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Assessment of the Protective Effect of the Methnolic Extract from *Salvia verbenaca* Roots against Oxidative Damage Induced by Hydrogen Peroxide (H_2O_2)

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ABSTRACT
Objectives: *Salvia verbenaca* is medicinal plant has been traditionally used by Algerian people to treat wounds and abscesses emptied to facilitate their healing. This works aimed to detect the cytotoxicity and the protective activities of the methnolic extract from *Salvia verbenaca* root against oxidative stress induced by H_2O_2 (200µM).
Methods: this research was carried out on human monocytic leukemia cells (THP-1) using the
following assays, thiazolyl blue tetrazolium bromide (MTT) assay, single cell gel electrophoresis (comet) assay and 2,7-Dichlorodihydrofluorescien Diacetate (H2DCFDA) assay.
Results: the findings of MTT assay showed that the concentrations of *Salvia verbenaca* less than 500 µg/mL exhibited a non-cytotoxic effect on the cell viability of THP-1 cells. Whereas two higher concentrations of extract (500 and 1000 µg/mL) manifested a cytotoxic activity, as evidenced significant decrease in the cell viability of THP-1 cells. The H2DCF-DA results revealed that *Salvia verbenaca* root extract (1 and 10 µg/mL) exhibited anti-oxidative stress effect on decreasing reactive oxygen species (ROS) amount produced by H_2O_2 in THP-1 cells. The finding of comet assay revealed a significant decrease in tail length and % DNA in comet tail after the treatment with *Salvia verbenaca* root extract and THP-1 nucleus appeared without degradation.
Conclusion: the *Salvia verbenaca* root extract can prevent strongly ROS production and DNA breakage induced by H_2O_2 in THP-1 cells.

Key words: *Salvia verbenaca* root, THP-1 cells, ROS amount, Comet assay.

INTRODUCTION

Salvia species have many uses in food, cosmetics, and pharmaceutical industries [1]. The genus Salvia is probably the largest genera in the Lamiaceae family with nearly 1000 species distributed around the world, which are mainly found in the Mediterranean basin, South-East Asia, and Central and South America [2]. Essential oils and concentrates from *Salvia* species have had numerous natural properties including antimicrobial, hypoglycemic, antiphlogistic, antituberculous anti-inflammatory and cancer prevention activities [3-5]. Algeria has eighteen species of *Salvia* genus among which is *Salvia verbenaca* that is commonly called Meryiamia or khiyata and is used with other medicinal herbs to treat cold [6]. Flowering leaves and tops have stomachic, stimulating [7], tonic, vulnerary, and anti-rheumatic activities [8].
This species is used to make tonic and stimulating infusions [9]. Freshly chopped leaves are also applied as a poultice on the infected wounds and emptied abscesses to facilitate their healing [10].

Oxidative stress is basically induced by reactive oxygen species (ROS) which react principally with proteins, lipids and DNA[11]. It is known that ROS promote a decrease in glutathione (GSH) substance, and change of reproductive hormones, oxidative DNA damages, genetic transformation, DNA strand rupture and chromosomal modifications [12]. Oxidative stress to DNA is real problem as DNA cannot be resynthesized or corrected. ROS is mainly represented by hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). ROS is generated from the oxidative metabolism of mitochondria and different endogenous sources for example inflammatory cells and peroxisomes [13]. ROS can attach easily to DNA with strong nucleophilic sites on nucleobases because of their higher reactivity. Many mutagenic agents , such as base alterations or base adhesion, can be produced through the responses with either the DNA bases or the desoxyribose sugars [14]. Moreover, oxidative damage to DNA may conduct to mutations that activate oncogenes or inactivate tumor suppressor genes as well as changing of gene expression [15].
Many studies were focused on the biological activity of the aerial parts of *Salvia* species. However, the present work has shed new light on the biological activities of *Salvia verbenaca* root extract. According to our knowledge, this is the first *in vitro* study to determine the cytotoxic and the protective activities of the methanolic extract from *Salvia verbenaca* roots against H_2O_2-induced oxidative stress.

MATERIAL AND METHODS

Salvia verbenaca as plant sample

Salvia verbenaca plants were collected from Oued Abid-Batna, the roots were dried for 15 days at room temperature, and then they were powdered by a blender.

Extraction

One hundred grams of *Salvia verbenaca* powder roots were macerated in a hydroalcoholic mixture (MeOH-H_2O, (7:3 v/v) for 24 h. The extraction was repeated three times and the three hydroalcoholic extracts were mixed then concentrated to dryness in a rotary vacuum evaporator. The crude extract was weighed, and stored at 4°C until use [16].

Cell culture

Human monocytic leukemia cell line THP-1 (Rockville, MD, USA) was grown in RPMI-1640 medium containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/mL streptomycin, and 50 U/mL penicillin (Sigma, Milan, Italy). The

cells were kept up at 37 °C in an air-humidified atmosphere of 95% and 5% CO_2.The cell concentration was maintained in the flasks at 5×10^5 cells/ mL.
THP-1 cells were cultivated in 24 or 96-well plates then incubated for 24 h with H_2O_2 (200 µM) with or without the plant extract which were added to the culture medium 30 minutes earlier of H_2O_2 addition. 50 mg/mL of the extract was dissolved in DMSO (1%) and used as mother concentration.

Evaluation of extract cytotoxicity

The MTT (3- (4,5- dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) test was used to evaluate the cytotoxicity of different concentrations (1, 10, 50, 100, 500 and 1000 µg/mL) of *Salvia verbenaca* extract. Then, the non-cytotoxic concentrations of *Salvia verbenaca* were selected for further assays. THP-1 cells were seeded in 96-well plates (5×10^4 cells/ mL) and treated with H_2O_2 (200 µM) for 24 h with or without *Salvia verbenaca* extract. THP-1 cells were washed with phosphate buffer solution (PBS) and then transferred in another 96 well plates and incubated with MTT (0.5 mg/mL) for 4 h at 37 °C. THP-1 cells were placed in 100 µl of acidic isopropanol (0.04 M HCl in absolute isopropanol) for 1h to dissolve insoluble formazan crystals[17]. A microplate spectrophotometer (Tecan Italia, ColognoMonzese, Italy) was used to read the optical density in each well at the wavelength 540 nm. Cell viability (%) was estimated by the absorbance proportion of the treated cell to untreated cells ×100. The experiment was made in triplicate, with three wells for each treatment (n=9).

Evaluation of ROS production

The 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) test was performed to quantify ROS amount produced by THP-1 cells. The cells were grown in 12-well plates ($5x10^5$ cells/mL) and treated with two concentrations of *Salvia verbenaca* extract (1 and 10 µg/mL) with and without H_2O_2 for 24 h at 37 °C. The cells were washed twice with PBS and incubated for 30 min with H2DCF-DA (5 µM) at 37 °C in 5% CO_2 incubator. Then, THP-1 cells were suspended again in 200 mL of PBS containing 0.1 M K_2PO_4 and 0.5% Triton X-100. The samples were placed in a 96-well plate with a transparent bottom [18]. The oxidation of H2DCF-DA was estimated by a fluorescence microplate reader, the excitation-emission filters were fixed at 485 and 480 nm respectively. The quantity of ROS was expressed as a percentage of the fluorescence intensity of THP-1 cells. The experiment was made in triplicate, with three wells for each treatment (n=9).

Evaluation of DNA breakage

The DNA breakage was assessed by comet assay as previously described by Di Pietro (2008)[19]. The undamaged DNA migrates in the gel at a slower rate and remains within the confines of the nucleoid to appear as an intact comet head. DNA breaks migrate relatively faster and form a comet-like tail whose fluorescent intensity and shape can be related to the level of damage [20]. DNA breakage can be quantified by measuring the length and % DNA in the tail of each comet. After 24 h of treatment with *Salvia verbenaca* extract with and without H_2O_2, THP-1 cells ($5x10^4$ cells/mL) were washed twice with PBS Then, the cells were mixed with 0.5 % low melting point agarose at 37 °C at the concentration of 10×10^4 cells/mL. Cell suspension (50 µL) was pipetted onto the pre-coated glass slides, immediately covered with covers lips, and placed at 4 °C for 15 min, the covers lips were carefully removed and another 50 µL of the low melting agarose (0.5%) was deposited onto the previous cell-containing layer and allowed to solidify. The slides were put in a lysis solution (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, pH 10, 4 °C) for 1 h. At that point the samples were incubated in electrophoresis support (10 N NaOH, 200 mM EDTA, pH >13) for 20 min for DNA loosening up, followed by an 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 to remove the alkaline buffer. The slides were stained with

ethidium bromide then analyzed at 10×40 magnification by 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 analysis was calculated by ANOVA (one-way analysis of variance), the difference between groups means was analyzed by Tukey's post hoc test using SPSS software. The data were reported as the mean ± Standard Error, $p < 0.05$ is considered significant.

RESULTS

Cytotoxicity

Cell viability study was performed to avoid any cytotoxic concentrations of the extract from *Salvia verbenaca* root on THP-1 cells. The result of the MTT test showed that the treatment with 200 µM of H_2O_2 was significantly decreased the number of viable cells in THP-1 ($p<0.05$). As shown in Figure (1), H_2O_2 (200 µM) was able to enhance 33% of cell death and only 67% of cells were viable. Further, four concentrations (1, 10, 50 and 100 µg/mL) of *Salvia verbenaca* extract did not decrease the cell viability of THP-1 cells. However, the two higher concentrations of the extract (500 and 1000 µg/mL) decreased significantly the percentage of cell viability of THP-1 cells. The most cytotoxic concentration of the extract was 1000 µg/mL which caused 70% of cell death and 30% of cell viability ($p<0.001$).

Production of ROS

The oxidative stress was detected by measuring the intracellular ROS level using the H2DCF-DA assay. The results showed that H_2O_2 (200 µM) promoted an augmentation in ROS level in THP-1 cells after 24h of treatment ($p<0.001$). The anti-oxidative effect of *Salvia verbenaca* extract was tested for only two lower concentrations (1 and 10 µg/mL). The incubation of cells with two concentrations of the extract in the absence of H_2O_2 did not cause any augmentation in the intracellular ROS levels of THP-1 cells. However, the treated THP-1 cells with extract (1 and 10 µg/mL) with H_2O_2 (200 µM) showed a significant decrease in the intracellular ROS level especially with the concentration 10 µg/mL (Figure 2).

DNA breakage

DNA induced by H_2O_2 and the ability of *Salvia verbenaca* extract to protect THP-1 against DNA damage was evaluated by comet assay (Figure 3). The amount of DNA in the head and tail were the parameters used to measure DNA damage level. As shown in Figure (3), THP-1 cells incubated with H_2O_2 for 24h showed a highly increase in DNA breakage ($p <0.001$). As expected, THP-1 cells treated alone with *Salvia verbenaca* root extract (1 and 10 µg/mL) did not promote an increase in the comet parameters ($p>0.05$), tail length and % of DNA in the tail were not significant compared to those of control).

Interestingly, *Salvia verbenaca* significantly ($p <0.001$) decreased DNA breakage (tail length, % DNA in comet head and tail) induced by H_2O_2 in THP-1 cells in a concentration-dependent manner.

THP-1 cells after exposition to H_2O_2 (200 µM) showed fragmented head and apparent tail (comet aspect), however after 24h of treatment with *Salvia verbenaca* root extract (1 and 10 µg/ml), cells appeared with spherical aspect (low fragmentation). The extract had the ability to protect cells against DNA breakage enhanced by H_2O_2 (Figure 4).

DISCUSSION

The present work was established to test the ability of *Salvia verbenaca* root extract to reduce ROS generation and prevent DNA breakage engendered by H_2O_2 in THP-1 cells. To achieve this goal, THP-1 cells were incubated with two lower concentrations of *Salvia verbenaca* root

extract for 24 h. Cytotoxicity findings showed that the tested concentrations less than 500 µg/mL were non-cytotoxic as evidenced by promoting the % of cells viability of more than 100%. Noteworthy, these concentrations had higher cytotoxic effects on THP-1 cells than H_2O_2. Especially the concentration 1000 µg/mL with only 30% of cell viability. These data referred that the higher concentrations of *Salvia verbenaca* may have anticancer activity toward THP-1 cells. Many data have already detected the cytotoxicity of some *Salvia* species, however the concentration in which *Salvia* species extract become cytotoxic differs from species to another.

Poyraz et al. (2017) [21] who evaluated the cytotoxic activities of *Salvia aethiopis* L. and *S. ceratophylla* L in mouse embryonic fibroblast cell line (NIH/3T3) using MTT assay. The tested methanol and ethyl acetate extracts for both species showed a significantly increase of toxicity depending on time and concentration however ethyl acetate extract was more cytotoxic.

According to Gateva et al. (2015) [22] *Salvia officinalis* extract exerted a cytotoxic effect at the concentration 100 µg/mL in *Hordeum vulgare* root meristematic cells ($p<0.05$). *Salvia officinalis* extract was appeared to have a cytotoxic impact in human lymphocytes especially at the concentrations 50 and 100 µg/mL ($p< 0.01$). The *in vivo* study conducted by Vujošević and Blagojević (2004)[23] revealed that *Salvia officinalis* extract had a cytotoxic effect in a in mammalian bone marrow at the concentration (100 µl/kg).

The ability of *Salvia verbenaca* extract to reduce ROS generation was measured using H_2DCFDA assay, H_2O_2 was used as a positive control. Hydrogen peroxide (H_2O_2) is recognized as a model of ROS production, it generates hydroxyl radicals (OH) in the presence of transition metal ions. H_2O_2 is delivered endogenously by a few physiological procedures, during oxidative phosphorylation [24]. H_2O_2 can certainly reach the nucleus and interact with DNA [25]. These radicals attack the DNA from the sugar residue of the spine of the DNA, prompting single strand breaks. They additionally change purines and pyrimidines to their hydroxyl derivatives, for example, 8-hydroxyguanine [26].

The obtained data indicated that *Salvia verbenaca* extract reduced significantly the intracellular amount of ROS produced by H_2O_2 after 24h of treatment ($P<0.05$), this reduction was observed with the tested concentrations (1 and 10µg/mL) which showed a very similarity effect against ROS with or without H_2O_2. Both concentrations were not cytotoxic and promoted cell viability. We suggest that this ability to reduce ROS is related to the antioxidant activity of *Salvia verbenaca* extract, already evaluated by Nassar et al. (2015) [27]. The extract showed a very high antioxidant activity at low concentrations. Other studies concerning *Salvia* species showed a similar data, according to Chang et al. (2016) [28] the root extract of *Salvia miltiorrhiza* belonging to *Salvia* genus exhibits cancer prevention agent, antiapoptotic and antioxidant effects. The extract was able to reduce ROS generation by inhibiting oxidases, decreasing superoxide production, inhibiting oxidative alteration of LDL and enhancing mitochondrial oxidative stress. As well it increases the activities of antioxidant enzymes such as glutathione peroxidase, MnSOD and catalase [28].

It has been shown that sage could increase the content of the antioxidant enzyme GSH in Caco-2 cells and HeoG2 cells [29,30] which reduce ROS production and protect Caco-2 cells from H_2O_2 cytotoxicity [29].

Comet assay was used to measure DNA breakage with or without *Salvia verbenaca* extract, H_2O_2 After 24h of exposition enhanced significantly DNA breakage and THP-1 nuclei appeared highly fragmented, DNA breakage was indicated mainly by an augmentation of the tail length and the % of DNA comet tail compared to untreated cells. Regarding the effect of *Salvia verbenaca* extract, the breakage of DNA was almost absent either with and without H_2O_2, the extract revealed a very potent activity to protect nuclei against DNA breakage, the effectiveness of both concentrations (1 and 10 µg/mL) was expressed by highly significant

decrease in tail length and DNA percentage in each nuclei ($P<0.001$), all nuclei appeared with low breakage (almost spherical shape). These results concord with those reported by Bani Hani and Bayachou (2014) [31] showed that the incubation of HEK-293 cells with 100 µM/L H_2O_2 for 3 h increase the intrinsic cellular DNA oxidation. However the treatment of cells with 100 µL *Salvia fruticosa* extract in the presence of 100 µM/L of H_2O_2 for 3h showed a significant decrease in the intrinsic cellular DNA oxidation. These results suggest that *Salvia fruticosa* extract may enhance the activity of DNA repair machinery.

In vitro studies have indicated that *Salvia* species were potentially antimutagen, antidiabetic, antiangiogenic and gastroprotective [32-35].

The major polyphenols of *Salvia* species are reported to be rosmarinic acid, caffeic acid, carnosol, and carnosic acid [36]. Another study of Fotovvat et al. (2018) [37] has shown the presence of five phenolic compounds (rosmarinic acid, salvianolic acid A, salvianolic acid B, carnosic acid and caffeic acid) with different content in the roots of 41 populations from 27 *Salvia* species, however the most abundant compounds were rosmarinic and caffeic acids. According to Renzulli et al. (2004) [38] rosmarinic acid from sage was found to offer a cytoprotective effect *in vitro* against ochratoxin A (OTA) and aflatoxin B1 (AFB1) causing cell damage, through the inhibition of ROS production, DNA and protein synthesis induced by toxins. The *in vitro* study released by Tumur et al. (2015) [39] showed that rosmarinic acid could reduce cell viabity of HNSCC tumoral cell line, and regulate their proliferation by blocking the signaling of epidermal growth factor increases ROS. Carnosic acid and carnosol were found to inhibit ROS formation and human leukocyte elastase secretion, both polyphenols could attenuate the pro-inflammatory leukotrienes formation in intact PMNL[40]. We mention that there is a rarity of data and studies regarding the chemical composition of *Salvia verbenaca* roots in special and its biological activities in general.

CONCLUSION

Salvia verbenaca root extract at the concentration 1 and 10 µg/ml was able to promote cell viability, reduce ROS production and decrease DNA breakage induced by H_2O_2. The potential mechanisms behind these DNA protective properties could be related to the potent antioxidant activity of this species as it is belonging to the Lamiaceae family, as well as to their content of polyphenols. Other complementary studies have to be planned in order to determine the major secondary metabolites in *Salvia verbenaca* roots.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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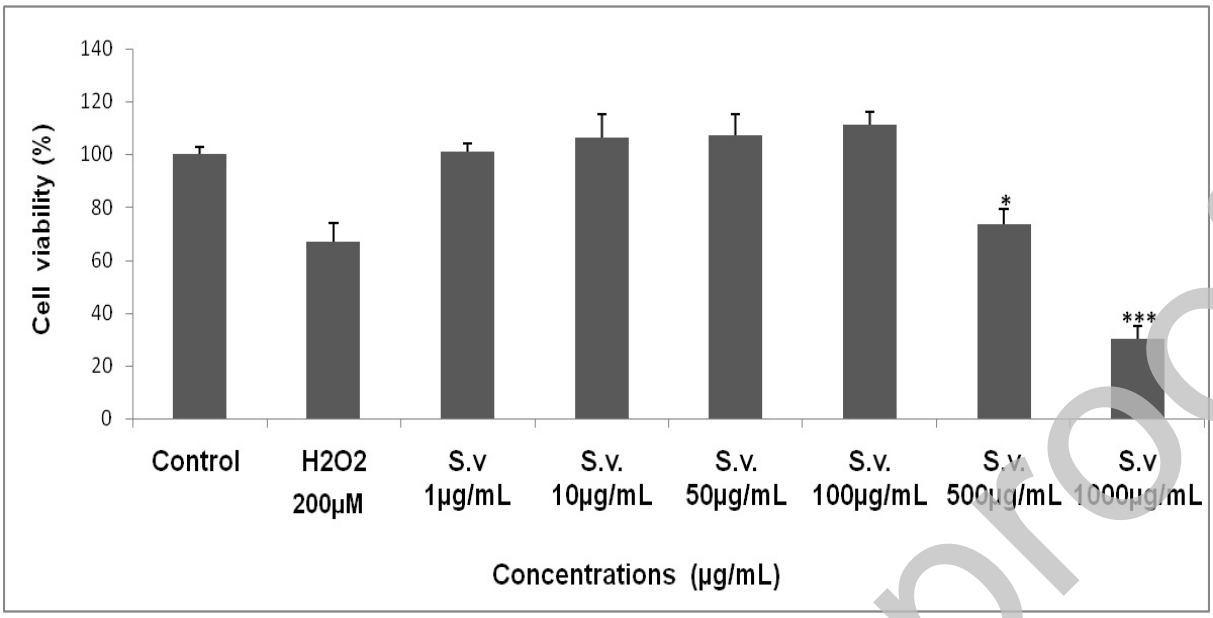

Figure 1. Effect of methanolic extract from *Salvia verbenaca* roots (S.v) on THP-1 cells viability using MTT assay. The concentrations less than 500 µg/mL did not affect cell viability, the higher concentrations (500 and 1000 µg/mL) were significantly cytotoxic. Data presented as mean ±SE (n=9). *$p<0.05$ and ***$p<0.001$ compared to control cells.

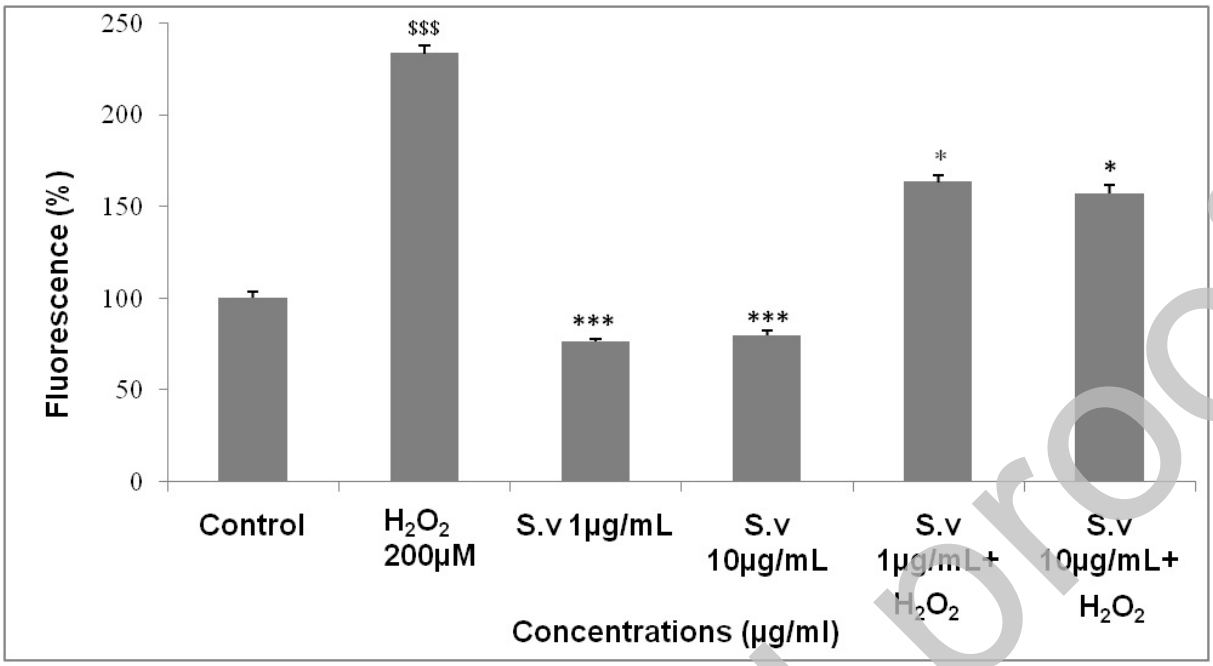

Figure 2. Effect of methanolic extract from *Salvia verbenaca* roots (S.v) extract on the level of the intracellular ROS induced by H_2O_2 in THP-1 cells using H2DCF-DA assay. The cells ($5x10^5$ cells/ mL/ 12-well plate) were incubated with *Salvia verbenaca* (1 and 10 µg/mL) with or without H_2O_2 (200 µM) for 24h. Data presented as mean±S.E (n=9). $^{\$\$\$}p<0.001$, H_2O_2 compared to control, ***p<0.001 and *p<0.05 *Salvia verbenaca* groups compared to H_2O_2.

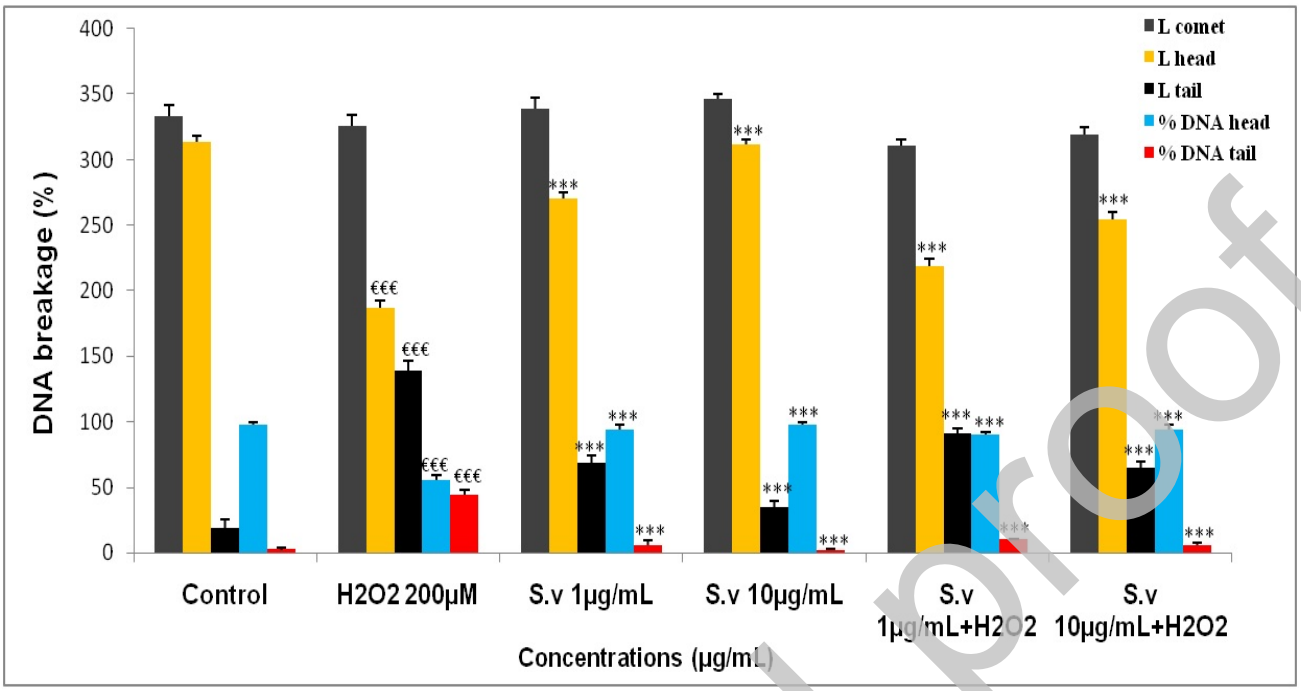

Figure 3. Effect of methanolic extract from *Salvia verbenaca* roots (S.v) extract on the level of DNA damage induced by H_2O_2 in THP-1 cells using comet assay. The cells ($5x10^4$) were incubated with *Salvia verbenaca* (1 and 10 µg/mL) with or without H_2O_2 (200 µM) for 24h. DNA damage was expressed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

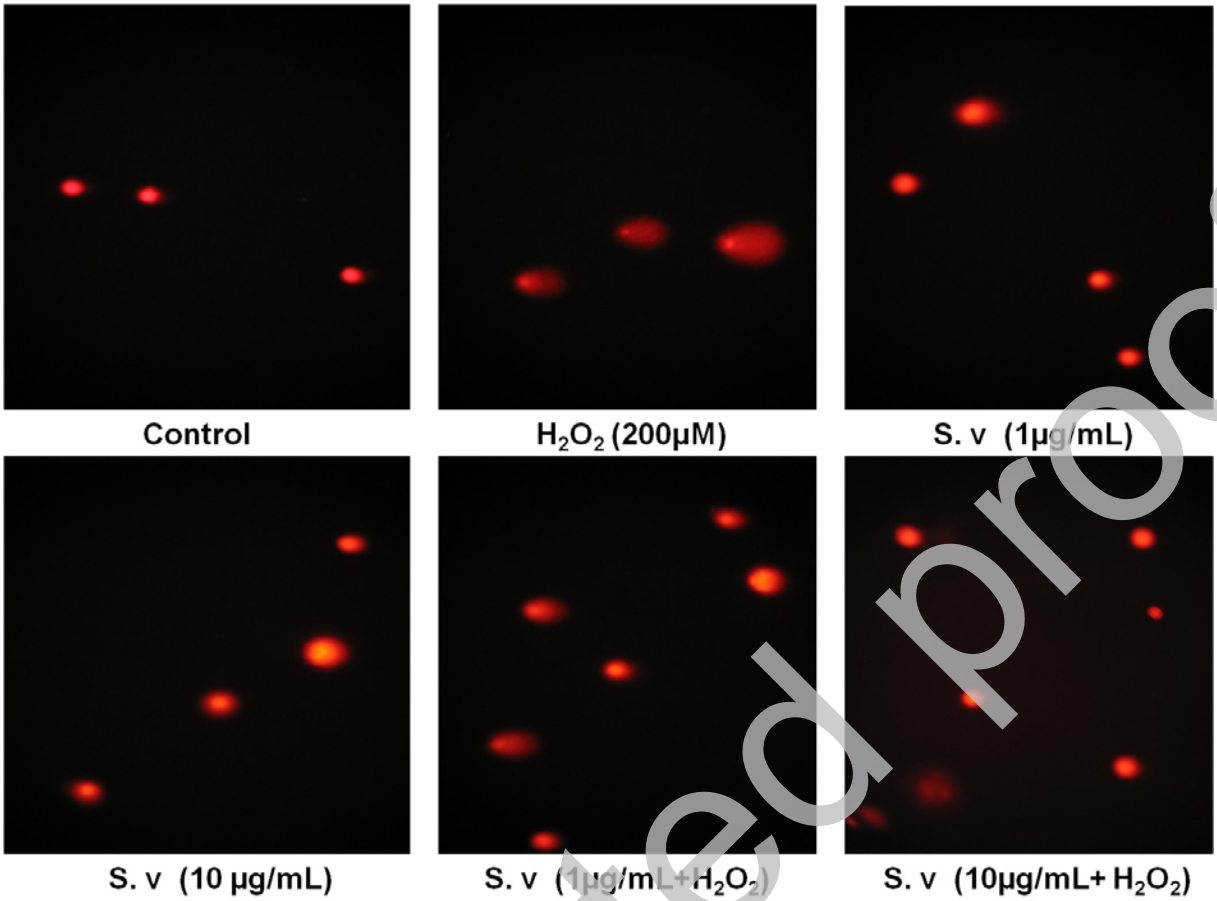

Figure 4. Fluorescence photomicrographs of THP-1 cells in comet assay (original magnification 10×40) showing: H_2O_2-treated cells exhibited high DNA breakage (comet tail-like appearance), *S. verbenaca*-treated cells showed no DNA degradation (spherical mass), cells treated with H_2O_2 and two concentrations of *S. verbenaca* (1 and10 µg/mL) appeared low to very low DNA breakage.