Biomedical & Pharmacology Journal, September 2018. Vol. **11**(3), p. 1239-1245

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

Bahaa Al-Trad[1]*, Mahmoud A Al –Qudah[2], Mazhar Al Zoubi[3], AlaaAl-Masri[1], Riyadh Muhaidat[1], Janti qar[1], Ghada Alomari[1] and Nizar I. Alrabadi[4]

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

http://dx.doi.org/10.13005/bpj/1485

(Received: 04 August 2018; accepted: 25 August 2018)

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 species[1,2]. Oxidative stress has been interconnected to numerous chronic diseases[3]. For instance, increasing evidence suggested a pathological impact of oxidative stress in the development of complications of the two major types of diabetes mellitus[1].

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)[4,2]. 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[4,2].

Basically, antioxidants counteract the oxidation of biological molecules by delaying or inhibition mechanism[3]. 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[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 [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 [7]. *Ephedra* is a genus of the family *Ephedraceae* consisting of of 50–65 species among which are shrubs, vines, but rarely small trees [8]. *Ephedra alte* C. A. Mey (synonym *Ephedra aphylla Forssk*) is one of the common species in different Middle East countries[8]. Results of previous studies on the biological activity of the plant indicted that the extracts from different *Ephedra* species have antibacterial, antifungal and antioxidant activities[9-11]. 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 $CHCl_3$ and H_2O (1:1) solvent system. After the separation of $CHCl_3$ and H_2O phases, the dried $CHCl_3$ 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 [12].

Total phenolic and flavonoid contents analysis

Folin-Ciocalteu assay was used to evaluate and analyze the total phenolic contents as described before[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[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[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**) decolonization assay was determined as described before[15]. The ABTS working solution was freshly prepared by mixing equal quantities of 7 mM of ABTS and 2.4 mM of ($K_2S_2O_8$) solutions which was incubated for 16 hours at (2-3^oC) in dark conditions. Before using, the working solution was

diluted with d.H2O 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 3mL of ABTS working solution and 5 minutes incubation, different concentrations of the stem extract were applied (5-500 ¼g/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 ¼g/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 ¼g/mL) was added to 250 ¼l of 6 mM $FeSO_4$, followed by addition of 0.5 mL of 6 mM H_2O_2. 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 intraperitoneally (i.p) and 200, 500, 1000, 2000 mg/kg given orally. The mortilty 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 Amplite TM Fluorimetric Catalase Assay Kit (AAT 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 IC_{50} 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[3]. The oxidative stress has been shown to have high impact in many disorders like neurodegenerative diseases, cancer and diabetes[19,1,3,6]. Since scavenging of free radicals could inhibit the harmful effect of free radicals and stop the spreading of oxidation[4], the antioxidants contents from plant extract through their scavenging activity are valuable for management of those diseases[6].

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 [6]. 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[20], for *Ephedra sarcocarpa* growing in Iran was 709.18 mg catechin equivalent/g extract[10], for *Ephedra laristanica* was 513 µmol gallic acid/g extract[11] and for *Ephedra strobilacea* was 504.9 ± 41.51 ¼mol eq catechin/g extracts and 114.61 ± 15.13 ¼mol eq catechin/g extracts for the wild plants and callus, respectively[9]. 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[14].

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 ¼g/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 ¼g/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*

	DPPH		ABTS		ferrous chelating		hydroxyl radical	
C(µg/ml)	BE	VC	BE	VC	BE	EDTA	BE	VC
5	9.6±1.0	45.6±0.3	7.65±1.5	14.1±0.5	22.8±0.5	24.1±0.1	21.2±0.3	6.25±0.2
10	17.9±0.2	77.9±0.7	10.4±0.8	57.6±0.6	22.9±0.4	35.4±0.1	32.4±0.5	34.81±0.1
50	34.4±0.1	94.9±0.2	42.4±0.1	99±0.1	29.6±0.7	65.1±0.2	43.9±0.9	55.47±0.3
100	62.5±0.6	96.7±0.2	71.3±0.3	99.2±0.1	33.7±0.4	82.8±0.1	56.4±1.0	96.09±0.2
500	86.1±0.5	96.9±0.1	98.7±0.1	99.6±0.1	73.6±0.2	95.1±0.3	92.9±1.0	98.69±0.2

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*

Antioxidant activity	IC50 values (µg/ml)	
	BE	VC
DPPH	66.4±0.55	1.6±0.03
ABTS	50.2±1.2	11.2 ± 0.45
hydroxyl radical	43.5±1.14	28.2±1.3
	BE	EDTA
ferrous chelating	77.1±1.1	21.8 ± 0.18

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.

Phytochemical components, including the phenolic and flavonoids, are important compounds that determine the plants antioxidant capacity, mainly due to their redox properties[21,5]. The high antioxidant activity of *Ephedra alte* extract can be explained by the presence of the hydroxyl groups in the phenolic compounds[22]. It has been shown previously that phenolic compounds provide the major contribution to the antioxidant activity of the methanolic extracts of *Ephedra sarcocarpa* measured by the DPPH assay[10]. Therefore, the

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[2]. Superoxide dismutase is one of the major mechanisms of defense against oxygen-derived free radicals, by converting superoxide radicals to H_2O_2, while CAT is a key enzyme of the enzymatic antioxidant systems which dismantling H_2O_2 to water and oxygen[2,23]. 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.

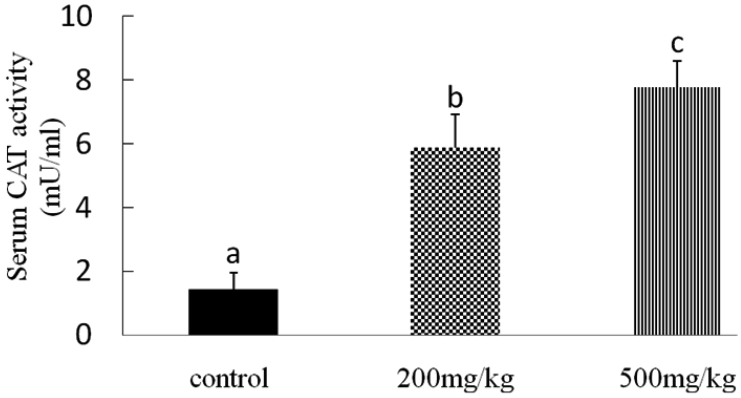

Fig. 1. Effect of butanolic extract treatment (200 mg/kg and 500 mg/kg, orally for 12 days) on the serum CAT enzyme activity. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$).. Abbreviations: CAT, catalase

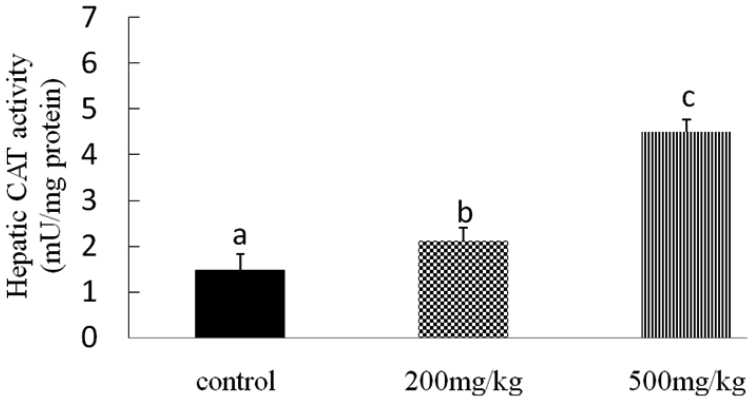

Fig. 2. Effect of butanolic extract treatment (200 mg/kg and 500 mg/kg, orally for 12 days) on the hepatic CAT enzyme activity. Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$).. Abbreviations: CAT, catalase

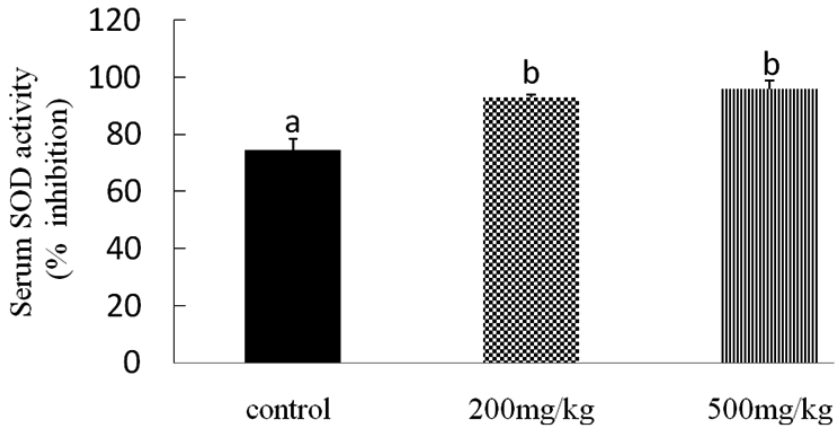

Fig. 3. Effect of butanolic extract treatment (200 mg/kg and 500 mg/kg, orally for 12 days) on the serum SOD enzyme activity (inhibition rate %). Data represent the mean ± SEM. Means with different superscript letters are significantly different from one another ($P < 0.05$). Abbreviations: SOD, superoxide dismutase

Finally, in the present study acute oral toxicity study showed that the LD_{50} value of stem butanol extraction of *Ephedra alte* was found to be more than 2000 mg/kg body weight for oral administration and more than 500mg/kg body weight for i.p administration. This indicates that stem butanol extraction of *Ephedra alte* might be non-toxic and safe when administered orally or i.p.

ACKNOWLEDGMENTS

We are very thankful to the Deanship of Research and Graduate Studies at Yarmouk University for their financial support under Grant Number 2/2017.

CONCLUSIONS

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

REFERENCES

1. Maritim A. C, Sanders A and Watkins JB. Diabetes, oxidative stress, and antioxidants: a review. *Journal of Biochemical and Molecular Toxicology*., **17**: 24-38 (2003).
2. Nimse S. B and Pal D. Free radicals, natural antioxidants, and their reaction mechanisms. *Royal Society of Chemistry Advances*., **5**: 27986-28006 (2015).
3. Willcox J. K, Ash S. L and Catignani G. L. Antioxidants and prevention of chronic disease. *Critical Reviews in food Science and Nutrition*., **44**: 275-295 (2004).
4. Kryston T. B, Georgiev A. B, Pissis P and Georgakilas AG. Role of oxidative stress and DNA damage in human carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*., **711**: 193-201 (2011).
5. Rice-Evans C, Miller N and Paganga G. Antioxidant properties of phenolic compounds. *Trends in Plant Science*., **2**: 152-159 (1997).
6. Pandey K. B and Rizvi S. I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev*., **2**: 270-278 (2009).
7. Al-Khalil S. A survey of plants used in Jordanian traditional medicine. *International Journal of Pharmacognosy*., **33**: 317-323 (1995).
8. Qasem J. R. Ephedra alte (joint pine): an invasive, problematic weedy species in forestry and fruit tree orchards in Jordan. *The Scientific World Journal.,* 2012: 10 (2012).
9. Parsaeimehr A, Sargsyan E and Javidnia K. A comparative study of the antibacterial, antifungal and antioxidant activity and total content of phenolic compounds of cell cultures and wild plants of three endemic species of Ephedra. *Molecules*., **15**: 1668-1678 (2010).
10. Rustaiyan A, Javidnia K, Farjam M. H, Aboee-Mehrizi F and Ezzatzadeh E. Antimicrobial and

antioxidant activity of the Ephedra sarcocarpa growing in Iran. *Journal of Medicinal Plants Research*., **5**: 4251-4255 (2011).

11. Rustaiyan A, Javidnia K, Farjam MH, Mohammadi MK and Mohammadi N. Total phenols, antioxidant potential and antimicrobial activity of the methanolic extracts of Ephedra laristanica. *Journal of Medicinal Plants Research*., **5**: 5713-5717 (2011).
12. Siddiqui A and Ali M. Pratical Pharmaceutical Chemistry.1st Edition. *CBS Publish and distributors*. New Delhi. 1997: 126-13.
13. Singleton L, Orthofer R and Lamuela-Raventos R. Analysis of total phenols and other oxidation substrates and antioxidant by means of folin-ciocalteu reaget. *Methods Enzymology*., **299**: 152-178 (1999).
14. Al-Rimawi F, Abu-Lafi S, Abbadi J, Alamarneh AA, Sawahreh R. A and Odeh I. Analysis of phenolic and flavonoids of wild Ephedra alata plant extract by lc/pda and lc/ms and their anti-oxidant activity. *African Journal of Traditional, Complementary and Alternative Medicines*., **14**: 130-141 (2017).
15. Al-Qudah, M. A, Al-Ghoul A. M, Trawenh I. N, Al-Jaber H. I, Al Shboul T. M, Abu Zarga M. H and Abu orabi S. T. Antioxidant Activity and Chemical Composition of Essential Oils from Jordanian Ononis Natrix L. and Ononis Sicula Guss. *Journal of Biologically Active Products from Nature*., **4**: 52-61 (2014).
16. Mathew S and Abraham T. E. In vitro antioxidant activity and scavenging effects of Cinnamomum verum leaf extract assayed by different methodologies. *Food Chem Toxicol*., **44**: 198–206 (2006).
17. Barker W. D and Antia U. A study of the use of Ephedra in the manufacture of methamphetamine. *Forensic science international*., **166**: 102-109 (2007).
18. Wink M. Modes of action of herbal medicines and plant secondary metabolites. *Medicines*., **2**: 251–286 (2015).
19. Valko M, Rhodes CJ, Moncol J, Izakovic M and Mazura M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Che*mico-biological Interactions*., **160**: 1-40 (2006).
20. Dehkordi N. V, Kachouie M. A, Pirbalouti A. G, Malekpoor F and Rabei, M. Total phenolic content, antioxidant and antibacterial activities of the extract of Ephedra procera fisch. et mey. *Acta poloniae pharmaceutica*., **72**: 341-345 (2015).
21. Labud J, Buckova M, Heilerova L, Silhar S and Stepanek I. Evaluation of the redox properties and anti/pro-oxidant effects of selected flavonoids by means of a DNA-based electrochemical biosensor. *Analytical and bioanalytical chemistry*., **376**: 168-173 (2003).
22. Mathew S, Abraham T. E and Zakaria Z. A. Reactivity of phenolic compounds towards free radicals under in vitro conditions. *J Food Sci Technol*., **52**: 5790-2798 (2015).
23. Winterbourn C. C. Superoxide as an intracellular radical sink. *Free Radical Biology and Medicine*., **14**: 85-90 (1993).