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Research Article

# ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF *SALIX AEGYPTIACA* BARK EXTRACT ATTRIBUTE TO ITS ANTICANCER EFFICACY

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### ABSTRACT

*Salix aegyptiaca* has been used traditionally from the ancient time for its beneficial effects. The protective action of this plant is not well understood so far. Oxidative stress and inflammation are generally linked with carcinogenesis. In the present study, antioxidant, anti-inflammatory and anticancer activities of hydroethanolic bark extract of *Salix aegyptiaca* have been studied. The results of DPPH assay indicated the free radical scavenging ability of the bark extract (2-20 µg/ml). The inhibition of ·OH radical induced damage of pBR322 plasmid DNA by the extract (20 µg/ml) depicted its antioxidant property. This bark extract (5-800 µg/ml) exhibited the anti-inflammatory activity against heat induced protein denaturation. The medicinal property of *S. aegyptiaca* could be attributed to its free radicals scavenging ability and anti-inflammatory activity. Further, the extract (5-40 µg/ml) has shown anticancer activity against human hepatocellular carcinoma (HepG2) cells. However, the underlying mechanisms related to its anticancer effect need to be explored.

**Keywords:** *Salix aegyptiaca*, antioxidants, inflammation and hepatocellular carcinoma.

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## 1. INTRODUCTION

Oxidative stress refers to the increased level of free radicals mainly reactive oxygen species (ROS) along with reactive nitrogen species (RNS) which cause damage to main cellular constituents like DNA, lipid and protein. Oxidative damage could lead to the initiation and development of several health complications such as diabetes, Alzheimer's disease, atherosclerosis, cardiovascular problems and various types of cancers<sup>1,2</sup>. ROS apart from damaging the cellular macromolecules, also lead to the secretion of chemotactic factors, stimulation of inflammatory process and several growth factors, and activation of many transcription factors and oncogenes<sup>3</sup>. Mitochondria, peroxisomes, cytochrome P450 metabolism and inflammatory responses are the

endogenous sources of ROS generation<sup>4</sup>. The endogenous ROS production is scavenged by endogenous and exogenous antioxidants to avoid their detrimental effects.

Liver cancer is one of most common cancers worldwide. The developing countries in South-East Asia and Africa account for majority of these cases (more than 83% of total liver cancer cases) and cases are being increased in the industrially developed states<sup>5</sup>. According to GLOBOCAN, 2012, liver cancer has been reported as fifth most common cancer found in men and ninth in women. It ranked second according to the cancer mortality rate worldwide<sup>6</sup>. In 2012, there were around 782,500 new cases and 745,500 deaths found due to liver cancer globally<sup>7</sup>. *Salix aegyptiaca*, commonly known as Musk willow, is a diecious flowering plant. It

is mainly cultivated in province of Iran<sup>8</sup>. From ancient period, it has been traditionally used in Iran as cardioprotectant, gastroprotectant, laxative, sedative, nervonic, hypnotic, antihelminthic and vermifuge etc.<sup>9</sup>. Some recent reports showed anticancer activity of bark extract of *S. aegyptiaca* against human colon cancer cell lines and mouse model<sup>10,11,12</sup>.

In the present study, an attempt was made to study the free radical scavenging activity of hydroethanolic extract of *S. aegyptiaca* bark using DPPH assay, and its protecting role was also assessed by utilizing a method based on the induction of DNA damage in the form of single strand break by Fenton reagent generated  $\cdot\text{OH}$  radicals. The anti-inflammatory activity of this extract was also tested against heat induced protein denaturation. Further, the anticancer activity of the extract against human hepatocellular carcinoma cells, HepG2 was analyzed using cell viability assay.

## 2. MATERIALS AND METHODS

### 2.1 Cell lines and reagents

HepG2, a human hepatocellular carcinoma cell line, was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were grown in Dulbecco's modified Eagles medium (DMEM; Sigma, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Himedia, India) at 37°C with 5% CO<sub>2</sub>. *S. aegyptiaca* bark extract was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the medium was kept 0.1% (v/v) in all treatments including control. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO were obtained from Sigma (Saint Louis, MO, USA). pBR322 plasmid DNA was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The rest of the chemicals used were procured from local firms (India) and were of highest purity grade.

### 2.2 Preparation of hydroethanolic extract of *S. aegyptiaca* stem bark

The stem bark of *S. aegyptiaca* was collected from the garden in Qazwin, Iran. The plant was identified by the botanist on the morphological basis and deposited (Sample no. 20150217) in the Department of Biotechnology, Jamia Millia Islamia, New Delhi, India. The bark was shade dried and powdered 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 continuous 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 R-300) at 45°C followed by the desiccation through lyophilizer.

### 2.3 Determination of antioxidant activity by DPPH assay

The free radical scavenging activities of *S. aegyptiaca* bark extract was measured by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay as carried out by G. C. Yen with slight modification<sup>13</sup>. Briefly, 1.3 ml of methanolic solution of DPPH (100 μM) was added to

varying concentration of *S. aegyptiaca* bark extract (2-20 μg/ml). The reaction was incubated at room temperature for 30 min in dark. The absorbance of the residual DPPH solution was determined at 517 nm in a UV-1800 spectrophotometer (Shimadzu Corp). BHT (Butylhydroxytoluene) was used as positive control. The percent DPPH radicals scavenging effect was calculated using following formula,

% DPPH scavenging effect =

$$[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$$

Where, Abs<sub>control</sub> is the absorbance of DPPH radical + methanol and Abs<sub>sample</sub> is absorbance of DPPH radical + sample extract/standard

### 2.4 Determination of antioxidant activity by DNA nicking assay

DNA nicking assay was carried out as described by Lee *et al* 2002<sup>14</sup>. Fenton reagent was used to generate the hydroxyl free radicals ( $\cdot\text{OH}$ ) which reacts with pBR322 plasmid DNA and damages its structure. *S. aegyptiaca* bark extract (20 μg/ml) were added to the reaction mixture (20 μl) containing 0.5 μg of pBR322 and Fenton reagent (30 mM H<sub>2</sub>O<sub>2</sub>, 50 μM ascorbic acid, 80 μM FeCl<sub>3</sub>). After incubation of 30 min at 37 °C, analysis was done by running the samples on 1% agarose gel electrophoresis (Mini Sub-Cell GT, Biorad) followed by ethidium bromide staining. Gel documentation system (FluorChem HD2) was used to visualize the bands. The band intensity was analyzed by ImageJ software (NIH, USA).

### 2.5 Determination of anti-inflammatory activity by protein denaturation assay

The anti-inflammatory activity of *S. aegyptiaca* bark extract was studied by the method described by H. M. Arif Ullah<sup>15</sup>. The final reaction mixture contained 0.2 ml of egg albumin, 2.8 ml of phosphate buffer saline (PBS) and 2 ml of varying concentrations of the *S. aegyptiaca* bark extract. The final concentration of extract was kept 50-800 μg/ml. Sodium diclofenac was used as positive control. The tubes containing the mixture were incubated at 37 °C for 15 min followed by heating at 70 °C for 5 min. The tubes were allowed to cool and the absorbance was measured at 660 nm. The percent inhibition of protein denaturation was calculated by using the following formula,

$$\% \text{ Inhibition of protein denaturation} = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$$

Where, Abs<sub>control</sub> is the absorbance of control and Abs<sub>sample</sub> is absorbance of extract/standard.

### 2.6 Determination of cell viability by MTT assay

Five thousand HepG2 cells were seeded in 96 well culture plate and incubated for 24 h. Cells were then treated with DMSO or 5-40 μg/ml of *S. aegyptiaca* bark extract for 48 h. Then 100 μl of MTT solution (5mg/ml in PBS) was added to each well and incubated in 5 % CO<sub>2</sub> incubator for 4 h. The MTT solution was removed without disturbing the cells and 100 μl DMSO was added to each well to dissolve formazan crystals. After

incubation of 10 min in dark, the wells were read in ELISA plate reader at 570 nm wavelength<sup>16</sup>.

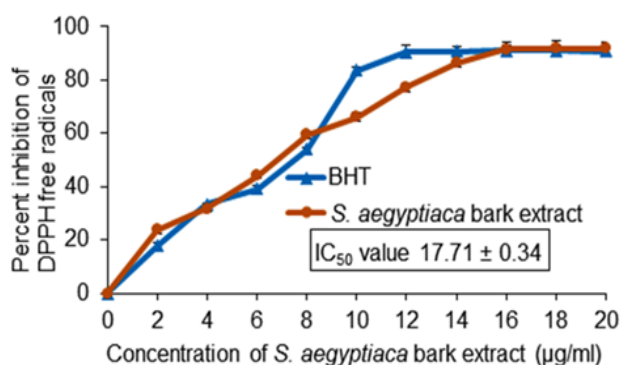
### 2.7 Statistical analysis

The values were presented as mean  $\pm$  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.

## 3. RESULTS

### 3.1 *S. aegyptiaca* bark extract scavenges DPPH free radicals

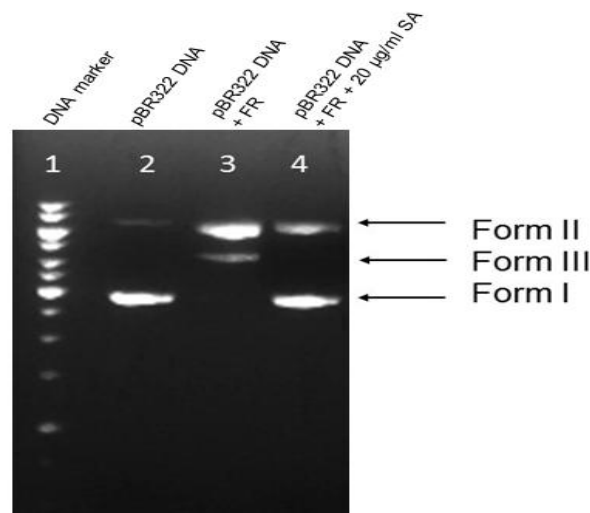
A varying range of *S. aegyptiaca* bark extract concentration (2-20  $\mu\text{g/ml}$ ) exhibited the scavenging of DPPH radicals in concentration dependent manner. The  $\text{IC}_{50}$  value of the extract was found to be  $17.71 \pm 0.34$ . BHT was used as positive control for comparison. The results are shown in **Figure 1**.



**Figure 1:** Free radical scavenging property of *S. aegyptiaca* barks extract. Data represents percent inhibition of DPPH free radicals through DPPH assay. Methanolic solution of DPPH was added to different concentrations of *S. aegyptiaca* bark extract (2-20  $\mu\text{g/ml}$ ). The reaction was incubated at room temperature for 30 min in dark. The absorbance of the residual DPPH solution was determined at 517 nm. BHT was used as positive control. Values are represented as mean  $\pm$  SEM of three samples. Abbreviations: BHT; Butylhydroxytoluene.

### 3.2 *S. aegyptiaca* bark extract protects DNA against $\cdot\text{OH}$ radicals

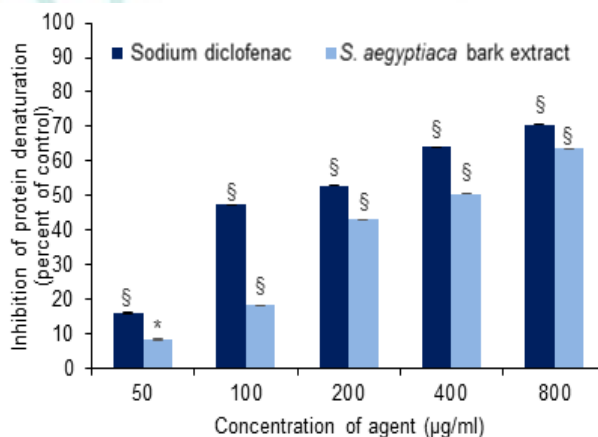
*S. aegyptiaca* bark extract (20  $\mu\text{g/ml}$ ) exhibited protective role against DNA damage induced by  $\cdot\text{OH}$  radicals. For this assay, 20  $\mu\text{g/ml}$  concentration was selected on the basis of  $\text{IC}_{50}$  value ( $17.71 \pm 0.34$ ) of this extract which was determined by DPPH assay. The damage was determined in terms of conversion of Form I of DNA (super coiled) into its Form II (relaxed) and Form III (linear). When the supercoiled pBR322 plasmid DNA incubated in presence of the Fenton reagent, the Form I of DNA was changed into for II (Lane: 3). The extent of conversion into Form III was relatively very small. The bark extract inhibited the conversion of Form I into Form II (Lane: 4) against Fenton reagent. The results can be seen in **Figure 2**.



**Figure 2:** Antioxidant activity of *S. aegyptiaca* barks extract against  $\cdot\text{OH}$  radicals causing DNA nick. Fenton reagent was added to 0.5  $\mu\text{g}$  of pBR322 plasmid DNA in absence or presence of *S. aegyptiaca* bark extract. The mixture was incubated for 30 min at 37°C. After incubation the samples were analyzed by 1% agarose gel electrophoresis followed by ethidium bromide staining. Abbreviations: FR; Fenton reagent and SA; *S. aegyptiaca* bark extract.

### 3.3 *S. aegyptiaca* bark extract exhibits anti-inflammatory activity

The anti-inflammatory activity of the extract (50-800  $\mu\text{g/ml}$ ) was determined by assessing its effect on inhibition of heat induced protein denaturation. The extract significantly inhibited the protein denaturation in concentration-dependent manner by 8.28 % ( $p < 0.05$ ), 18.25 % ( $p < 0.005$ ), 43.09 % ( $p < 0.005$ ), 50.53 % ( $p < 0.005$ ) and 63.48 % ( $p < 0.005$ ) with the treatment 50, 100, 200, 400 and 800  $\mu\text{g/ml}$  of *S. aegyptiaca* bark extract. The results are depicted in **Figure 3**.

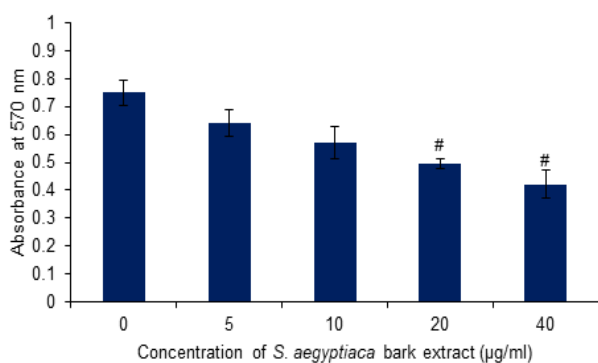


**Figure 3:** Anti-inflammatory activity of *S. aegyptiaca* barks extract. The final reaction mixture contained 0.2 ml of egg albumin, 2.8 ml of phosphate buffer saline (PBS) and 2 ml of varying concentrations of the *S. aegyptiaca* bark extract (50-800  $\mu\text{g/ml}$ ). The tubes containing reaction mixture were incubated at 37 °C for 15 min followed by heating at 70 °C for 5 min. The tubes were allowed to cool and absorbance was measured at 660 nm. Sodium diclofenac was used as a positive control. Values are represented as percent of

control calculated by mean of three samples.  $^{\#}(p < 0.005)$  represents significant changes relative to control.

### 3.4 *S. aegyptiaca* bark extract decreases the cancer cell viability

The treatment of *S. aegyptiaca* bark extract on HepG2 cells exhibited the decrease in cell viability at 48 h determined by MTT assay. The cell viability of HepG2 cells was significantly decreased by 33.79 % ( $p < 0.01$ ) and 43.78 % ( $p < 0.01$ ) with the treatment of 20 and 40  $\mu\text{g/ml}$  of bark extract, respectively. The results are shown in **Figure 4**.



**Figure 4:** Inhibitory effect of *S. aegyptiaca* barks extract on viability of HepG2 cells. Cells were treated with DMSO or 5-40  $\mu\text{g/ml}$  of *S. aegyptiaca* bark extract for 48 h. Then, 100  $\mu\text{l}$  of MTT solution (5 mg/ml in PBS) was added to each well and incubated in 5 %  $\text{CO}_2$  incubator for 4 h. The MTT solution was removed and 100  $\mu\text{l}$  DMSO was added to each well to dissolve formazan crystals. After incubation of 10 min in dark, the wells were read in ELISA plate reader at 570 nm. Values are represented as mean  $\pm$  SEM of three samples.  $^{\#}(p < 0.01)$  represents significant changes relative to control.

## 4. DISCUSSION

ROS like  $^1\text{O}_2$  (singlet oxygen),  $\text{O}_2^{\cdot-}$  (superoxide),  $\text{H}_2\text{O}_2$  (hydrogen peroxide),  $\cdot\text{OH}$  (hydroxyl),  $\text{NO}\cdot$  (nitric oxide),  $\text{HO}_2\cdot$  (per hydroxyl),  $\text{ROO}\cdot$  (peroxyl) and  $\text{RO}\cdot$  (alkoxyl) are produced in mammalian body and result in the development of oxidative stress<sup>17</sup>. This excessive generation of ROS consequently leads to the cellular damage, which finally cause the induction of many pathological complications. *S. aegyptiaca*, being a plant with medicinal value, is supposed to possess antioxidant activity. Expectedly, in the present study, hydroethanolic extract of *S. aegyptiaca* scavenged the DPPH free radicals which indicated its antioxidant activity.

The DNA damage is considered to be one of the critical events in the cell damage. The  $\cdot\text{OH}$  free radicals almost exclusively interact with DNA and cause strand breaks by removing the phosphate group from its backbone<sup>18</sup>. In the present study, the supercoiled pBR322 plasmid DNA was incubated in presence of the Fenton reagent along with or without the bark extract. Fenton reagent produces the  $\cdot\text{OH}$  free radicals which attack on supercoiled DNA (Form I) to convert it into relaxed DNA (Form II). The bark extract was found to inhibit the conversion of Form I into Form II (indicative of the

single strand breaks). The protection of supercoiled DNA structure (Form I) could have occurred due to inactivation of  $\cdot\text{OH}$  free radicals. The main detrimental effects are performed by  $\cdot\text{OH}$  free radicals being the most reactive free radical<sup>19</sup> with a shortest biological half-life<sup>20</sup>. From the above findings, it could be concluded that *S. aegyptiaca* has property to scavenge  $\cdot\text{OH}$  and other free radicals. The antioxidant activity of this bark extract may be attributed by the presence of various phenolic compounds such as gallic acid, caffeic acid, vanillin, p-coumaric acid, myricetin, catechin, epigallocatechin gallate, rutin and quercetin, which were determined by high performance liquid chromatography in bark extract of *S. aegyptiaca*<sup>21</sup>.

Now, it is well established that initiation and progression of many cancers originate from inflammation, hence identified as a hallmark of cancer thereby it has been turned out as one of the main focus for drug development studies. The inflammatory cells markedly contribute to the tumor microenvironment, promoting the neoplastic process and aiding in the cell proliferation, survival and migration<sup>22</sup>. The secretion of chemokines, cytokines, certain growth factors, and proteases by inflammatory cells could lead to the initiation and promotion of cancer<sup>23</sup>. In our study, *S. aegyptiaca* bark extract showed anti-inflammatory activity which might be ascribed to the anticancer potential of this extract as it exhibited decrease in cell viability of human hepatocellular carcinoma cells, HepG2. Hepatocellular carcinoma is the most common type of primary liver cancer (PLC) that accounts for approximately 75-90 % of total PLC<sup>24</sup> and a major health issue in developing countries.

The underlying mechanisms related to anticancer efficacy of *S. aegyptiaca* bark extract are yet to be explored, however some studies showed that the inhibition of colon cancer cells proliferation was mediated through G1/S cell cycle arrest and induction of apoptosis through p53 dependent pathway. Other pathways include the inhibition of PI3K/Akt and MAP Kinase pathways, and inhibition of epithelial to mesenchymal transition with the treatment of *S. aegyptiaca* bark extract<sup>11,12</sup>. Ethanolic extract of *S. aegyptiaca* bark exhibited chemopreventive efficacy against 1,2-dimethylhydrazine induced colon carcinogenesis in mice through antioxidant and anti-inflammatory activities<sup>10</sup>. The above findings strongly indicate that *S. aegyptiaca* bark extract may show anticancer efficacy against hepatocellular carcinoma in animal models.

## 5. CONCLUSION

Overall, the present study suggests the potential of *S. aegyptiaca* bark extract to scavenge the free radicals, inhibit the inflammation and cell viability of hepatocellular carcinoma cells, HepG2. The antioxidant and anti-inflammatory activity of this extract might be responsible for its efficacy against several health complications including cancer.

### Conflict of interest

The authors declare that they have no conflict of interest.

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