salvia verbenaca*, commonly known as Meryiamia or khiyata, is used in combination with other medicinal herbs to treat cold.[6] Flowering leaves and tops of this plant exhibit stomachic, stimulating,[7] tonic, vulnerary, and antirheumatic effects.[8]

S. verbenaca is used to prepare tonics and stimulating infusions.[9] Freshly chopped leaves of this plant are applied as a poultice on the infected wounds and emptied abscesses to facilitate healing.[10]

Oxidative stress is generally induced by the generation of reactive oxygen species (ROS), which principally react with proteins, lipids, and DNA.[11] ROS are known to promote decrease in glutathione (GSH), changes in hormones, oxidative DNA damage, genetic transformation, DNA chain rupture, and chromosomal modifications.[12]

Oxidative damage to DNA poses a serious problem as DNA cannot be resynthesized or corrected. ROS mainly include free radicals like hydroxyl radicals (•OH), superoxide anions ($O_2^{\bullet-}$) and non-radical molecules like hydrogen peroxide (H_2O_2). Primarily, ROS are generated from oxidative metabolism in mitochondria. The other endogenous sources of ROS include inflammatory cells and peroxisomes.[13] ROS can attach easily to DNA, which is mainly mediated via higher reactivity of ROS with strong nucleophilic sites present on nucleobases. Various mutations, like base alterations or base adhesion, can be produced via interaction of mutagenic agents with DNA bases or deoxyribose sugar.[14] Moreover, oxidative damage to DNA might induce mutations, resulting in activation of oncogenes or inactivation of tumor suppressor genes as well as alterations in gene expression.[15]

Several studies have explored the biological activity of the aerial parts of *Salvia* species. The present study aimed to evaluate biological activities of *S. verbenaca* root extract. This is the first report for *in vitro* evaluation of cytotoxic and protective effects of the methanolic extract of *S. verbenaca* roots against H_2O_2-induced oxidative stress.

MATERIAL AND METHODS

Salvia verbenaca as plant sample

S. verbenaca plants were collected from Oued Abid-Batna. The roots of the plants were dried for 15 days at room temperature and converted into fine powder using a blender.

Extraction

For extraction, 100 mg of powdered roots were macerated in a hydroalcoholic mixture (MeOH:H_2O, 7:3 v/v) for 24 h. The extraction process was repeated twice. The hydroalcoholic extracts were mixed and concentrated using a rotary vacuum evaporator. The crude extract was weighed and stored at 4°C until further use.[16]

Cell culture

Human monocytic leukemia cell line THP-1 (Rockville, MD, USA) was grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/mL streptomycin, and 50 U/mL penicillin (Sigma, Milan, Italy). The cells were cultured in T75 flasks at cell density of $5x10^5$ cells/mL and maintained at 37°C in a humidified chamber under 5% CO_2.

For assay, THP-1 cells were seeded in 24- or 96-well plates and treated with H_2O_2 (200 µM) for 24h either alone or in the presence of the extract. The extracts were added to the culture medium 30 mins prior to the addition of H_2O_2. Stock solution (50 mg/mL) of the extract was prepared by dissolving extract in dimethyl sulfoxide (DMSO) (1%).

Evaluation of cytotoxicity of plant extract

To evaluate the cytotoxic effects of *S. verbenaca*, THP-1 cells were treated with six concentrations of the extract (1, 10, 50, 100, 500, and 1000 µg/mL) and cytotoxicity was measured using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Generally, MTT assay involves conversion of yellow tetrazolium salt to purple formazan crystals in viable cells by the action of NAD(P)H-dependent oxidoreductase enzymes. The resulting insoluble formazan crystals are dissolved using DMSO and cell viability/proliferation is measured in terms of absorbance of purple colored solution at 570 nm.

The concentration at which the root extract was non-cytotoxicity was identified and used for further studies. To evaluate the protective effects of *S. verbenaca*, THP-1 cells were seeded in a 96-well plate at a cell density of $5x10^4$ cells/mL and treated with H_2O_2 (200 µM) for 24 h both in the presence and absence of the extract. Further, the cells were washed with phosphate buffer solution (PBS), transferred intoa fresh 96-well plate, and incubated with MTT (0.5 mg/mL) for 4 h at 37°C. The formazan crystals were dissolved using100 µL of acidic isopropanol (0.04 M HCl in absolute isopropanol) and the cells were incubated for 1 h.[17] The absorbance of the samples was measured at 540 nm using a microplate spectrophotometer (Tecan Italia, ColognoMonzese, Italy). The cell viability (%) was estimated using the formula, [(absorbance of treated cells/absorbance of untreated cells) ×100]. The experiment was performedin triplicates, with three wells per treatment (n=9).

Evaluation of ROS production

The production of ROS was evaluated using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) a non-fluorescent probe which crosses the plasma membrane before being cleaved into DCFH by intracellular esterases. DCFH can then be oxidized to fluorescent DCF by ROS.

In a 12-well plate, the cells were seeded at a density of $5x10^5$ cells/mL and treated with *S. verbenaca* extract (1 and 10 µg/mL), both in the presence and absence of H_2O_2. The cells were incubated for 24 h at 37°C. After 24 h, the cells were washed twice with PBS and ROS production was estimated using H2DCFDA (5 µM). The cells were incubated at 37°C for 30 min in a humidified incubator under 5% CO_2. Following this, the cells were suspended again in 200 mL PBS containing 0.1 M K_2PO_4 and 0.5% Triton X-100. The samples were transferred into a 96-well plate with transparent bottom.[18] The oxidation of H2DCFDA was estimated in terms of fluorescence using a microplate reader, with excitation-emission filters fixed at 485 and 480 nm, respectively. The amount of ROS produced was expressed in term of percentage of fluorescence intensity of THP-1 cells. The experiment was performed in triplicates, with three wells per treatment (n=9).

Evaluation of DNA breakage

H_2O_2-induced DNA breakage was assessed using comet assay, according to the protocol described by Di Pietro et al.[19] The comet assay works on the principle that undamaged DNA migrates in the gel at a slow rate and remains within the confines of the nucleoid when current is applied. Thus, it appears as an intact comet head. In comparison to this, the broken DNA migrates at a faster rate and forms a comet-like tail. The fluorescent intensity and shape of this tail can be used to measure level of damage.[20] DNA breakage can be quantified by measuring the length and % DNA in the tail of each comet. In a 96-well plate, THP-1 cells were seeded at a density of $5x10^4$ cells/mL and treated with *S. verbenaca* extract with and without H_2O_2. After 24 h, the cells were washed twice with PBS. Following this, the cells were mixed with 0.5% low melting point agarose at 37°C to achieve a concentration of $10x10^4$ cells/mL. Further, 50 µL of this cell suspension was loaded on to pre-coated glass slides and immediately covered with covers slips. These slides were incubated at 4°C for 15 min. Further, the covers slips were carefully removed and additional 50 µL of low melting agarose (0.5%) was added onto the previously coated cell layer. The samples were allowed to solidify in ice surface for 5 min. The slides were immersed into lysis solution (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, pH 10, 4°C) for 1 h. Further, the slides were incubated in an electrophoresis solution (10 N NaOH, 200 mM EDTA, pH >13) for 20 min to ensure unwinding of DNA. Post winding, the DNA was subjected to electrophoresis in a similar buffer for 30 min (25 V, 300 mA).

After electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris, pH 7.5), three times for 5 min each. The slides were stained with ethidium bromide and analyzed at 10x40 magnification using an epifluorescence microscope DM IRB (Leica Microsystem, Heidelberg, Mannheim, Germany) with an integrated digital camera (Canon Power Shot S50, Milan, Italy).

Statistical analyses

All statistical analyses were performed using One-Way ANOVA. The difference between the means of the group was analyzed by Tukey's post-hoc test using SPSS software. The results were reported as the mean±standard error and the analysis was considered significant for $p<0.05$.

RESULTS

Cytotoxicity

MTT assay was used to evaluate the cytotoxic effects of six concentrations of *S. verbenaca* root extract. As shown in Figure 1, the treatment of THP-1 cells with 200 µM of H_2O_2 resulted in a significant reduction in cell viability ($p<0.05$). After 24 h of treatment with H_2O_2 (200 µM), only 67% of the cells were viable. For *S. verbenaca* extract, four concentrations (1, 10, 50 and 100 µg/mL) did not show any significant cytotoxicity. However, use of 500 and 1000 µg/mL of the extract resulted in a significant decrease in cell viability. *S. verbenaca* extract at 1000 µg/mL concentration resulted in 70% cell death ($p<0.001$). Thus, *S. verbenaca* was found to be safe at doses ≤100 µg/mL.

Production of ROS

H_2O_2-induced oxidative stress was evaluated in terms of intracellular levels of ROS, assessed using H2DCFDA assay. As shown in Figure 2 the treatment of THP-1 cells with H_2O_2 (200 µM) alone promoted the production of ROS ($p<0.001$). To evaluate the antioxidative effect of *S. verbenaca* extract, the cells were treated with 1 and 10 µg/mL of the extract along with H_2O_2. The presence of the extract resulted in a significant reduction in the intracellular levels of ROS as compared to the cells treated with H_2O_2 alone. The protective effect of the root extract was found to be more pronounced at the concentration of 10 µg/mL. The use of *S. verbenaca* extract alone did not show any augmentation in the intracellular ROS levels (Figure 2).

DNA breakage

Comet assay was used to evaluate H_2O_2-induced DNA breakage as well as the ability of *S. verbenaca* extract to protect THP-1 cells against this DNA damage (Figure 3). DNA damage was measured in terms of the amount of DNA present in the comet head and tail. As shown in Figure 3, the treatment of THP-1 cells with H_2O_2 for 24 h resulted in a significant increase in DNA

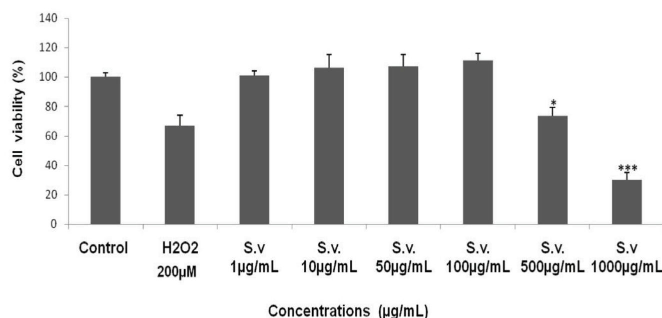

Figure 1. Effect of methanolic extract obtained from *Salvia verbenaca* roots (S.v) on the cell viability of THP-1 cells studied using MTT assay. No cytotoxic effects were observed for the root extractat concentrations <500 µg/mL, however, higher concentrations (500 and 1000 µg/mL) of the extract resulted in significant cytotoxicity. Data presented as mean±SE (n=9).*$p<0.05$ and ***$p<0.001$ compared tocontrol cells

MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide, SE: Standard error

breakage (p<0.001). As expected, THP-1 cells treated only with *S. verbenaca* root extract (1 and 10 μg/mL) did not promote any increase in the comet parameters (p>0.05). These cells showed no significant changes in the length and % DNA ofthe tail as compared to the control.

Interestingly, *S. verbenaca* resulted in a significant (p<0.001) and dose-dependent reduction in DNA breakage induced by H_2O_2 in THP-1 cells.

THP-1 cells treated with H_2O_2 alone were characterized by fragmented head and apparent tail (comet aspect). However, treatment of the cells with the extract (1 and 10 μg/mL) resulted in lower fragmentation and the cells displayed a spherical aspect. Thus, *S. verbenaca* root extract exhibited ability to protect THP-1 cells against DNA breakage induced by H_2O_2 (Figure 4).

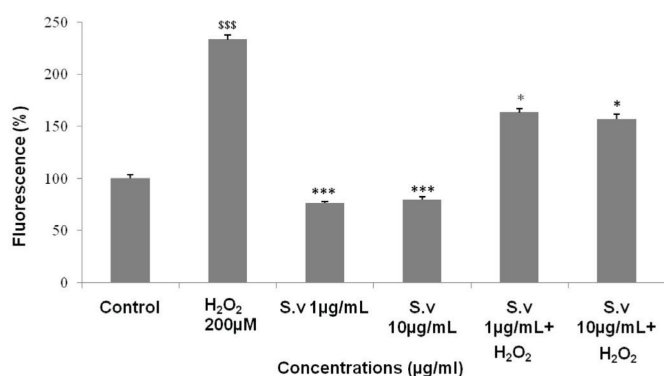

Figure 2. Effect of methanolic extract of *Salvia verbenaca* roots (S.v) on the intracellular levels of ROS induced by H_2O_2 in THP-1 cells studied using H2DCFDA assay. THP-1 cells (5x10^5 cells/mL/12-well plate) were incubated with *S. verbenaca* (1 and 10 μg/mL) with or without H_2O_2 (200 μM) for 24 h. Data presented as mean±SE (n=9). $$$p<0.001, H_2O_2 compared to control, ***p<0.001 and *p<0.05 *S. verbenaca* treated groups compared to H_2O_2

ROS: Reactive oxygen species, H2DCFDA: 2,7-dichlorodihydrofluorescien diacetate, SE: Standard error

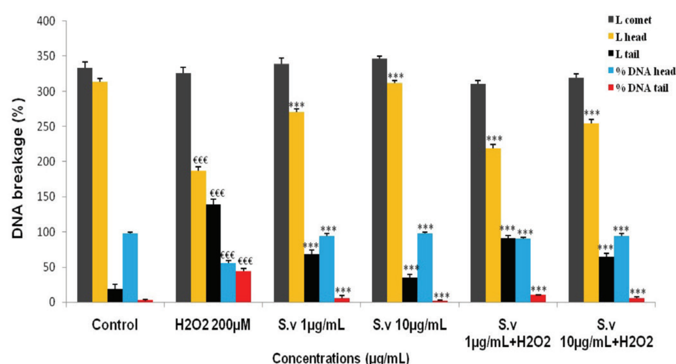

Figure 3. Effect of methanolic extract of *Salvia verbenaca* roots (S.v) on the level of DNA damage induced by H_2O_2 in THP-1 cells studied using comet assay. THP-1 cells (5x10^4 cells/mL) were incubated with *S. verbenaca* (1 and 10 μg/mL) with or without H_2O_2 (200 μM) for 24 h. DNA damage was expressed in terms of % of DNA content in comet head and tail. Data presented as mean±SE (n>50). $$$p<0.001, H_2O_2 compared to control, ***p<0.001 and *p<0.05 *S. verbenaca* treated groups compared to H_2O_2

SE: Standard error

DISCUSSION

The present study aimed toevaluate the ability of *S. verbenaca* root extract to ameliorate H_2O_2-induced ROS generation and DNA breakage. To establish the protective effects of the extract, THP-1 cells were incubated with 1 and 10 μg/mL of the extract for 24 h. *S. verbenaca* extract (at concentrations <500 μg/mL) was found to non-cytotoxic and promoted cell growth, as indicated by cell viability of >100%. Interestingly, *S. verbenaca* at concentrations >500 μg/mL was found to be more cytotoxic as compared to H_2O_2. In fact, the use of 1000 μg/mL of the extract resulted in 30% viability. Thus, all these results indicated anticancerous activity of higher doses of *S. verbenaca* toward THP-1 cells. Several previous studies have reported cytotoxic effects of various *Salvia* species, however, the concentration at which these extracts exert cytotoxic effects is species dependent.

Poyraz et al.[21] evaluated the cytotoxic activities of *S. aethiopis* L. and *S. ceratophylla* L. in mouse embryonic fibroblast cell line

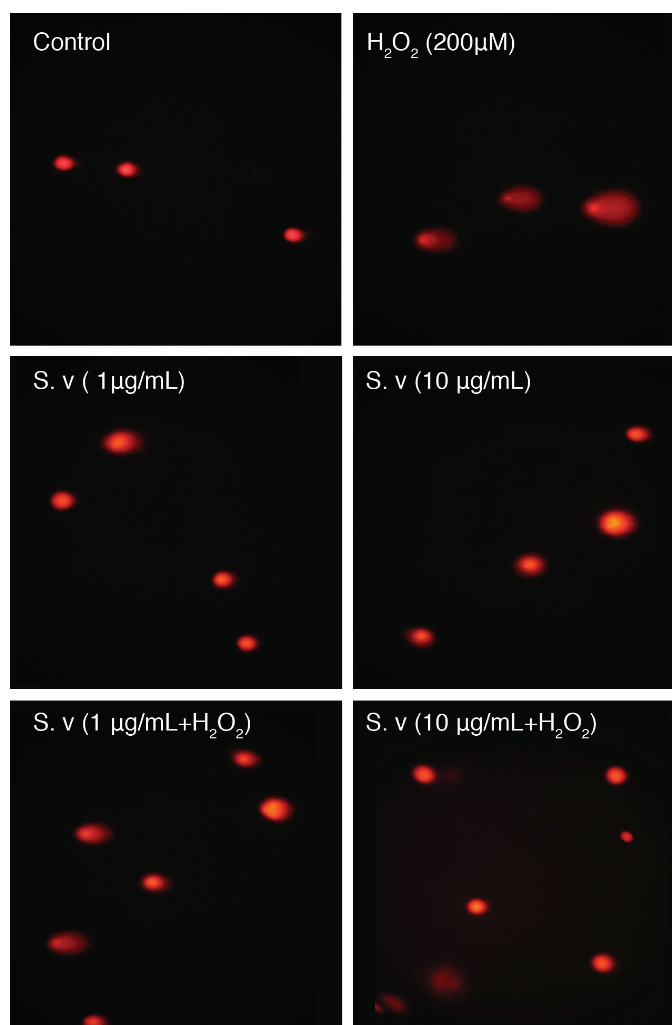

Figure 4. Fluorescence photomicrographs for DNA breakage in THP-1 cells studied using comet assay (original magnification 10x40). H_2O_2-treated cells exhibited high levels of DNA breakage (comet tail-like appearance), *S. verbenaca*-treated cells showed no DNA degradation (spherical mass), the cells treated with H_2O_2 and *S. verbenaca* (1 and 10 μg/mL) showedlow to very low levels of DNA breakage

(NIH/3T3) using MTT assay. The use of methanol and ethyl acetate extracts for both species resulted in a significant dose- and time-dependent increase intoxicity. However, the ethyl acetate extracts were found to be more cytotoxic.

Gateva et al.[22] reported the cytotoxic effects of *S. officinalis* extract at the concentration of 100 µg/mL in *Hordeum vulgare* root meristematic cells ($p<0.05$). Additionally, *S. officinalis* extract was shown to exert cytotoxic effect on human lymphocytes, especially at the concentrations of 50 and 100 µg/mL ($p<0.01$). *In vivo* study conducted by Vujošević and Blagojević[23] reported cytotoxic effects of *S. officinalis* extract, at the concentration of 100 µL/kg, toward mammalian bone marrow.

The ability of *S. verbenaca* extract to reduce H_2O_2-induced ROS generation was measured using H2DCFDA assay. H_2O_2 was used as a positive control. H_2O_2 is widely used as a model for ROS production. It generates hydroxyl radicals (•OH) in the presence of transition metal ions. Generally, H_2O_2 is delivered endogenously via certain physiological processes during oxidative phosphorylation.[24] It can enter the nucleus and interact with DNA.[25] These radicals can attack the sugar residues present in the DNA backbone, prompting single strand breaks. Additionally, these radicals can change purines and pyrimidines to their hydroxyl derivatives, for example 8-hydroxyguanine.[26]

The present study showed that the treatment of the cells with *S. verbenaca* extract (1 and 10 µg/mL) resulted in a significant reduction in the intracellular levels of ROS produced by H_2O_2 ($p<0.05$). The protective effects of *S. verbenaca* (1 and 10 µg/mL) against ROS production were found to be similar both in the presence or absence of H_2O_2. For both concentrations, *S. verbenaca* showed no cytotoxicity and promoted cell viability. The ability of *S. verbenaca* extract to reduce ROS production might be attributed to its antioxidant activity. In aprevious study, Nassar et al.[27] reported the presence of high antioxidant activity in *S. verbenaca* even at low concentrations. Similar results were reported in several studies involving *Salvia* species. Chang et al.[28] reported that the root extracts of *S. miltiorrhiza* of *Salvia* genus exhibited antiapoptotic and antioxidant effects. This root extract was endowed with cancer preventing properties. *S. miltiorrhiza* extract mediated reduction in ROS generation was achieved by inhibition of oxidases, decrease in superoxide production, inhibition of oxidative alteration of low-density lipoprotein, and promotion of mitochondrial oxidative stress. This was accompanied by an increase in the enzymatic activity of antioxidant enzymes, such as GSH peroxidase, MnSOD, and catalase.[28]

It has been previously shown that the use of sage induced an increase in the amount of antioxidant enzyme GSH in Caco-2 and HepG2 cells.[29,30] This antioxidant enzyme reduced ROS production and provided protection against H_2O_2-induced cytotoxicity in Caco-2 cells.[29]

Comet assay was used to measure H_2O_2 mediated DNA breakage in THP-1 cells. The assay also investigated the ability of *S. verbenaca* extract to protect the cells against this H_2O_2-induced DNA breakage. The treatment of the cells with H_2O_2 for 24 h resulted in a significant increase in DNA breakage and THP-1 nuclei were found to be highly fragmented. In H_2O_2 treated cells, DNA fragmentation was indicated by the augmentation of tail length and % DNA content in the comet tail as compared to the untreated cells. Interestingly, no DNA breakage was observed in the cells treated with the extract, both in the presence and absence of H_2O_2. All these results suggested that the extract was endowed with excellent ability to protect the cell nuclei against DNA breakage. The efficacy of the extract (1 and 10 µg/mL) was indicated by a significant decrease in the tail length and % DNA content in each nucleus ($p<0.001$). In addition to this, all cell nuclei were characterized by a nearly spherical shape having low breakage. These results were in concordance with the findings of Bani Hani and Bayachou.[31] The study showed that the incubation of HEK-293 cells with 100 µM/L of H_2O_2 for 3 h resulted in a significant increase in the intrinsic cellular DNA oxidation. However, the use of 100 µL of *S. fruticose* extract in the presence of 100 µM/L of H_2O_2 for 3 h resulted in a significant decrease in the intrinsic cellular DNA oxidation. These results suggested that *S. fruticose* extract might enhance the activity of DNA repair machinery.

Several *in vitro* studies have previously suggested that *Salvia* species are endowed with antimutagenic, antidiabetic, antiangiogenic, and gastroprotective properties.[32-35]

The major polyphenols found in *Salvia* species include rosmarinic acid, caffeic acid, carnosol, and carnosic acid.[36] In another study, Fotovvat et al.[37] reported the occurrence of five phenolic compounds (rosmarinic acid, salvianolic acid A, salvianolic acid B, carnosic acid, and caffeic acid) at different concentration in the roots of 41 populations of 27 *Salvia* species. However, rosmarinic and caffeic acids were found to be most abundant among these five compounds. Renzulli et al.[38] reported that rosmarinic acid present insage offered cytoprotective effect against *in vitro* cell damage induced by ochratoxin A and aflatoxin B1. Rosmarinic acid in sageacted via inhibition of toxin-induced ROS production and DNA and protein synthesis. *In vitro* study conducted by Tumur et al.[39] showed that rosmarinic acid could reduce the cell viability of HNSCC tumoral cell line. Rosmarinic acid regulated the proliferation of the cells by blocking the signaling pathway of epidermal growth factor and increased ROS levels.[39] In another study, carnosic acid and carnosol were found to inhibit ROS production and secretion of human leukocyte elastase. In addition to this, both polyphenols could attenuate the generation of proinflammatory leukotrienes in intact PMNL.[40]

Currently, very limited information is available regarding the chemical composition of *S. verbenaca* roots and its biological activities.

CONCLUSION

In the present study, *S. verbenaca* root extract was found to exhibit protective activity against H_2O_2-induced oxidative damage. The treatment of THP-1 cells with 1 and 10 µg/mL of the extract resulted in amelioration of H_2O_2-induced cytotoxicity, ROS production, and DNA breakage. Since *S. verbenaca* belongs to

the Lamiaceae family, the DNA protective effects of the extract could be attributed to its antioxidant activity. Additionally, the polyphenols present in this plant might further contribute to this protective ability. Future studies aimed at the identification of secondary metabolites present in *S. verbenaca* roots might provide better understanding regarding its protective ability.

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