Determination of antioxidant potential of *Salix aegyptiaca* L. through biochemical analysis

Mohd Nauman, RK Kale, Preeti Dhruve & Rana P. Singh*
School of Life Sciences, Jawaharlal Nehru University, New Delhi – 110 067, India

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*Salix aegyptiaca* L., commonly called musk willow, is a medicinal herb in use since ancient time. However, information on its mode of protective action is scanty. Considering its significance and wide applications, here, we explored the same using its hydroethanolic bark extract. The results of nitric oxide assay indicated the free radical scavenging ability of the bark extract. In the *ex vivo* study, the extract of bark was found to exert protective effects against protein and membrane damage caused by the Fenton’s reagent in the liver homogenate of C57BL/6 mice. In *in vivo* studies, the specific activities of enzymes involved in antioxidant function and the level of reduced glutathione (GSH) enhanced in the liver of six weeks old C57BL/6 mice treated with extract of bark. On the other hand, the oxidative damage in the liver determined in terms of TBARS was decreased significantly. The concomitant increase in the free radical metabolizing enzymes and the content of GSH; and inhibition of oxidative damage is suggestive of possibility of enhanced antioxidant potential of animals. The bark extract also enhanced the specific activities of phase I and phase II enzymes, which would likely to contribute in the detoxification. In conclusion, the *S. aegyptiaca* scavenges the free radicals, elevates the endogenous antioxidant status and detoxifies the toxic agents which determine its beneficial effects.

**Keywords**: Antioxidants, Musk willow, Oxidative stress, Reactive oxygen species

*Salix aegyptiaca*, commonly known as musk willow is a member of *Salicaceae* plant family and mainly cultivated in province of Iran. This plant is a traditional herb, particularly in Middle East region, used as a cardioprotectant, gastroprotector, sedative, laxative, hypnotic, nervous, anthelmintic and vermifuge etc. Anticancer activity of ethanolic bark extract of *S. aegyptiaca* against colon cancer *in vitro* and *in vivo* has been reported earlier. Though the medicinal properties of *S. aegyptiaca* show its importance in human health, the mechanisms of its beneficial effects are not well understood. The reactive oxygen species (ROS) are known to be involved in the cause and complication of several pathophysiological conditions. The ROS produced in the animal body interact with biomolecules, and alter their structure and function. If not repaired, these changes could result in damage and death of cell. Continuation of such events leads to enhanced oxidative stress and subsequently causes diseases. The inactivation of ROS and augmentation of cells prevent the adverse effects of oxidative stress. The presence of antioxidants in *S. aegyptiaca* may have some beneficial effect by scavenging ROS and modulating the enzyme system involved in their metabolism as well as other systems with detoxification function. Therefore, in the present study, we examined the hydroethanolic extract of bark for its hepatoprotective effects in the liver of mice.

**Materials and Methods**

**Chemicals**

Ascorbic acid, sodium nitroprusside (SNP), guanidine hydrochloride, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), oxidized GSH (GSSG), pyrogallol, 2,6-dichlorophenol-indophenol (DCPIP), potassium ferricyanide, triton X-100, ethylenediaminetetraacetic acid (EDTA), sodium pyruvate, thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The rest of the chemicals used were procured from local firms (India) and were of highest purity grade.

**Preparation of hydroethanolic extract of *S. aegyptiaca* bark**

The bark of *S. aegyptiaca* was procured from the garden in Qazwin, Iran. The plant was identified on the morphological basis by Dr. Mohammad Irfan

*Correspondence:
E-mail: ranaps@hotmail.com (RPS); mnomanq@gmail.com (MN)
Qureshi, Assistant Professor, Jamia Millia Islamia, New Delhi, India and deposited as sample no. 20150217 in the departmental herbarium. The bark was dried and powered to make a hydroethanolic extract (water:ethanol; 20:80 v/v). The powder was dissolved in solvent with ratio of 1:10 (w/v). The solution was kept on shaking at 30°C for 48 h followed by the filtration through Whatman (No. 1) filter paper. The supernatant was concentrated using rotary evaporator (Buchi, Singapore) at 45°C followed by the desiccation through lyophiliser (Labconco Corp, USA). The resulting powder was stored at –20°C till the end of experiments.

**Determination of antioxidant activity by nitric oxide assay**

This assay was performed to investigate free radical scavenging property of *S. aegyptiaca* bark extract following the protocol of Kumaran & Karunakaran et al. Sodium nitroprusside (10 mM) was dissolved in phosphate buffer saline and added to different concentrations of *S. aegyptiaca* bark extract (2-20 µg/mL) dissolved in water and incubated for 150 min at 25°C. The reaction mixture without *S. aegyptiaca* bark extract served as control. After the completion of incubation, 0.5 mL of Griess reagent [1% sulfanilamide, 2% H3PO4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride] was added to each test tube and absorbance of coloured product was recorded at 546 nm in UV-1800 Spectrophotometer (Shimadzu Corp, Japan). Ascorbic acid was used as positive control.

**Animals**

In the present study, six weeks old male C57BL/6 mice were used for ex vivo and in vivo studies. The animals were kept in the Central Laboratory Animal Resources (Jawaharlal Nehru University, New Delhi) with a 12 h light/dark cycle and given standard food pellets and drinking water *ad libitum*. The animals were under observation throughout the experimentation, for body weight, food and water consumption, or any other sign of health toxicity. The experiments were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and Jawaharlal Nehru University Institutional Animal Ethics Committee (IAEC). The experiments were performed as per their guidelines.

**Ex vivo studies**

The free radicals scavenging activity of was determined in an ex vivo system as described by Uddin et al. The animals were first euthanized, and their livers were excised and perfused with 0.9% saline. The livers were blot dried and 20% w/v liver tissue homogenate (LH) was prepared in phosphate buffer (25 mM, pH 7.4) using an electric homogenizer (Model no. RQ-127A, Remi, India). Free radicals were generated in homogenate using Fenton’s reagent (0.5 mM FeSO4; 0.5 mM H2O2; 1:1). Control group contained 4 mL liver homogenate (LH) + 8 mL double distilled water (DDW); positive control group, 4 mL LH + 4 mL Fenton’s reagent + 4 mL DDW, while the remaining groups were mixed with *S. aegyptiaca* bark extract, giving the final reaction mixture as 4 mL LH + 4 mL Fenton’s reagent + 4 mL of drug with two concentrations (400 and 800 µg/mL) each. The test tubes of reaction mixture were incubated at 37°C for 4 h. The tubes were centrifuged at 10000 rpm for 20 min (Hitachi, Japan). The resultant supernatant was then transferred into ultracentrifugation tubes and again centrifugated at 105000xg for 60 min in ultracentrifuge (Beckman Coulter, US). The cytosolic fraction supernatant was used to study protein carbonyl assay and whereas the pellet that represents microsomes were used to determine the peroxidative damage.

**Estimation of protein carbonyl content**

Protein carbonyl content was estimated according to method of Fagan et al. The protein carbonyl formation was expressed as moles of dinitrophenyl hydrazine (DNPH) incorporated/100 mg protein using a molar extinction coefficient of 21 mM−1 cm−1.

**Estimation of peroxidative damage**

The microsomes were resuspended into homogenizing buffer and used to study the peroxidative damage by thiobarbituric acid reactive substances (TBARS) method as described by Varshney & Kale. The result is expressed as nmole malondialdehyde (MDA) formed/mg protein.

**In vivo studies**

Modulatory effect of *S. aegyptiaca* bark extract (40 and 80 mg/kg body wt.) on the reduced glutathione content, formation of malonaldehyde, specific activities of antioxidant enzymes and drug/carcinogen metabolizing enzymes of phase I and phase II systems was determined in the liver of mice. A suspension of *S. aegyptiaca* bark extract was made in 0.5% carboxymethyl cellulose (CMC, w/v) and given through oral gavage on alternate days for 14 days to C57BL/6 mice. The mice were divided randomly into three experimental groups: Group I
(n=6) mice were fed with 0.5% CMC (vehicle) and served as control, Group II (n=6) and Group III (n=6) mice were fed with 40 and 80 mg/kg body wt. of S. aegyptiaca bark extract, respectively.

Preparation of homogenate, cytosol and microsomes
After two weeks treatment of S. aegyptiaca bark extract the animals were euthanized. The liver was perfused immediately with ice-cold NaCl (0.9%) and rinsed in chilled 0.15 M of Tris-KCl buffer (pH 7.4). The liver was then blotted dry, weighed quickly and homogenized in ice cold 0.15 M Tris-KCl buffer (pH 7.4) to get 10% (w/v) homogenate. An aliquot (0.5 mL) was separated to study the reduced glutathione content (GSH). The remainder homogenate was processed to prepare microsomes and cytosolic fraction as described earlier in ex vivo section. The specific activities of glutathione-S-transferase (GST), DT-diaphorase (DTD), and antioxidant enzymes were determined in cytosolic fraction. The specific activities of cytochrome P450 reductase, cytochrome b5 reductase and level of malonaldehyde were measured in microsomes.

Determination of reduced glutathione
Reduced glutathione (GSH) content was estimated as the total non-protein sulphydryl using the method of Moron et al. Reduced glutathione (GSH) was used as a standard to calculate nmole of –SH content/g tissue.

Determination of superoxide dismutase and catalase activities
The specific activity of SOD was determined following the method of Marklund & Marklund that involves the inhibition of autooxidation of pyrogallol at pH 8.0. A single unit of enzyme was defined as the quantity of superoxide dismutase required to produce 50% inhibition of autooxidation. The specific activity of SOD is expressed as µmole/mg protein. The specific activity of catalase was determined according to the method of Aebl by analysing the disappearance of H₂O₂. The activity was calculated using the extinction coefficient as 40 μmol⁻¹ cm⁻¹. The specific activity of catalase is expressed as moles of H₂O₂ reduced/min/mg protein.

Determination of glutathione reductase and glutathione peroxidase activities
The specific activity of GR was determined according to the method of Carlberg & Mannervik. One unit of enzyme activity was defined as nmol of NADPH consumed/min/mg protein, based on an extinction coefficient of 6.22 mM⁻¹ cm⁻¹. The specific activity of GPx was determined by the method of Paglia & Valentine. One unit of enzyme activity has been defined as nmoles of NADPH consumed/min/mg protein based on an extinction coefficient of 6.22 mM⁻¹ cm⁻¹.

Determination of NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase activities
The specific activity of NADPH-cytochrome P450 reductase was determined according to Omura & Takesue with some modifications, measuring the rate of oxidation of NADPH at 340 nm. The enzyme activity was calculated using extinction coefficient 6.22 mM⁻¹ cm⁻¹. One unit of enzyme activity is defined as that causing the oxidation of 1 mol of NADPH per minute. The specific activity of NADH-cytochrome b5 reductase was measured following the method of Mihara and Sato with some modifications, measuring the rate of reduction of potassium ferricyanide at 420 nm by NADH. The enzyme activity was calculated using the extinction coefficient of 1.02 mM⁻¹ cm⁻¹. One unit of enzyme activity is defined as that causing the reduction of 1 mol of ferricyanide per minute.

Determination of glutathione S-transferase and DT-diaphorase activities
The specific activity of cytosolic GST was determined according to the method of Habig et al. The specific activity was expressed as micromoles of GSH–CDNB conjugate formed/min/mg protein using the extinction coefficient 9.6 mM⁻¹ cm⁻¹. The specific activity of DTD was determined carrying out the method of Ernster et al. The activity was calculated using extinction coefficient 21 mM⁻¹ cm⁻¹. One unit of enzyme activity is defined as amount of enzyme required to reduce one micromole of DCPIP per minute.

Estimation of peroxidative damage and Protein
Lipid peroxidation was estimated in microsomes, following the method of Varshney & Kale which has been described in the ex vivo section. Protein content of the samples was determined using Bradford’s reagent with BSA as standard at 595 nm.

Statistical analysis
The values were presented as mean ± SEM. The mean and significance of the differences between the data pairs was calculated by t-test (SigmaPlot 8.0). A value of P <0.05 was considered significant.

Results
Nitric oxide scavenging activity
Salix aegyptiaca bark extract exhibited nitric oxide (NO’) scavenging property. These NO’ free radicals
were generated by the addition of sodium nitroprusside, dissolved in PBS at physiological pH. The addition of S. aegyptiaca bark extract inhibited the production of nitrite ions which were estimated by adding Griess reagent (Fig. 1). The IC₅₀ value of the extract was found to be 17.71±0.34 µg/mL. Ascorbic acid was used as positive control for comparison.

**Protein carbonyl estimation**

In the ex vivo studies, the treatment of S. aegyptiaca bark extract showed a significant protection by 12.77% (P <0.05) at concentration of 800 µg/mL against protein carbonyl damage caused by 'OH free radicals, generated by the Fenton’s reagent (Fig. 2).

**Peroxidative damage**

Inhibition of peroxidative damage by Salix aegyptiaca, in the ex vivo studies, was investigated in the microsomes. The peroxidation was initiated by addition of the Fenton’s reagent and determined in terms of TBARS formation. The S. aegyptiaca bark extract @800 µg/mL of showed a significant reduction in the formation TBARS by 21.34% (P <0.05) (Fig. 3).

**Reduced glutathione**

Reduced glutathione content was found to be significantly elevated by 1.19 fold (P <0.05) and 1.31 fold (P <0.01), respectively, when the animal treated with 40 and 80 mg/kg body wt. of S. aegyptiaca bark extract (Fig. 4A).

**Superoxide dismutase and catalase**

The animals treated with 40 and 80 mg/kg body wt. of S. aegyptiaca bark extract exhibited significant increase in the specific activity of SOD by 1.41 fold (P <0.05) and 1.58 fold (P <0.005), respectively (Fig. 4B). Similarly, specific activity of catalase was also found significantly elevated in animals treated with 40 and 80 mg/kg body wt. of the extract by 1.26 fold (P <0.05) and 1.32 fold (P <0.05), respectively (Fig. 4C).

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**Fig. 1** — Nitric oxide assay; percent inhibition of nitrite ion by Salix aegyptiaca bark extract. [Sodium nitroprusside was added to different concentrations of S. aegyptiaca bark extract (2-20 µg/mL) and incubated for 150 min at room temperature. After the completion of incubation, Griess reagent was added to each test tube and absorbance of coloured product was recorded at 546 nm. Ascorbic acid (2-20 µg/mL) was used as positive control. Values are represented as mean ± SEM of three samples]

**Fig. 2** — Protein carbonyl content; formation of protein carbonyls against Fenton reagent. [Salix aegyptiaca bark extract was added at concentration of 400-800 µg/mL. The test tubes were incubated for four hours at 37°C followed by two consecutive centrifugations. The supernatant was used for protein carbonyl assay. Abbreviations: FR, Fenton’s reagent; SA, S. aegyptiaca bark extract. Values are represented as mean ± SEM of three samples. *(P <0.05) represents significant changes relative to control]

**Fig. 3** — Peroxidative damage; formation of MDA against Fenton’s reagent. [Salix aegyptiaca bark extract was added at concentration of 400-800 µg/mL. The test tubes were incubated for four hours at 37°C followed by two consecutive centrifugations. The resulting microsome was used for peroxidative damage estimation. Abbreviations: FR, Fenton reagent; SA, S. aegyptiaca bark extract. Values are represented as mean ± SEM of three samples. *(P <0.05) represents significant changes relative to control]
Glutathione reductase and glutathione peroxidase

The specific activity of GR was found to be significantly increased by 1.22 fold \((P < 0.05)\) and 1.38 fold \((P < 0.05)\), respectively, in the animal treated with 40 and 80 mg/kg body wt. of \textit{S. aegyptiaca} bark extract (Fig. 4D). The specific activity of GPx was also found elevated by 1.29 fold \((P < 0.01)\) in the animals treated with 80 mg/kg body wt. of \textit{S. aegyptiaca} bark extract (Fig. 4E).

Cytochrome P450 reductase and cytochrome b5 reductase

Treatment of \textit{S. aegyptiaca} bark extract resulted in a significant increase in the specific activity of cytochrome P450 reductase by 1.25 fold \((P < 0.05)\) and 1.27 fold \((P < 0.005)\), respectively, when animals were treated with 40 and 80 mg/kg body wt. (Fig. 5A). The specific activity of cytochrome b5 reductase showed significant increase by 1.09 fold \((P < 0.01)\) in the animals treated with 80 mg/kg body wt. of \textit{S. aegyptiaca} bark extract (Fig. 5B).

Glutathione S-transferase and DT-diaphorase

The significant enhancement in the specific activity of glutathione S-transferase (GST) was found by 1.43 fold \((P < 0.05)\) and 1.91 fold \((P < 0.01)\) in the animals treated with 40 and 80 mg/kg body weight of \textit{S. aegyptiaca} bark extract, respectively (Fig. 5C). The specific activity of DT-diaphorase (DTD) was also found to be significantly elevated by 1.52 fold \((P < 0.05)\) and 1.63 fold \((P < 0.05)\), respectively, in the group treated with 40 and 80 mg/kg body wt. of \textit{S. aegyptiaca} bark extract (Fig. 5D).
The peroxidative damage was determined in the microsomes prepared from the liver of mice, where a significant decrease in peroxidative damage was seen by 33.73% (P <0.05) with the treatment of 80 mg/kg body wt. of S. aegyptiaca bark extract (Fig. 5E).

Discussion

Reactive oxygen species, such as O$_2^-$ (superoxide), OH (hydroxyl), HO$_2$ (per hydroxyl), O$_2$ (singlet oxygen), NO$^+$ (nitric oxide), ROO$^-$ (peroxyl), RO$^-$ (alkoxyl) and H$_2$O$_2$ (hydrogen peroxide) generated in the animal body cause oxidative stress leading to cellular damage, which if not repaired, may induce several pathological conditions and their complications. Since, Salix aegyptiaca has medicinal properties and antioxidant activity, it is expected to scavenge the free radicals and exhibit the protective role in biological systems. This possibility was examined in ex vivo studies, using the peroxidative damage and formation of protein carbonyl in microsomal and cytosolic fractions, respectively prepared from the liver homogenate and incubated in presence of Fenton’s reagent to generate the free radicals as well as using nitroprusside to get nitric oxide free radicals in chemical system.

The peroxidation, a free radical chain reaction initiated by OH and, propagated by ROO$^-$ and RO$, is a determinant to membrane damage. In case of proteins, the interaction with free radicals, particularly OH could lead to the formation of carbonyl which is used as a measure of protein damage. The nitric oxide assay is widely used to ascertain the antioxidant activity of a given agent. In the present study, the bark extract of S. aegyptiaca significantly inhibited the peroxidation, protein carbonyl formation as well as nitric oxide generation. These findings are suggestive of the free radical scavenging ability of the extract of bark. It would be pertinent to mention that the several primary and secondary free radicals are generated in the cells. However, among those OH free radicals mainly contribute to the detrimental effects, being most reactive (for example the rate of its addition to carbon double bond is $k = 4.8\times10^9$ dm$^3$ mol$^{-1}$s$^{-1}$) and also evident from its shortest biological half-life (1×10$^9$ s). It can be inferred from the above findings that S. aegyptiaca scavenges OH and other free radicals quite effectively and can be attributed to its medicinal properties.

In the animal body, free radicals are formed due to exposure to agents such as ionizing radiations and toxic chemicals as well as during the disturbed and normal metabolic processes like the release of electrons from the mitochondrial electron transport chain, conversion of molecular oxygen into superoxide by xanthine oxidase, β-oxidation in peroxisomes and others including inflammation, phagocytosis and arachidonate pathway and in turn cause an increase in oxidative stress and its burden. In response to such situation, the defense systems both enzymatic and non-enzymatic, present in the body metabolize the free radicals and their molecular products and prevent the oxidative burden and its consequent complications. Therefore, it was interesting to see whether S. aegyptiaca modulates the enzymatic and non-enzymatic defense system.

Mice were treated with the bark extract for 14 days and the specific activities of enzymes involved in the free radical metabolism, and the level of glutathione (GSH) as a representative of the non-enzymatic system were determined in the liver. The extract enhanced the specific activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) and the level of GSH as well. As a consequence, SOD is likely to dismutate O$_2^-$ to H$_2$O$_2$ and subsequently H$_2$O$_2$ into water and molecular oxygen by catalase and GPx. GR reduces the glutathione disulphides (GSSG) to GSH and helps to recycle and make it available for the antioxidant function of GPx. As GSH is known to be a free radical scavenger, its increased level would also contribute and enhance the free radical scavenging potential of S. aegyptiaca. Apart from the scavenging of free radicals, the extract of S. aegyptiaca augmented the metabolism of free radicals and restored the antioxidant potential of animals. It was supported by the lowered levels of peroxidative damage in the liver, under similar circumstances.

Since, the cytochrome P450 system, consisting of the phase I and phase II enzyme systems, metabolizes the toxic chemicals including carcinogens and prevents from their detrimental effects; it was sought to determine the possible link with medicinal properties of S. aegyptiaca. In the liver-of mice, fed with its bark extract for 14 days, the specific activities of cytochrome P450 reductase and cytochrome b5 reductase, important members of phase I enzymes system as well as of glutathione-S-transferase and DT-diphorase, members of the phase II enzymes system, elevated significantly. Under such conditions, the phase I enzymes metabolise the toxic agents and...
convert them into hydrophilic metabolites, to serve as substrate for phase II enzymes system which subsequently increase their polarity and hydrophilicity, and facilitate their rapid elimination from the body\textsuperscript{31}. DT-diaphorase which is member of phase II system, also functions as an antioxidant enzyme. Its enhanced specific activity in the liver of the mice treated with bark extract, is likely to accelerate the two-electron reduction of the xenobiotics, and reduce the quinones to prevent their conversion into semi quinones and provide protection from their reactive intermediate metabolites\textsuperscript{32} and in turn maintains the antioxidant status of the animals. The modulation of cytochrome P450 system by \textit{S. aegyptiaca} is suggestive of its chemopreventive efficacy against carcinogenesis and other toxic agents.

**Conclusion**

In this work we report that the extract of bark of \textit{Salix aegyptiaca} has an antioxidant activity and able to enhance the specific activities of superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPx) which metabolize the free radicals generated in the cells through the modulation of cytochrome P450 system. The extract was also found to exert protective action against the membrane, protein and DNA damage and support its ability to scavenge the free radicals. The enhanced specific activities of phase I and II enzymes due to the extract treatment indicate its potential to augment the chemopreventive efficacy of \textit{Salix aegyptiaca}.

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**Conflict of interest**

The authors declare no conflict of interests.

**References**


