**In-vitro and in-vivo Antioxidant Activity of the Butanolic Extract from Ephedra alte C. A. Mey**

Bahaa Al-Trad*, Mahmoud A Al –Qudah², Mazhar Al Zoubi³, AlaaAl-Masri¹, Riyadh Muhaidat¹, Janti qar¹, Ghada Alomari¹ and Nizar I. Alrabadi⁴

¹Department of Biological Sciences, Yarmouk University, Irbid, Jordan.
²Department of Chemistry, Yarmouk University, Irbid, Jordan.
³Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan.
⁴Department of Food Science and Nutrition, Faculty of Agriculture, Jerash University, Jerash, Jordan.
*Corresponding author E-mail : bahaa.tr@yu.edu.jo

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Previous studies indicated that the extracts from different Ephedra species have antibacterial, antifungal and antioxidant activities. However, none of the published report described the phytochemical components and the antioxidant capacities of Ephedra alte belonging to the family Ephedraceae. To evaluate the in-vitro and in-vivo antioxidant activities of the butanolic extract from the stem of Ephedra alte which grows wild in northern Jordan. Different concentrations of the butanolic extract from stem samples of Ephedra alte plant were subjected to four different in-vitro antioxidant assays (DPPH, ABTS, ferrous ion chelating and hydroxyl radical scavenging activities). The in-vivo effects of two different doses of the extract (200 mg/kg and 500 mg/kg, orally for 12 days) on the activities of serum and liver superoxide dismutase (SOD) and catalase (CAT) were measured in mice. The extract revealed a strong in-vitro antioxidant activity in a concentration-dependent manner. As well, significant increases in both liver and serum CAT enzyme activity and in serum SOD activity were observed in mice treated for 12 days with the extract. These results suggested that the butanolic extract from the stem of Ephedra alte exhibited a significant antioxidant activity both in-vitro and in-vivo that can be an important source of natural antioxidants.

**Keywords:** Antioxidant, Ephedra alte, in vitro, in vivo, butanol extract.

Oxidative stress in animal cells reflects the imbalance between the production of antioxidants and oxidants which consequently leads to a severe damage of the cellular compartments and increased lipid peroxidation due to the action of reactive species¹,². Oxidative stress has been interconnected to numerous chronic diseases³. For instance, increasing evidence suggested a pathological impact of oxidative stress in the development of complications of the two major types of diabetes mellitus⁴.

The main reactive species include ROS and RNS, reactive oxygen species and reactive nitrogen species, respectively. ROS and RNS are generated in human body due to external and internal physiological processes. However, the imbalanced production of the oxidants can lead to the damage of many biomolecules (proteins, lipids, and nucleic acid)⁵,⁶. Normally, animal cells are equipped with many defense mechanisms against oxidative stress including glutathione (GSH),
vitamins C and E, catalase (CAT), superoxide dismutase (SOD) and various peroxidases\textsuperscript{12}.

Basically, antioxidants counteract the oxidation of biological molecules by delaying or inhibition mechanism\textsuperscript{7}. Early modulations of oxidative stress by exogenous natural antioxidants and diet rich in vitamins have proven a beneficial effect in the protection against the oxidative stress induced damage\textsuperscript{2,5}. Plant origin polyphenols; have gained considerable attention due to their possible health benefits. Epidemiological studies showed an effective impact of polyphenol plant diets on the reduction of the incidence of cancers, diabetes, osteoporosis, cardiovascular and neurodegenerative disorders\textsuperscript{6}.

Jordanian traditional medicine included a list of more than 110 species from 49 plant families, mainly in the population of limited health care providers\textsuperscript{7}. Ephedra is a genus of the family Ephedraceae consisting of of 50–65 species among which are shrubs, vines, but rarely small trees\textsuperscript{8}. Ephedra alte C. A. Mey (synonym Ephedra aphylla Forssk) is one of the common species in different Middle East countries\textsuperscript{8}. Results of previous studies on the biological activity of the plant indicated that the extracts from different Ephedra species have antibacterial, antifungal and antioxidant activities\textsuperscript{8,11}. However, there is no published report on the phytochemical composition and the antioxidant capacities of Ephedra alte belonging to the family Ephedraceae. Therefore, the objective of the present study was to determine the total phenolic and total flavonoids content of the butanolic extract from the stem of Ephedra alte that grows wild in northern Jordan and to determine its in vitro and in vivo antioxidant capacity.

**MATERIALS AND METHODS**

**Reagents and plant material**

All reagents and chemicals were supplied and purchased from Sigma-Aldrich, USA unless otherwise specified. Ephedra alte was collected from the north of Jordan during spring of 2016. Plants material was identified by the plant taxonomist professor Ahmad El-oqlah from the Department of Biological Sciences, Yarmouk University.

**Preparation of crude extract fractions**

The fresh aerial parts were subjected to drying conditions at room temperature in a shady place for a month. Then the dried and powdered stems were subjected to extraction process using Soxhlet extractor with petroleum ether to remove the fatty acids, dried and then followed by methanol extraction. The rotary vacuum evaporator was applied for for sample concentration and drying. This residue was partitioned between CHCl\textsubscript{3} and H\textsubscript{2}O (1:1) solvent system. After the separation of CHCl\textsubscript{3} and H\textsubscript{2}O phases, the dried CHCl\textsubscript{3} fraction was partitioned between 10% aqueous methanol and hexane. The polar organic compounds were extracted from water by n-butanol.

**Phytochemical Analysis**

Crude fractions and distilled crude obtained from plants were screened for the presence of secondary metabolites of terpenes, saponins, flavonoids, tannins, alkaloids, anthraquinones, and cardiac glycosides following standard procedures described previously\textsuperscript{12}.

**Total phenolic and flavonoid contents analysis**

Folin-Ciocalteu assay was used to evaluate and analyze the total phenolic contents as described before\textsuperscript{13}. The results were expressed as mg/g gallic acid equivalent. The colorimetric aluminum chloride assay was used to evaluate and determine the total flavonoid content based on the previous description\textsuperscript{14} and expressed as mg/g quercetin.

**Antioxidant activity in vitro**

**DPPH radical scavenging assay**

The radical scavenging activity of the butanolic stem extract was determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, and Ascorbic acid (vitamin C) was used as a standard as described before\textsuperscript{15}. Briefly, 1 mL of different concentration (5-500 µg/mL) of the stem extract was added to 2 mL of 0.1mM of DPPH/methanol solution, followed by 30 minutes of incubation in dark conditions. Finally, the optical density was recorded at 517 nm against scavenger-free blank.

**ABTS assay**

For antioxidant activity by 2,2′-Azino–bis (3-ethylbenzoline-6- sulfonic acid) diammonium salt (ABTS) decolorization assay was determined as described before\textsuperscript{16}. The ABTS working solution was freshly prepared by mixing equal quantities of 7 mM of ABTS and 2.4 mM of (K\textsubscript{3}S\textsubscript{4}O\textsubscript{6})\textsubscript{2} solutions which was incubated for 16 hours at (2-3\textdegree)C in dark conditions. Before using, the working solution was
diluted with dH2O for obtaining an absorbance equals 0.75 ± 0.02 at 734 nm. The antioxidant assay reaction was performed by adding 1 mL of the stem extract to 3 mL of ABTS working solution and 5 minutes incubation, different concentrations of the stem extract were applied (5-500 μg/mL). The optical density was measured at 734 nm against the blank.

**Ferrous ion (Fe^{2+}) chelating assay**

Ferrous ions chelating activity was conducted as described before with modification^{16}. Briefly, 3 mL of stem extract at different concentrations (5-500 μg/mL) was added to 0.25 mL of 2 mM FeCl2 solution. 0.2 mL of 5 mM ferrozine solution was added for reaction initiation and left at room temperature for 10 minutes. EDTA solution was used as a positive control. Finally, the optical density was measured at 562 nm against the blank.

**Hydroxyl radical assay**

Salicylic acid was used to measure the hydroxyl radical formation according to the modified method of^{16}. 1mL of the stem extract solution at different concentrations (5-500 μg/mL) was added to 250 μl of 6 mM FeSO4, followed by addition of 0.5 mL of 6 mM H2O2. The reaction mixtures were subjected to shaking followed by standing for 10 min. Then 1mL of 6 mM salicylic acid was added and incubated for 30 min at room temperature. Vitamin C was used as a positive control. The optical density was measured at 510 nm against the blank.

**In Vivo Experiment**

**Acute toxicity**

The Institutional Ethics Committee at the Department of Biological Sciences, Yarmouk University approved all animal procedures and protocols. Different doses from the butanolic Ephedra alte stem extract were given to the mice (weighing 25-30 g; n=5/group) as follow: 50, 100, 200 mg/kg intraperitoneal (i.p) and 200, 500, 1000, 2000 mg/kg given orally. The mortality and any sign of toxicity were observed regularly for the first 24 hrs and daily for 14 day.

**Animal treatment**

Twenty-eight adult males Swiss albino mice, 8 weeks old and weighing ~25-30 g were kept in the animal house at Yarmouk University under controlled conditions at 21 - 23°C on an illumination schedule of 12 hours of light. Standard pellet food and water were provided *ad libitum*. Mice were divided into three groups (n=7 in each group): Control and Ephedra alte extract treated groups (200 mg/kg and 500 mg/kg, orally for 12d). At the end of the experiments, the animals were weighed and anesthetized with ether, blood was collected, and the liver was rapidly excised and stored in liquid nitrogen.

**In vivo antioxidant activity**

Serum was isolated from blood samples by centrifugation at 3000 rpm for 6 min at 4°C. The liver was homogenized in phosphate buffer saline. After centrifugation at 15000 rpm for 15 min at 4°C, the supernatant was used for oxidative stress assessment. Serum and hepatic supernatant CAT and SOD enzyme activity were measured using Amplex TM Fluorimetric Catalase Assay Kit (AAE Bioquest, USA) and SOD determination kit (Sigma–Aldrich, USA) according to the manufacturer’s instructions.

**Calculations and Statistical analysis**

*In-vitro* data were recorded as means ± SEM of triplicate measurements. Scavenging or chelating effect (%) was calculated as the following: % = (control absorbance – sample absorbance/ control absorbance) × 100. The IC50 values were calculated by the linear regression method of plots of the percent of antiradical activity against the concentration of the tested compounds. Statistical analyses of *in-vivo* data were calculated using the SPSS version 19.0 for Windows (SPSS Inc., Chicago, IL). *P* values were determined using one-way ANOVA followed by LSD. Differences were considered significant if *P* < 0.05.

**RESULTS AND DISCUSSION**

The phytochemical screening showed that the butanolic extract from the stem of *Ephedra alte* is rich in tannins, flavonoids, saponins, alkaloids, and glycosides which support the concept that *Ephedra alte* may have medical benefit. In line with that, it is well known that the *Ephedra* is a source of natural alkaloids products such as ephedrine that has been used medicinally to treat asthma, sinusitis and rhinitis^{17,18}. Additionally, pure isolated alkaloids are used as essential medicinal agents for their pain killer, antispasmodic and bactericidal effects^{18}. Oxidative stress is generated when the
free radicals and oxidants are produced in excess which can damage many biological molecules that are important for cell functions. The oxidative stress has been shown to have high impact in many disorders like neurodegenerative diseases, cancer and diabetes. Since scavenging of free radicals could inhibit the harmful effect of free radicals and stop the spreading of oxidation, the antioxidants contents from plant extract through their scavenging activity are valuable for management of those diseases.

Scientific evidence suggests that the flavonoids and phenolic acids, the most studied groups of polyphenols, play an essential role in protecting cell constituents against oxidative damage. In the present study, the butanolic extract from the stem of Ephedra alte had a total phenolic (404.001±5.53 mg/g gallic acid) and flavonoid (40.73±6.59 mg/g quercetin) contents. In previous studies, the total phenolic content of Ephedra procera was 718 mg tannic acid/g, and 404.001±5.53 mg/g gallic acid for Ephedra sarcocarpa growing in Iran was 709.18 mg catechin equivalent/g extract, for Ephedra laristanica was 513 μmol gallic acid/g extract and for Ephedra strobilacea was 504.9 ± 41.51 μmol eq catechin/g extracts and 114.61 ± 15.13 μmol eq catechin/g extracts for the wild plants and callus, respectively. Recent studies showed that the flavonoids of Ephedra alata growing in Palestine was in the range of 4.2 to 19.5 mg catechin/g and the phenolic content range from 30 to 101 mg gallic acid/g.

In the current study, the in-vitro antioxidant activities of the butanolic extract from the stem of Ephedra alte were assessed against DPPH, ABTS and hydroxyl radicals. The ferrous ion chelating activity of the extracts was also determined. The butanolic extract showed different levels of radicals scavenging activity in a dose-dependent manner over the range of 5–500 μg/mL concentration (Table 1), indicating the high antioxidative capacity of the extract. The IC$_{50}$, the concentration of the sample required to inhibit 50% of radical, of the extract were 66.4, 50.2, 43.5, 77.1 μg/mL for DPPH, ABTS, hydroxyl radicals and the ferrous ion chelating activity, respectively (Table 2).

### Table 1. Antioxidant activity (%) of the butanolic extract from the stem of Ephedra alte

<table>
<thead>
<tr>
<th>C(μg/ml)</th>
<th>DPPH</th>
<th>ABTS</th>
<th>hydroxyl radical</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE</td>
<td>VC</td>
<td>BE</td>
</tr>
<tr>
<td>5</td>
<td>9.6±1.0</td>
<td>45.6±0.3</td>
<td>7.65±1.5</td>
</tr>
<tr>
<td>10</td>
<td>17.9±0.2</td>
<td>77.9±0.7</td>
<td>10.4±0.8</td>
</tr>
<tr>
<td>50</td>
<td>34.4±0.1</td>
<td>94.9±0.2</td>
<td>42.4±0.1</td>
</tr>
<tr>
<td>100</td>
<td>62.5±0.6</td>
<td>96.7±0.2</td>
<td>71.3±0.3</td>
</tr>
<tr>
<td>500</td>
<td>86.1±0.5</td>
<td>96.9±0.1</td>
<td>98.7±0.1</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM. Abbreviations: BE, butanolic extract; VC, vitamin c; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); EDTA, ethylenediaminetetraacetic acid.

### Table 2. IC50 (μg/ml) of the butanolic extract from the stem of Ephedra alte

<table>
<thead>
<tr>
<th>Antioxidant activity</th>
<th>IC50 values (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BE</td>
</tr>
<tr>
<td>DPPH</td>
<td>66.4±0.55</td>
</tr>
<tr>
<td>ABTS</td>
<td>50.2±1.2</td>
</tr>
<tr>
<td>hydroxyl radical</td>
<td>43.5±1.14</td>
</tr>
<tr>
<td>BE</td>
<td>EDTA</td>
</tr>
<tr>
<td>ferrous chelating</td>
<td>77.1±1.11</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM. Abbreviations: BE, butanolic extract; VC, vitamin c; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); EDTA, ethylenediaminetetraacetic acid.
high phenolic constituents of the butanol extract of stem of *Ephedra alte* are responsible for its high antioxidative capacities.

Further confirmation of the antioxidant activity was conducted *in vivo* for the stem butanol extract. Biologically, the harmful effects of the ROS are defended by *in vivo* built-in mechanisms which involve enzymatic and non-enzymatic defense mechanisms. For instance, enzymatic antioxidant systems, CAT, GSH-Px, and SOD are the three important antioxidant enzymes which have an important role as a defense process that protects cells from the reactive oxygen species. Superoxide dismutase is one of the major mechanisms of defense against oxygen-derived free radicals, by converting superoxide radicals to H$_2$O$_2$, while CAT is a key enzyme of the enzymatic antioxidant systems which dismantling H$_2$O$_2$ to water and oxygen. Our study recorded a significant dose-dependent increase in CAT level in both liver homogenate and serum samples ($P < 0.05$; Fig 1 and 2) and in serum SOD level ($P < 0.05$; Fig 3) after 12d treatment with the stem butanol *Ephedra alte* extract in mice. However, no effect of the extract on hepatic SOD activity was observed (data not shown). Such data, coupled with the *in vitro* results indicated that the stem butanol extract of *Ephedra alte* could be an important source of natural compounds with antioxidant capacity.

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**Fig. 1.** Effect of butanolic extract treatment (200 mg/kg and 500 mg/kg, orally for 12 days) on the serum CAT enzyme activity. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: CAT, catalase

**Fig. 2.** Effect of butanolic extract treatment (200 mg/kg and 500 mg/kg, orally for 12 days) on the hepatic CAT enzyme activity. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: CAT, catalase
Finally, in the present study acute oral toxicity study showed that the LD\textsubscript{50} value of stem butanol extraction of \textit{Ephedra alte} was found to be more than 2000 mg/kg body weight for oral administration and more than 500 mg/kg body weight for i.p administration. This indicates that stem butanol extraction of \textit{Ephedra alte} might be non-toxic and safe when administered orally or i.p.

ACKNOWLEDGMENTS

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CONCLUSIONS

The butanolic extract from the stem of \textit{Ephedra alte} showed high phenolic contents and exhibited high antioxidant activity both \textit{in vitro} and \textit{in vivo} which nominating the use of \textit{Ephedra alte} as an important source for natural antioxidants.

REFERENCES

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