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Research Article

PROTECTIVE EFFECT OF ALGERIAN *SALVIA VERBENACA* EXTRACT AGAINST INFLAMMATION AND OXIDATIVE STRESS

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ABSTRACT. This study aims to examine the anti-inflammatory and antioxidant effects of polyphenolic extract of *Salvia verbenaca* L. using *in vivo* and *in vitro* models. The *in vivo* anti-inflammatory activity was evaluated using carrageenan-induced mice paw edema method. The *in vivo* antioxidant effect of the extract was explored by measuring oxidative stress (MDA, CAT, GSH and SOD) parameters. The *in vitro* anti-inflammatory activity was performed using protein denaturation and membrane stabilization assays. DPPH and hydrogen peroxide tests were used for the *in vitro* antioxidant evaluation. The highest percentage of edema inhibition was 75.03% at the concentration of 500 mg/kg. Significant increases ($p<0.05$) in the activities of CAT, SOD, GSH and significant decreases in the MDA level activity were showed. The extract was found to possess an *in vitro* anti-inflammatory activity by inhibiting the heat induced protein denaturation and red blood cells membrane stabilization with the IC_{50} values of 133.21±0.89 and 160.62±1.60 µg/mL, respectively. This study demonstrated that extract exhibited high free radical scavenging activity as showed by the low IC_{50} values for DPPH (115.41±1.40 µg/mL) and for H_2O_2 (180.39±1.77 µg/mL) scavenging assays. Our findings suggest that *Salvia verbenaca* could be tested as a drug candidate against oxidative stress and inflammation diseases.

Keywords: *Salvia verbenaca L, polyphenols, anti-inflammatory effect, antioxidant effect.*

INTRODUCTION

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as the increase of vascular permeability, increase of protein denaturation and membrane alteration. Kinins, prostaglandins, and histamine are released when tissue cells are wounded. These molecules have the role of chemical messengers that stimulate some of the natural body defense cells, this is known as chemotaxis. Non-steroidal anti-inflammatory drugs (NSAIDs) are largely used for the clinical treatment of

inflammatory diseases. The cyclooxygenase enzyme that catalyzes the biosynthesis of prostaglandins and thromboxane from arachidonic acid is inhibited by these compounds. It has also been reported that reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and peroxynitrite contribute to the process of inflammation in several tissues [1, 2]. The inflammatory tissue damage is due to the liberation of reactive oxygen species from phagocytes invading the inflammation sites [3]. Reactive oxygen species (ROS) play a crucial role in the pathogenesis of various diseases, such as neurodegenerative disorders, cardiovascular diseases, atherosclerosis, cataracts, inflammation and cancer [4]. Thus, antioxidant compounds and/or lipid peroxidation inhibitors may play an important role in the prevention of these inflammatory diseases [5]. Although steroidal anti-inflammatory drugs and non-steroidal anti-inflammatory drugs NSAIDs are currently used to treat acute inflammation, these agents carry the risk of gastro-intestinal toxicity, cardiovascular and other toxicity for prolonged use [6]. For this reason, there is a need of anti-inflammatory drugs having less severe side effects to be used. Therefore, in recent time, more interest is shown in alternative and natural drugs for treatment of various diseases such as inflammation. *Lamiaceae* is one of the most widely used families as a source of bioactive molecules with high antioxidant and anti-inflammatory potentials. The genus *Salvia*, with about 900 species throughout the world, is one of the most widespread members of this family. The traditional medical practices of the *Salvia* species have been studied all over the world [7] for a wide range of actions including epilepsy, colds, bronchitis and tuberculosis [8] as well as biological activities such as antioxidant, antimicrobial [9] anti-inflammatory [10], antidiabetic [11], antitumor [12], anti-cancer [13] and antiviral activities [14]. Additionally, Kamatou et al. [15] reported that some *Salvia* species shows a good antimycobacterial activity. Some members of this genus have also an economic importance as flavouring agents in perfumery and cosmetics [16]. *Salvia* species are rich in phenolic compounds, which in most cases are responsible for the pharmacological properties of these plants. Some bioactive compounds have been isolated and various phenolic acids and flavonoids have been considered in literature. *Salvia verbenaca* L., also known as Om lemdhamedh in Algerian traditional medicine, is one of the most popular plant remedies. The aerial part of the plant is usually used in the curative activity of wounds.

To the best of our knowledge, there are few reports carried out on evaluation of the *in vivo* activities of the plant phenolic compounds, and no reports were found in Algeria about the *in vivo* antioxidant activity. Therefore, this study was conducted, to determine the total phenol and flavonoids contents of *S. verbenaca* L., as well as to assess its *in vivo* and *in vitro* anti-inflammatory activities and effects on oxidative stress.

MATERIALS AND METHODS

Plant material and extraction

The leaves of *Salvia verbenaca* L. were harvested from Ouled Rabeh region in Jijel, in the North East of Algeria, during the month of April (2019). The material was identified and authenticated by the Department of Environment and Agronomy Sciences of the University of Jijel. Fresh intact leaves of *Salvia verbenaca* L. were shade-dried and ground in an electrical grinder (Sayona model: Sy-601, China) into a fine powder. A total of 100 g of plant powder was extracted using 1000 mL of methanol (80%) at 25 °C for 48 hours. The solvent was then filtered, defatted with hexane, and evaporated at 40 °C

using a Rotavapor (Heidolph, Laborot 4003). Concentrated extract was stored in a refrigerator (4 °C) until used [17].

Determination of total phenolic content

The amount of total phenolics in the sample was determined with the Folin-Ciocalteu reagent using the method of Othman et al. [18]. An amount of 0.2 mL of the sample was added to 1.5 mL (1/10 dilution) of the Folin-Ciocalteu reagent. The solutions were mixed and incubated at 25 °C for 5 min and then 1.5 mL of 7.5% sodium carbonate (Na_2CO_3) solution was added. The resultant mixture was stirred and incubated at 25°C for 90 minutes in the dark. The absorbance was then measured at 725 nm. Gallic acid was used to make the calibration curve. Total polyphenols content was expressed as Gallic Acid Equivalents (GAE) per g of crude extract. The test was carried out in triplicate and the mean values were calculated.

Determination of total flavonoid content

The quantitative estimation of the total flavonoids contained in the extract was carried out by the aluminum trichloride ($AlCl_3$) method [19]. 1.5 mL of the polyphenolic extract (2mg/mL) was added to an equal volume of 2% $AlCl_3$ solution. The mixture was stirred vigorously, and the absorbance was measured at 430 nm after 30 minutes of incubation in the dark at 25°C. Quercetin was used to make the standard calibration curve, and the flavonoids content was expressed as Quercetin Equivalents (QE) per g of crude extract. The test was carried out in triplicate.

In vivo assays

Test animals

Swiss albino mice of either sex weighing between 25 to 30 g obtained from the Pasteur Institute of Algiers was used for the present study. The mice were housed in polypropylene cages and kept under standard laboratory conditions (temperature 25-20 °C) with dark and light cycle (12/12 hours). All of the animals had free access to water and were fed standard commercial mice chew pallets. All of the experiments were carried out according to the ethical guidelines for the care and use of laboratory animals.

Acute toxicity

The acute toxicity test was performed to evaluate any possible toxicity for *Salvia verbenaca* L. The extract was given in the single dose of 5000 mg/kg of body weight (bw = body weight) orally to different groups of mice, each group consisting of five mice. A normal control was also run parallel which was receiving normal saline (10 mL/kg). The mice were observed for any brutal effects and mortality for 1, 4, and 24 h after treatment. Animals were further observed for up to seven days for any signs of delayed toxicity and mortality.

Anti-inflammatory activity: Carrageenan-induced mice paw edema

Effect of *S. verbenaca* L. extract on carrageenan-induced inflammation was carried out as described by Winter et al. [20]. This experiment involved five groups of five mice each. The mice were fasted overnight and had free access to water prior to the experiment. The experimental design was as follows:

Group 1: normal control mice received distilled water (vehicle).

Group 2: inflammatory control mice received distilled water.

Group 3: inflammatory mice received *S. verbenaca* L. leaves extract at a concentration of 250 mg/kg bw

Group 4: inflammatory mice received *S. verbenaca* L. leaves extract at a concentration of 500 mg/kg bw.

Group 5: inflammatory mice received 2-[2-(2,6-dichloroanilino) phenyl]acetic acid (standard) at a concentration of 50 mg/kg bw.

Where bw corresponds to body weight.

Carrageenan-induced paw inflammation in mice was produced by injecting carrageenan (1%, 50 µL), subcutaneously into the plantar surface of right hind paw of each mouse after 1 h administration of the respective drug treatment to each group. The edema volume was measured 1 h before, and 1, 2, 3 and 4 h after carrageenan injection using a calibrated digital thickness gauge (Shanghai, China). The percentage of inhibition of inflammation was calculated as stated below:

$$\% \text{ Inhibition } = \frac{(P_t - P_0)}{P_0}.100$$

Equ. 1

P_t : volume of the right hind paw after carrageenan treatment.
P_0: volume of the right hind paw before carrageenan treatment.

Preparation of the cytosolic fraction

The cytosolic fraction was extracted according to the method described by Iqbal et al. [21]. The animals were sacrificed and their livers were excised and rinsed in ice-cold normal saline. The liver tissues were then cut into small pieces, weighed and homogenized (homogenization with 3 volumes of 0.1 M phosphate buffer; pH 7.4 containing 1.17% KCl). The homogenate fraction was centrifuged at 800 rpm for 15 min at 4 °C. After centrifugation of the resultant supernatants at 9600 rpm at 4 °C for 45 min, the final supernatant containing the cytosolic enzymes was obtained. The protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Measurement of Malondialdehyde (MDA) levels

Malondialdehyde (MDA), a secondary product of lipid peroxidation, reacts with thiobarbituric acid at pH 3.5. The red pigment produced was extracted in n-butanol–pyridine mixture, and estimated by measuring the absorbance at 532 nm [22]. A volume of 1 mL of mice liver tissue, 0.5 of TCA (20%) and 1 mL of TBA (0.67%) were added to form a mixture. The mixture was heated in boiling water (water bath) for 15 minutes and then cooled. After cooling, 4 mL of n-butanol was added and mixed. The mixture was then centrifuged at 3000 rpm for 15 minutes. The optical density of the supernatant was recorded at 532 nm. A standard curve was obtained with a known amount of 1.1.3.3.-tetraethoxypropane, using the same assay procedure. The MDA was expressed in µmol/g of tissue.

Measurement of catalase activity (CAT)

Catalase activity was estimated by determining the decomposition of H_2O_2 at 240 nm in an assay mixture containing phosphate buffer [23]. For the assay, in a measuring cuvette, a substrate solution composed of 1 mL of phosphate buffer (0.1 M, pH 7.4), 0.950 mL of hydrogen peroxide (0.019 M) and 0.025 mL of the enzymatic source was prepared. Catalase activity is calculated by following the decomposition of H_2O_2 measured as a decrease in absorbance at 240 nm every minute for two minutes. Activity of catalase was expressed as units/mg protein according to the following formula:

$$\text{Units/mg protein} = (2.3033/T) \log (A_1 / A_2) / \textit{m}\text{g protein}$$

Equ. 2

A_1: absorbance at time 0 min.
A_2: absorbance at time 1 min.
T: time interval in minute

Measurement of glutathione (GSH) levels

Glutathione was estimated using Ellman's reagent (5,5dithiobis-(2- nitrobenzoic acid) [DTNB]). The sulphydryl groups present in glutathione form a colored complex with DTNB, which was measured colorimetrically at 412 nm [24]. Briefly, 1 g liver tissues were homogenized with three volumes of 5% TCA. The contents were mixed and centrifuged at 2000 rpm for 15 minutes. After centrifugation, 50 µL of the resultant supernatant was diluted in 10 mL phosphate buffer (0.1 M, pH = 8), and to this mixture was added 20 µL of 0.01 M DTNB. Solutions were measured at 412 nm after incubation for 15 minutes, against a blank prepared under the same conditions with TCA (5%). The GSH level was expressed as mmol/g liver.

Measurement of superoxide dismutase activity (SOD)

The activity of SOD was evaluated according to the procedure of Beauchamp and Fridovich [25]. In the procedure, SOD assay was estimated by the reaction mixture which contained phosphate buffer (pH 7.8, 50m M), riboflavin (2×10^{-6} M), sodium cyanide (2×10^{-5} M), methionine (10^{-2}M), EDTA (6.6×10^{-3} M), and nitroblue tetrazolium (NBT 1.76×10^{-4} M). 5 µL of the cytosolic fraction was added to the reaction mixture. The resulting mixture is subsequently illuminated for 10 minutes by fluorescent lamps. The changes in absorbance were determined at 560 nm using a UV spectrophotometer. The amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions was defined as one unit of SOD activity. The result for SOD activity was expressed as Units/mg protein.

$$\%\ \text{Inhibition} = \frac{DO_{control} - DO_{sample}}{DO_{control}} . 100$$

Equ. 3

$$\text{SOD Units/mg protein} = \%\ \text{inhibition} \times 6.35$$

Equ. 4

In vitro assays

Inhibition of protein denaturation

Inhibition of protein denaturation was evaluated by the method of Mizushima and Kobayashi [26] and Sakat et al. [27] with small modifications. 500 µL of 1% BSA was added to 100 µL of plant extract (50, 100, 250, 500 µg/mL). The pH of the reaction mixture was adjusted using a small amount of 1N HCl. This mixture was then incubated at 37 ºC for 20 minutes, followed by heating at 51 °C for 20 minutes. The resulting solution was cooled down at 25°C and absorbance was recorded at 660 nm. 2-[2-(2,6-dichloroanilino) phenyl]acetic acid was taken as a positive control. The experiment was performed in triplicate. The percentage of protein denaturation inhibition was estimated as follows:

$$\% \text{ inhibition} = \frac{(\text{Abs Control} - \text{Abs Sample})\text{X } 100}{\text{Abs control}}$$

Equ. 5

The extract concentration providing 50% inhibition (IC_{50}) was calculated and obtained by interpolation from linear regression analysis.

Human red blood cell membrane stabilization method

Human red blood suspension (HRBCs) was prepared using blood from a healthy human volunteer who had not taken any NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) for two weeks prior to the experiment. The collected blood was mixed with an equal volume of Alsever solution (dextrose 2%, sodium citrate 0.8%, citric acid 0.05%, sodium chloride 0.42%, and distilled water 100 mL) and centrifuged with isosaline at 3000 rpm for 10 minutes. After washing the red blood clot three times with isosaline, the volume of blood was measured and reconstituted as 10% v/v suspension with isosaline (0.85%, pH 7.2). Different concentrations of extract (50, 100, 250, 500 µg/mL), standard drug 2-[2-(2,6-dichloroanilino) phenyl]acetic acid, and control were separately mixed with 1 mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of HRBC suspension. All assay mixtures were incubated at for 30 minutes at 37 °C and centrifuged at 3000 rpm. After decantation of the supernatant liquid, the haemoglobin content was estimated via a spectrophotometer at 560 nm [28]. The experiment was repeated three times, and the percentage of protection was estimated using the formula below:

$$\% \text{ protection}=100-\left[\left(\frac{\text{Optical density of sample}}{\text{Optical density of control}}\right)\times 100\right]$$

Equ. 6

Linear regression was performed to determine 50% inhibitory concentration (IC_{50}).

DPPH free radical scavenging activity

The extract scavenging activity against DPPH• free radicals was assessed according to the method of Brand–Williams et al. [29]. Briefly, a 100 µL volume of the extract at different concentrations (50, 100, 250, 500 µg/mL), was diluted two-fold in methanol, then it was mixed with 2.9 mL of 0.025 g/l DPPH in methanol. The mixture was shaken

vigorously and allowed to stand at 25°C in the dark for 30 min. The absorbance of stable DPPH• was measured at 517 nm using Shimadzu UV mini 1240 spectrophotometer. The DPPH• control (containing no sample) was prepared using the same procedure. Ascorbic acid (vitamin C) at the same concentrations as the studied extract was used as the positive control. The experiment was repeated three times, and the ability to scavenge the DPPH radical was calculated using the formula:

$$\text{DPPH scavenging activity (\%)} = \frac{A_0 - A_1}{A_0}. 100$$

Equ. 7

A_0: is the absorbance of the control.
A_1: is the absorbance of the sample.

Interpolation from linear regression analysis was used to quantify the extract concentration that provided 50% inhibition (IC_{50}).

Hydrogen peroxide radical scavenging activity

The ability of *Salvia verbenaca* leaves extract to neutralize the hydrogen peroxide was determined according to the method of Brand-Williams et al. [29]. Test tubes were prepared with 2.0 mL of plant extract at different concentrations (50, 100, 250 and 500 µg/mL) and 1.5 mL of H_2O_2 solution (40 mM) was prepared in buffer phosphate (pH 7.4, 0.1 M). A control, that contains only H_2O_2, was prepared. Ascorbic acid was used as positive control. The absorbance was recorded at 230 nm after an incubation period of 10 minutes. The experiment was performed in triplicate and the percentage of neutralization of H_2O_2 was calculated using the following formula:

$$H_2O_2 \text{ Inhibition (\%)} = \frac{A_0 - A_1}{A_0}. 100$$

Equ. 8

A_0: is the absorbance of the control
A_1: is the absorbance of the sample

The extract concentration providing 50% inhibition (IC_{50}) was calculated and obtained by interpolation from linear regression analysis.

Statistical analysis

Experimental results are given as mean±standard deviation (SD). For *in vivo* experiments n = 5, and for *in vitro* experiments n = 3. Data were compared on the basis of the mean values and differences among means were tested using a Tukey–Kramer HSD (Software JMP version 7.0). Values of $p<0.05$ were statistically considered as significant.

RESULTS AND DISCUSSION

Determination of total phenolic and flavonoid contents

The total phenolic contents of the extract were determined using the Folin-Ciocalteu reagent in comparison with standard gallic acid, and the result was expressed in terms of mg gallic acid per g of crude extract. The results in Table 1 indicate that *Salvia verbenaca* L. leaves contain an important amount of polyphenols that is 425.14±0.73 mg GAE/g

crude extract. Colorimetric estimation of the total flavonoids proved that *Salvia verbenaca* L. significant quantities of flavonoids (89.54±0.86 mg QE / g CE).

Table 1. *Total phenols and flavonoid contents of Salvia verbenava L. leaves extract.*

Total phenols and flavonoid contents	Concentration
Total phenolic content (mg GAE/ crude extract)	425.14±0.73
Total flavonoid content (mg QE/g crude extract)	89.54±0.86

In vivo assays

Acute toxicity

Oral administration of *Salvia verbenaca* L. extract at 5000 mg/kg did not produce any mortality. The extract did not produce significant changes in behavior during the time of observation.

Effect of Salvia verbenaca on Carrageenan-induced mice paw edema

The results of *in-vivo* anti-inflammatory activity of two concentrations 250 and 500 mg/kg bw of *Salvia verbenaca* leaves extract and standard on carrageenan induced paw edema in mice are given in Table 2. Anti-inflammatory effect of the extract was evaluated after sub plantar injection of carrageenan in mice. The extract showed dose dependent inhibition of increase in paw edema throughout the experiment. The 2-[2-(2,6-dichloroanilino) phenyl]acetic acid showed statistically a significant ($p<0.05$) inhibition (81.57%) of paw edema at 4 h after carrageenan injection compared with the extract at a dose of 500 mg/kg bw (75.03%).

Table 2. *Effect of Salvia verbenaca L. leaves extract on carrageenan-induced mice paw edema volume.*

Treatment	Doses (mg/kg bw)	Right hind paw volume (% inhibition)			
		1 h	2 h	3 h	4 h
Control	–	2.69±0.01	3.18± 0.03	3.28 ±0.02	3.37±0.03
Standard	50	1.49±0.04^{cd} (49.55%)	1.31±0.05^{ef} (57.88%)	1.03±0.04^{h} (68.39%)	0.61±0.05^{j} (81.57%)
Extract	250	1.83 ±0.01^{a} (38.06%)	1.62±0.02^{bc} (47.80%)	1.42±0.01^{de} (56.50%)	1.15±0.03^{gh} (65.06%)
	500	1.70 ± 0.02^{ab} (42.45 %)	1.44±0.03^{d} (53.48 %)	1.20±0.03^{fg} (63.21 %)	0.82± 0.04^{j} (75.03 %)

n = 5; Means followed by different letter are significantly different (p < 0.05).

Effect of extract on MDA level

Fig. 1 illustrates the effect of the extract on the variation of MDA cytosolic level in hepatocytes of inflammatory mice pretreated or not with the polyphenolic extract. According to the results; a significant increase in MDA was recorded in inflammatory mice (113.5±0.68 µmol) compared to the normal control group (41.37±1.22 µmol). In addition, no significant variation in MDA was observed in inflammatory mice receiving the standard (45.56±0.98 µmol) compared to those pretreated with *Salvia verbenaca* L. extract at a dose of 500 mg / kg (46.79±0.78 µmol).

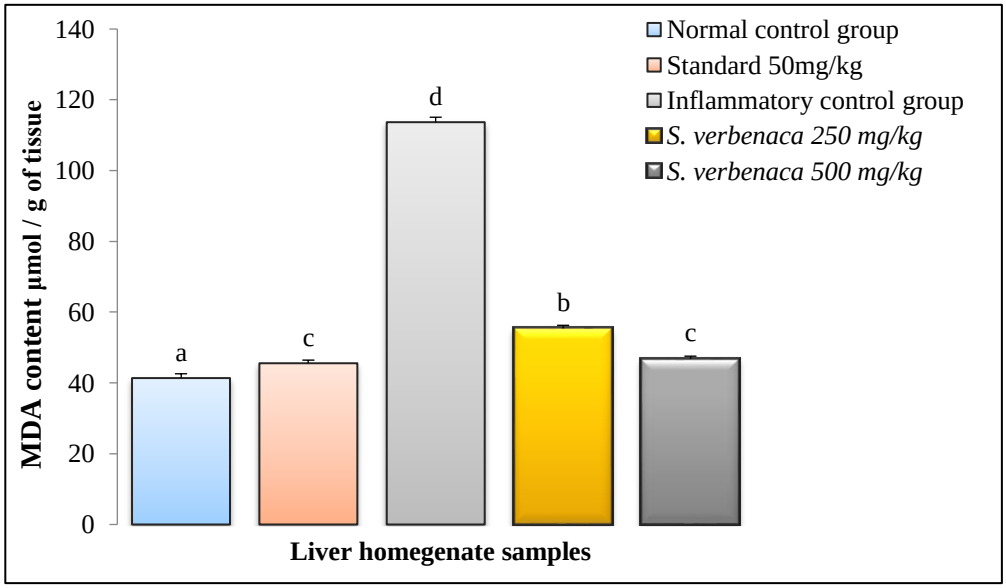

***Fig. 1**. Effect of 250 and 500 mg/kg Salvia verbenaca L. extract and controls on the MDA levels. Values are mean ± SD. n = 5 in each group. Different letters indicate that samples are significantly different ($p<0.05$).*

Effect of extract on CAT and SOD activities

Fig. 2 and Fig. 3 show the mean values of cytosolic CAT and SOD levels in mice liver homogenate samples. CAT and SOD levels were increased significantly ($p<0.05$) in pretreated groups with extract and standard in comparison to that of the inflammatory control group. The highest levels of these enzymes were recorded in mice receiving *Salvia verbenaca* L. extract at a dose of 500 mg/kg. These levels are statistically comparable to the standard.

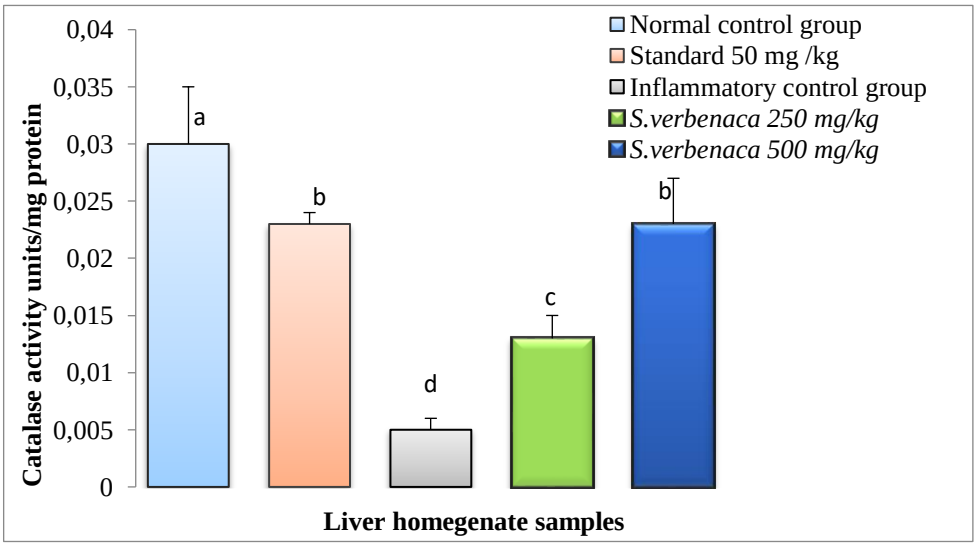

***Fig. 2**. Effect of 250 and 500 mg/kg Salvia verbenaca L. extract and controls on the CAT levels. Values are mean±SD. n=5 in each group. Different letters indicate that samples are significantly different ($p<0.05$).*

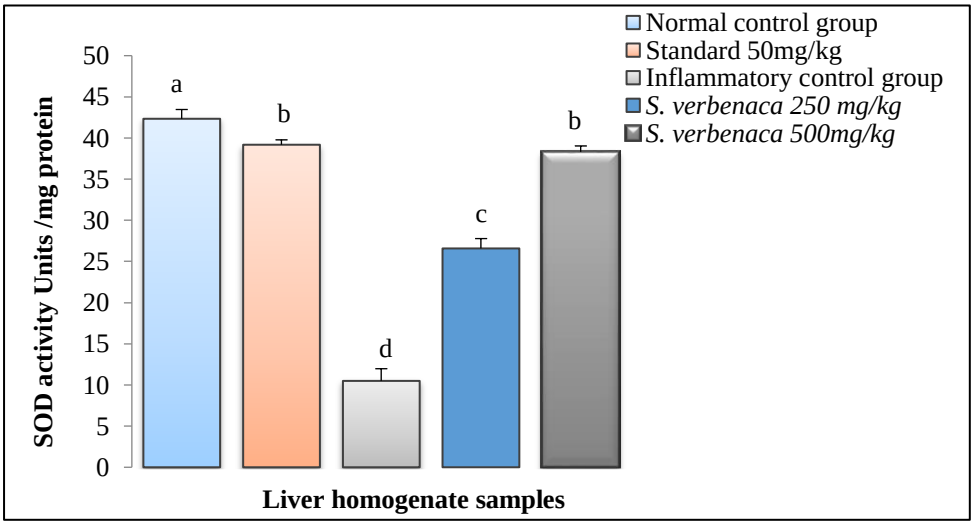

***Fig.3**. Effect of 250 and 500 mg/kg Salvia verbenaca L. extract and controls on the SOD levels. Values are mean±SD. n=5 in each group.*
Different letters indicate that samples are significantly different ($p<0.05$).

Effect of extract on GSH level

Fig. 4 shows the activity of GSH in the liver of normal control and experimental groups of mice. GSH level in the inflammatory group (0.047±0.003 mmol) was considerably lower than that of the normal control group (0.195±0.004 mmol). On the other hand, no significant variation in GSH was observed in the mice receiving standard and those pretreated with the extract at a dose of 250 and 500 mg/kg, their respective values of 0.191±0.0035 mmol, 0.187±0.008 mmol and 0.189±0.0061 show no significant difference from that of the normal control group.

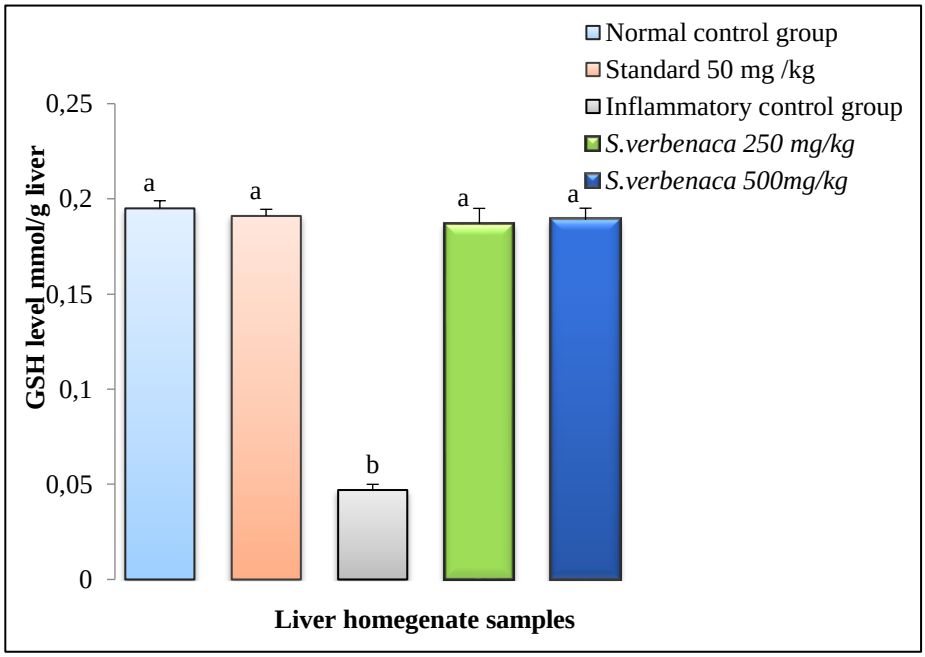

Fig. 4*. Effect of 250 and 500 mg/kg Salvia verbenaca L. extract and controls on the GSH levels. Values are mean±SD. n 5 in each group. Different letters indicate that samples are significantly different (p .05).*

In vitro assays

Effect of extract on protein denaturation

Effects of *Salvia verbenaca* L. leaves extract and standard on protein denaturation are shown in Table 3. Results showed that 50–500 µg/mL *Salvia verbenaca* L. leaves extract and standard inhibited heat-induced BSA denaturation occur in a concentration-dependent manner. Maximum inhibition of extract, 70.64±0.56% was observed at 500 µg/mL. The IC_{50} value was found to be 133.21±0.89 µg/mL. The standard showed the maximum inhibition 75.74±0.44% at the concentration of 500 µg/mL.

Effect of extract on stabilization of human red blood cell membrane

The effects of the extract and standard on stabilization of human red blood cell (RBC) membrane are presented in Table 3. The extract has the ability to stabilize the RBC membrane in hypotonic solution in a dose dependent manner. The percentage of protection of RBC membrane lysis by the extract of *Salvia verbenaca* L. was equal to 70.54±0.39% at 500 µg/mL which can be understood as a considerable value with an IC_{50} value 160.62±1.60 µg/mL when compared with that of the standard drug (76.30±0.32% at 500 µg/mL with an IC_{50} value 81.89±0.25 µg/mL).

Table 3. *Effect of Salvia verbenaca L. leaves extract and standard on protein denaturation and membrane stabilization activities*

Concentrations (µg/mL)	Protein denaturation (% inhibition)		Membrane stabilization (% protection)	
	Standard	Extract	Standard	Extract
50	45.48±0.37	42.31±0.20	42.51±0.50	39.53±0.52
100	53.74±0.86	50.37±0.25	55.62±0.46	47.40±0.30
250	66.25±0.53	58.18±0.13	63.82±0.15	59.40±0.48
500	75.74±0.44	70.64±0.56	76.30±0.32	70.54±0.39
IC_{50} (µg/mL)	63.61±1.53	133.21±0.89	81.89±0.25	160.62±1.60

The results are expressed as means±SD (n=3). Linear regression analysis was used to calculate IC50 value.

DPPH radical scavenging activity

Table 4 shows the DPPH radical scavenging capacity of the extract and ascorbic acid. The results reveal that the percentage inhibition of DPPH radical increased proportionally with concentration. The extract showed maximum activity of 72.04±0.28% at 500 µg/mL, whereas the positive standard exhibited 87.46±0.43% inhibition. The IC_{50} values of the extract and the standard were determined. The obtained values were: 115.41±1.40 µg/mL and 65.88±1.82 µg/mL for extract and standard respectively. The results showed that *Salvia verbenaca* L. extract has a good DPPH radical scavenging effect.

Hydrogen peroxide scavenging activity

The results of scavenging activity of hydrogen peroxide by the extract and standard are illustrated in Table 4. As shown, *Salvia verbenaca* L. leaves extract demonstrated a hydrogen peroxide decomposition activity of a concentration dependent manner with an IC_{50} of 180.39±1.77 µg/mL. However, the IC_{50} value of the standard was 62.63±2.02 µg/mL.

Table 4. *Scavenging activities of Salvia verbenaca L. extract and ascorbic acid by DPPH and H_2O_2 assays.*

Concentrations (µg/mL)	DPPH (% inhibition)		Hydrogen peroxide (% inhibition)	
	Ascorbic acid	Extract	Ascorbic acid	Extract
50	42.58±0.28	41.3±0.28	43.83±1.20	38.71±0.47
100	54.42±0.67	50.64±0.42	53.18±1.44	46.20±0.55
250	76.31±0.53	64.14±0.32	76.36±0.63	58.41±0.55
500	87.46±0.43	72.04±0.28	84.28±1.62	67.54±0.37
IC_{50} (µg/mL)	65.88±1.82	115.41±1.40	62.63±2.02	180.39±1.77

The results are expressed as means±SD (n=3). Linear regression analysis was used to calculate IC_{50} value.

Identification and isolation of medicinal products from plants have been an ever-increasing area of interest in the field of drug discovery [30]. In this study, the protective effects of polyphenolic extract of *Salvia verbenaca* L. leaves against inflammation and oxidative stress using *in vitro* and *in vivo* models were evaluated. In the study of the *in vivo* anti-inflammatory activity, the leaves extract of *Salvia verbenaca* L. was detected against carrageenan-induced acute inflammation. Carrageenan-induced hind paw edema is a model that has been used to assess the anti-inflammatory properties of numerous natural and synthetic products, as well as to discover the possible pathways of inflammation [31, 32].

Carrageenan is a phlogistic, non-antigenic agent and is devoid of apparent systemic effect. It is also believed that the experimental model exhibited a high degree of reproducibility in acute phase inflammation. Thus, carrageenan-induced paw edema, is a frequently used method for the screening of acute inflammatory potentials of various natural products [32]. Several studies indicated that injection of carrageenan causes the release of several chemical mediators which are responsible for the inflammatory process. This inflammatory response is biphasic. The primary section starts (within the first 2 hours after carrageenan injection) with chemical mediators corresponding to histamine and serotonin, while in the in second or delayed phase (after 2 hours of carrageenan injection) prostaglandins and lysosome enzymes performs their position in the inflammation procedure [33]. In the present investigation, a significant inhibitory activity was shown by *Salvia verbenaca* L. leaves extract particularly the oral pretreatment of 500 mg/kg over a period of 4 hours in carrageenan-induced inflammation. The peak of this activity was observed during the late phase. These results correlate with previous studies, showing that 4 hours after the carrageenan injection is the moment where its maximum effect is manifested and the moment where the anti-inflammatory activity of the test product is best observed [34]. In addition, the observed strong inhibition of the edema in the late phase suggests that the main mechanism of anti-inflammatory effect of extract may be due to the inhibition of prostaglandin biosynthesis, which is similar to that produced by non-steroidal anti-inflammatory drugs such as 2-[2-(2,6-dichloroanilino) phenyl]acetic acid. However, the exact mechanism of prostaglandin synthesis inhibition could be a potential future perspective. Similar to medicinal plants that are used in folk medicine, the anti-inflammatory activity of *Salvia vebenaca* L. leaves extract could be due to its phytochemical compounds such as polyphenolic compounds. The phytochemical study detected large amounts of phenolic compound in our extract. It has been demonstrated in previous studies that plants with high polyphenol contents present good anti-inflammatory activity [35, 36]. Also, many studies have shown that many flavonoids such as rutin, quercetin, and luteolin, biflavonoids, and related polyphenols, produced significant antinociceptive and/or anti-inflammatory activities [37, 38]. From another side, local inflammation caused by carrageenan was associated with the generation of reactive oxygen species (ROS), and it could play an important role in the genesis of oxidative stress [39]. Previous research reported that reactive oxygen species overproduction may lead to increasing lipid peroxidation [40]. In our study, a reduction in a dose dependent manner of the MDA level, is an important indicator of lipid peroxidation was observed in the extract treated groups when compared to the inflammatory control group.

According to the enzymatic antioxidant analysis, we found that a carrageenan injection caused a significant decrease in activity of SOD, CAT, and GSH in the inflammatory control group compared with the normal control group ($p<0.05$). This reduction in activity may be explained by high use of the produced enzymes that act as scavengers of free radicals generated during the inflammatory process.

Inversely, the oral pretreatment of *Salvia verbenaca* L. leaves extract and the reference product (2-[2-(2,6-dichloroanilino) phenyl]acetic acid) increased the activities of SOD, CAT and GSH, compared with the inflammatory control group. It should be noted that the extract effect particularly at 500 mg/kg is statistically comparable to the standard. These results suggest that the extract contains some phytochemical compounds with antioxidant activities which could contribute to the anti-inflammatory process. In our experiment, we found that the extract has highest amounts of phenolic compounds particularly flavonoids. Previous studies have confirmed that flavonoids are the major secondary metabolites class with several descriptions of antioxidant properties which confer a therapeutic potential with anti-inflammatory effect [41, 42, 43].

Moreover, the *in vitro* anti-inflammatory potential of *Salvia verbenaca* L. leaves extract was evaluated using two inflammation models: inhibition of protein denaturation and stabilization of erythrocyte membrane against hypotonicity induced hemolysis assays. In our investigation, the different concentrations of the extract exhibited inhibition of protein denaturation and stabilization of human red blood cell membrane in a concentration dependent manner with IC_{50} values of 133.21±0.89 µg/mL and 160.62±1.60 µg/mL respectively. These data indicate that *Salvia verbenaca* L. leaves extract could contain an anti-inflammatory property. Previous studies have demonstrated that plant extracts with anti-inflammatory property possess the capacity to inhibit protein denaturation and stabilize cell membrane against lysis [44, 45]. Proteins lose their tertiary and secondary structures as a result of protein denaturation due to application of external stress factors as heat, or compounds such as strong acids or bases, concentrated inorganic salts, or organic solvent. Proteins denaturation is a well-documented cause of inflammation [46]. A number of biological proteins lose their biological functions when it becomes denatured due to inflammation. Furthermore, lysosomal membrane breakage has been demonstrated to release pro-inflammatory indicators such as active neutrophils, proteases, and histamines at the local site of tissue injury during chronic inflammation [47, 48]. As a result, medicinal plant extracts that inhibit protein denaturation and protect cell membranes from lysis could be a promising source of anti-inflammatory medication candidates.

It is important to highlight that previous reports on the *in vivo* anti-inflammatory and antioxidant and also *in vitro* anti-inflammatory activities of *Salvia verbenaca* L. are very few or inexistent in the literature; this made the comparison of our results with those of previous studies very difficult. On the other hand, the *in vitro* antioxidant capacity of the *Salvia verbenaca* L. extract was evaluated using two assays. DPPH and hydrogen peroxide are common antioxidant tests used for the characterization of plant extracts.

In fact, the results of a single-assay can give only a reductive suggestion of the antioxidant properties of extracts. Therefore, an approach with multiple assays in screening works is highly advisable [49]. DPPH stable free radical method is a sensitive way to determine the antioxidant activity of plant extracts. By adding an electron or hydrogen radical, it can be transformed to a stable diamagnetic molecule. Our Results showed that scavenging activity of the extract was quite important (IC_{50} 115.41±1.40). However Belkhiri et al. [50] reported lower IC_{50} values. In the present study, there was

an increased scavenging activity of the DPPH radicals with increasing concentration of the plant extract which may indicate an increased ability to donate hydrogen ions resulting in a less turbid solution, which is proportional to the number of gained electrons [51, 52]. As a result of its hydrogen ion-donating ability, it is possible that *Salvia verbenaca* L. exhibits a DPPH scavenging activity by reducing the radical to the corresponding hydrazine molecule. Hydrogen peroxide is a weak oxidizing agent that can directly inactivate some enzymes, usually via oxidation of essential thiol (-SH) groups. Hydrogen peroxide can pass through cell membranes rapidly. Once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the cause of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. In our study, the extract demonstrated an ability to inhibit H_2O_2 radical (IC_{50} 180.39±1.77) in a concentration dependent manner. Our results are similar to other reports on the *in vitro* antioxidant activities of *Salvia verbenaca* L. such as the reports of Farhat et al. [53] and Mamache et al. [54], who stated that this plant has the aptitude of scavenging free radicals in varied *in vitro* models.

The present study has revealed that the *Salvia verbenaca* L. leave extract contains a considerable amount of phenolic compounds, thus it can be inferred that these phenolics are responsible for its noticeable antioxidant activity as assayed through various *in vitro* assays presented in this study. This is in accordance with other studies that found a strong link between total phenolic content and antioxidative activity in a variety of fruits, plants, and vegetables [55, 56].

CONCLUSION

Salvia verbenaca L. is a medicinal plant used in Algerian traditional medicine for the healing of wounds. From the results of the present study, the polyphenolic extract of this plant has shown considerable anti-inflammatory and antioxidant activities both in *in vivo* and *in vitro* models. This might be correlated with the presence of phenolic constituents and flavonoids in the extract. However, further investigations are required to isolate the active constituents responsible of the observed effects, and to elucidate the possible mechanisms responsible of the anti-inflammatory activity of the plant extract.

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: L.B., H.B., Design: L.B., H.B., Data Collection or Processing: L.B., H.B., N.A., Analysis or Interpretation: L.B., H.B., Literature Search: L.B., H.B., N.A., L.B., K.M., Writing: L.B., H.B., N.A.

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Abbreviations: *S. verbenaca* L: *Salvia Verbenaca* L. MDA: Malondialdehyde. CAT: Catalase. GSH: Glutathione. SOD: Superoxide dismutase. DPPH: Diphenyl-picryl-hydrazyl. H_2O_2: Hydrogen peroxideH_2O_2. NSAIDs: Non-steroidal anti-inflammatory drugs. GAE: Gallic Acid Equivalents. QE:

Quercetin Equivalents. Body weight: bw. BSA: Bovine serum albumin. TCA: Trichloroacetic acid. TBA: Thiobarbituric acid. DTNB:5,5dithiobis-(2- nitrobenzoic acid). NBT: Nitroblue tetrazolium. HRBCs: Human red blood suspension. RBC: Red blood cell.

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