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ORIGINAL ARTICLE

Antioxidant Activity and Phenolic and Flavonoid Contents of the Extract and Subfractions of *Euphorbia splendida* Mobayen

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ABSTRACT

Introduction: The harmful action of the free radicals which cause the oxidative stress can be blocked by antioxidant substances, and different plant extracts showed antioxidant activity. The aim of this study is was evaluation the antioxidant activity of total methanol extract (ME) and subfractions of *Euphorbia splendida* Mobayen. **Materials and Methods:** Aerial part of *E. splendida* was extracted by maceration with methanol and then subfractionated by liquid–liquid fractionation using petroleum ether, chloroform, ethyl acetate, and water. Antioxidant activity was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, reduction of ferric ions and ferrous ion chelating potential. Total phenolic contents (TPC) and total flavonoid contents (TFC) were estimated with Folin-Ciocaltue and aluminum chloride methods, respectively. **Results:** The findings revealed that *E. splendida* ME and subfractions showed a dose-dependent antioxidant activity. ME showed the highest antioxidant activity based on total reduction capability and ferrous ions chelating assay tests. Aqueous fraction and then ethyl acetate fraction showed the best IC_{50} in DPPH radical scavenging test in comparison to butylated hydroxytoluene. ME showed the highest value of TPC and TFC (270.74 ± 0.005 mg/g and 208.23 ± 0.007 mg/g, respectively). **Conclusion:** This study showed that the extract and subfractions of *E. splendida* have antioxidant activity. The antioxidant activity of the extract and fractions might be attributed to the presence of phenolic compounds. More studies are needed to determine the active antioxidant compounds of this plant.

Key words: 1,1-Diphenyl-1-picrylhydrazyl, antioxidant, *Euphorbia splendida,* flavonoid, fractions, free radicals

SUMMARY

- Total extract and subfractions of Euphorbia splendida showed antioxidant activity.

Abbreviations Used: TPC: Total phenolic content, TFC: Total flavonoid content, DPPH: 2, 2′- diphenyl-1-picrylhydrazyl, BHT: Butylated hydroxytoluene, EDTA: Ethylene Diamine Tetra Acetic acid, ME: Total methanol extract, EAF: Ethyl acetate fraction , AQF: Aqueous fraction, PEF: Pertolium ether fraction, CHF: Chloroformic fraction

***Euphorbia splendida* extract and fractions**

Antio xidant assay

Extract & fractions [1]	DPPH(IC50) (µg/ml)[2]	Chelating activity (%)[3]	Ferric reducing power (nm)[4]
ME	145.47±0.007	79±3.1	0.544±0.004
PEF	482.99±0.009	61.2±3.96	0.491±0.008
CHF	287.14±0.10	68.5±4.65	0.519±0.012
EAF	134.28±0.005	68±6.21	0.518±0.01
AQF	129.02±0.006	75±3.85	0.514±0.01
BHT	78.23±0.1	……	……
EDTA	……	99.07 ± 0.49	……

[1]ME= Total methanolic extract, PEF=pertolium ether fraction, EAF= Ethyl acetate fraction, AQF= Aquoes fraction, BHT= Butylated hydroxy toluene, EDTA= Ethylene diamine tetra acetic acid.

[2]The concentration (µg/ml) of the plant extracts for inhibition of 50% DPPH free radicals (IC_{50}).

[3] The percentage of iron chelating activity of the plant extracts in the concentration of 1000 µg/ml.

[4] Increased control absorbance by the plant extracts (600 µg/ml / (increased control absorbance by BHT 1000)

µg/ml) × 100

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INTRODUCTION

It has been shown that oxidative stress is one of the major causative factors in the induction of many chronic and degenerative diseases including atherosclerosis, ischemic heart disease, aging, diabetes mellitus, cancer, neurodegenerative diseases, and others.[1] The harmful action of the free radicals which cause the oxidative stress can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism.[2] Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activity.[3,4]

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Genus *Euphorbia* (Euphorbiaceae) comprising about 2000 species and spreads in Pakistan, India, and Iran and over 82 species of *Euphorbia* have been found in Iran.[5] Previous studies on *Euphorbia* species or their active components showed different biological effects such as cytotoxic, antitumor, antioxidant, antibacterial,[6] anti-inflammatory, and antinociceptive activities.[7-9] Up to now, antioxidant activity of some species of *Euphorbia* such as *Euphorbia helioscopia* and *Euphorbia hirta* has been reported.[10,11]

Euphorbia splendida Mobayen is a plant distributed in the West of Iran with 30–50 cm height.[12] our previous studies on *E. splendida* showed the presence of diterpenoid, triterpenoid, and flavonoid in this plant.[13,14] According to our investigation, antioxidant activity of this plant has not been studied so far. Therefore, in this study, antioxidant activity of the methanol extract (ME) and subfractions of *E. splendida* has been evaluated with different methods.

MATERIALS AND METHODS

Plant material

Fresh aerial parts of *E. splendida* were collected in May 2014 from Arak, Markazi province, Iran. The specimen was identified by Dr. M. Noori (the Biology Department, Faculty of Science, Arak University) and was deposited under voucher number CMK10 in the herbarium of the biology department.

Extraction and isolation

The aerial parts of plant (600 g) were dried, ground, and extracted with methanol by maceration. The extraction was repeated for three times (3 days for each time) in 25°C. Different fractions of the extract were obtained by liquid–liquid fractionation using water, petroleum ether (PE), chloroform, and ethyl acetate (1500 cc of each solvent). The extract and fractions were concentrated by rotary evaporator (IKA, Model, RV 10 D) and finally dried and stored in a clean, dark container and cool place. The antioxidant activity of the extract and fractions was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, total reduction capability, and ferrous ions chelating assays.

Chemicals

DPPH, butylated hydroxytoluene (BHT), and gallic acid were purchased from Sigma-Aldrich USA. Folin–Ciocalteu was obtained from Merck (Darmstadt, Germany). Potassium ferricyanide, potassium acetate, phosphate buffer, ferrous ammonium sulfate, ascorbic acid, aluminum chloride ($AlCl_3$), thrichloroacetic acid (TCA), ammonium molybdate, tannic acid, quercetin, acetyl acetone, and ferric chloride ($FeCl_3$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rutin, gallic acid, TCA, potassium ferricyanide, ferrozine, and BHT were purchased from Sigma, and iron (II) chloride was purchased from Aldrich. Methanol, chloroform, ethyl acetate, and PE were purchased from Merk (Darmstadt, Germany).

Total flavonoid content

The total flavonoid content (TFC) of *E. splendida* ME and subfractions was determined using $AlCl_3$ reagent.[15] Briefly, 2.5 mL of each sample (and/or rutin as the standard), previously dissolved in 90% ethanol, was mixed with 2.5 mL of $AlCl_3$ 2% solution in 90% ethanol. After 40 min, the absorbance of the produced yellow solution was measured at 425 nm. The TFC of the samples was calculated on the basis of a linear calibration curve obtained using rutin.

Total phenolic content

The content of total phenolic compounds in plant extract and fractions was determined by Folin–Ciocalteu method.[16] One milliliter of plant extract (concentration of 1 mg/mL) and fractions was dissolved in methanol and mixed with 5 mL Folin–Ciocalteu reagent and 4 mL (7.5 g/100 mL) sodium carbonate. After 1 h at room temperature, the absorption of clear solutions was read at 765 nm. For the preparation of calibration curve, different concentrations of gallic acid solution were mixed with the same reagents as described above. The amount of total phenolics was expressed as gallic acid equivalent (GAE) in milligrams per gram dry plant extract.

1,1-Diphenyl-2-picrylhydrazyl free radical scavenging assay

The free radical scavenging activity of the ME and subfractions was evaluated using DPPH methods.[17] Briefly, 1 mL of the sample solution with different concentrations (ranging from 50 to 1000 μg/mL) was mixed with 3 mL of DPPH methanol solution. The reaction mixtures were incubated at room temperature and allowed to react for 30 min in the dark. After 30 min, the absorbance values were measured at 517 nm and converted into a percentage of antioxidant activity. BHT was used as a positive standard control. The percentage inhibition of DPPH (%) was calculated as follows:

$$\text{Inhibition of DPPH \%} = \frac{\text{ODc} - (\text{ODs} - \text{ODs})}{\text{ODc}} \times 100$$

ODc = Control solution absorbance.

OD_b = Blank solution absorbance.

OD_S = Sample solution absorbance.

The concentration of sample required to scavenge 50% of the DPPH free radicals (IC_{50}) was determined from the curve of percentage inhibitions plotted against the respective concentration.

Ferric reducing antioxidant power assay

The total antioxidant potential of the ME and subfractions of *E. splendida* was determined according to method of Oyaizu.[18] Aliquot (0.25 mL) of samples solution at different concentrations (ranging from 25 to 600 μg/mL) was mixed with 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) solution of potassium ferricyanide. Then, all the mixtures were incubated in a water bath at 50°C for 20 min. Then, 2.5 mL of 10% (w/v) TCA solution was added and the mixture was then centrifuged at 3000 rpm for 10 min. A volume of 2.5 mL of the supernatant was combined with 2.5 mL of distilled water and 0.5 mL of a 0.1% (w/v) solution of $FeCl_3$. The absorbance was measured at 700 nm with a spectrophotometer uv-vis (UNICO Model, UV/VIS 2100). BHT was used as positive control. All the tests were done in triplicate and results were reported as mean ± standard deviation.

Metal chelating activity

The chelating of ferrous ions by the ME and subfractions of *E. splendida* was estimated by the method of Dinis *et al*., 1994.[19]

Table 1: Total flavonoid content and total phenolic content of *Euphorbia splendida* extract and fractions (data are mean±standard deviation)

Extract and fractions	TPC (mg/g)	TFC (mg/g)
ME	270.74±0.005	208.23±0.007
PEF	101.43±0.007	93.84±0.006
CHF	235.63±0.006	172.75±0.005
EAF	208.54±0.010	65.80±0.006
AQF	173.88±0.005	85.28±0.010

ME: Total methanolic extract; PEF: Petroleum ether fraction; CHF: Chloroformic fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction; TPC: Total phenolic content as mg/g plant sample in gallic acid equivalent; TFC: Total flavonoid content as mg/g plant sample in rutin equivalent

Briefly, 1 mL of each test sample (1 mg/mL) was taken and added to 0.5 mL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine into the mixture, which was then left at room temperature for 10 min and then the absorbance of the mixture was read at 562 nm.

RESULTS

Total flavonoid content and total phenolic content of the extract and subfractions of *Euphorbia splendida*

The total phenolic content (TPC) of the ME and subfractions calculated from regression equation of calibration curve ([y = 5.0121x, R^2 = 0.99]) and expressed in GAEs, varied between 101.43 and 270.74 mg GAE/g plant sample [Table 1]. The content of total flavonoids in plant samples (mg/g) calculated from regression equation of calibration curve (y = 5.3267x, R^2 = 0.99) was expressed in rutin equivalents (REs) varied between 65.80 and 208.23 mg RE/g plant sample [Table 1]. Total methanolic extract showed the highest TFC and TPC values.

DPPH free radical scavenging assay

ME of the aerial parts of *E. splendida* and aqueous, ethyl acetate, chloroform, and PE subfractions were analyzed for DPPH radical scavenging activity to obtain their concentrations to scavenge 50% DPPH (IC_{50}) as shown in Table 2. The aqueous and then ethyl acetate subfractions of *E. splendida* were shown the best results in inhibition of DPPH radical in comparison to standard (BHT). PE subfraction was shown the least activity with IC_{50} = 482.99 ± 0.01 µg/mL.

Metal chelating activity

Metal chelating activity (%) of the plant ME, subfractions, and ethylenediaminetetraacetic acid (EDTA) in concentrations of 100, 200, 400, 600, and 1000 µg/mL are shown in Figure 1. The ME and subfractions of *E. splendida* showed a dose-dependent antioxidant activity in this method comparing to EDTA. In concentration of 1000 µg/mL, ME showed the strongest activity (79±3.07%) and petroleum ether fraction (PEF) showed the lowest activity (61.2±3.96%) [Table 2].

Ferric reducing antioxidant power assay

In ferric reducing power assay, all the samples increased the absorbance of the control solution (0.210 ± 0.098) in 700 nm. As shown in Figure 2, all the concentrations had lower absorbance and consequently lower reduction capability toward BHT.

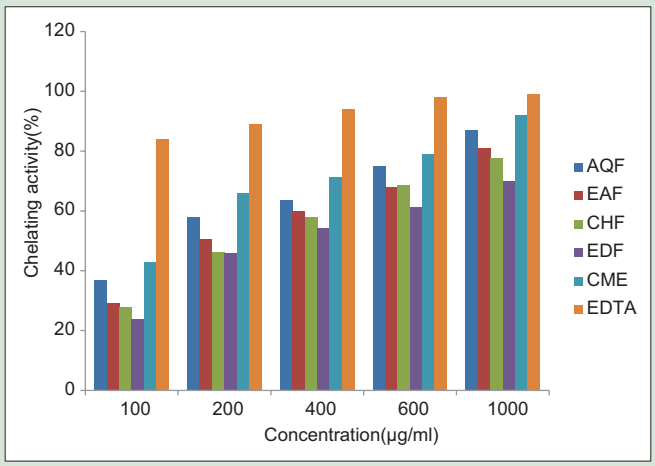

Figure 1: Iron chelating activity of the extract and subfractions of *Euphorbia splendida*. EDTA: Ethylenediaminetetraacetic acid; CME: Methanol extract; EDF: Petroleum ether fraction; CHF: Chloroform fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction

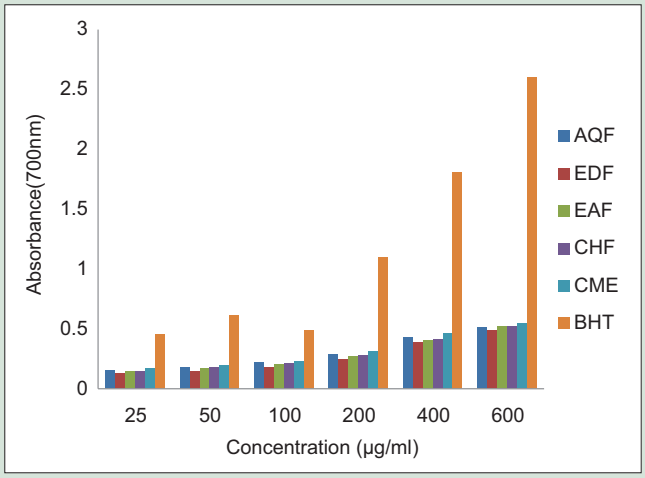

Figure 2: Ferric reducing power activity of the extract and subfractions of *Euphorbia splendida*. Standard; butylated hydroxytoluene. All the samples showed activity lower than butylated hydroxytoluene. Control absorbance: 0.210 ± 0.098. CME: Methanol extract; EDF: Petroleum ether fraction; CHF: Chloroform fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction

Table 2: Radical scavenging activity in the 2, 2′- diphenyl-1-picrylhydrazyl assay, metal chelating activity, and ferric reducing power activity of methanol extract and subfractions of *Euphorbia splendida* (mean±standard deviation)

Extract and fractions	DPPH (IC_{50}) (µg/ml)[a]	Chelating activity (%)[b]	Ferric reducing power (nm)[c]
ME	145.47±0.007	79±3.1	0.544±0.004
PEF	482.99±0.009	61.2±3.96	0.491±0.008
CHF	287.14±0.10	68.5±4.65	0.519±0.012
EAF	134.28±0.005	68±6.21	0.518±0.01
AQF	129.02±0.006	75±3.85	0.514±0.01
BHT	78.23±0.1	-	2.6±0.1
EDTA	-	99.07±0.49	-

[a]The concentration (µg/ml) of the plant extracts for inhibition of 50% DPPH free radicals (IC_{50}); [b]The percentage of iron chelating activity of the plant extracts in the concentration of 1000 µg/ml; [c]The absorbance of the plant samples (nm) in concentration of 600 µg/ml. ME: Total methanolic extract; PEF: Petroleum ether fraction; CHF: Chloroformic fraction; EAF: Ethyl acetate fraction; AQF: Aqueous fraction; BHT: Butylated hydroxytoluene; EDTA: Ethylenediaminetetraacetic acid; DPPH: 2, 2′- diphenyl-1-picrylhydrazyl

DISCUSSION

Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activity. Different species in genus *Euphorbia* showed antioxidant and free radical scavenging activity.[10,11] *E. splendida* is a plant which contains different chemical compounds,[13,14] and until now, it is not studied for antioxidant activity. Therefore, in the present study, antioxidant activity of the ME and subfractions of this plant was studied using DPPH free radical scavenging assay, metal chelating activity, and ferric reducing antioxidant power assay.

In DPPH method which is a good method to evaluate radical scavenging activity of the plants, the potency of *E. splendida* ME and subfractions was as below:

Aqueous fraction (AQF) > ethyl acetate fraction (EAF) > Total methanol extract (ME) > chloroformic fraction (CHF) > petroleum ether fraction (PEF).

The free radical scavenging activity was expressed as the effective concentration required for 50% of DPPH radical (DPPH) reduction (IC_{50}) obtained from a plot of graph of scavenging activity against the concentration of the extract and its fractions. The AQF showed the highest activity (IC_{50} = 129.02 ± 0.01 μg/mL) compared to other extract and fractions which was lower than IC_{50} of BHT (78.23 ± 0.1 μg/mL).

For the other tests, the order of potency of ME and subfractions was as below:

- Metal chelating activity: ME > AQF > CHF > EAF > PEF
- Total reduction capability: ME > CHF > EAF > AQF > PEF
- Phenolic contents: ME > CHF > EAF > AQF > PEF
- Flavonoid contents: ME > CHF > PEF > AQF > EAF.

The obtained results for total reduction capability are in agreement with the TPCs determined for extract and subfractions. There are acceptable correlation coefficients (R) between phenolic content and the data of DPPH scavenging activity, metal chelating activity, and total reduction capability of the plant extract and subfractions (R = −0.68, 0.73, and 0.95, respectively); such correlation with flavonoid content was R = −0.06, 0.51, and 0.69, respectively, which was not as significant as phenolic contents.

Different species of *Euphorbia* showed antioxidant activity in different antioxidant assays.[10,20] Comparing to *E. splendida*, some of *Euphorbia* species such as *E. hirta* showed lower IC_{50} value in DPPH scavenging assay which was comparable to standards such as BHT.[21] Hence, although *E. splendida* showed significant antioxidant activity in comparison to standards, especially in DPPH and metal chelating activity, this antioxidant activity may be lower than antioxidant activity of some *Euphorbia* species such as *E. hirta*. The extract and subfractions of this plant are found to have different levels of antioxidant activity and phenolic and flavonoid contents. There are some flavonoids such as quercetin and rutin which have been reported from *E. splendida*.[14] These flavonoids showed significant antioxidant activity in different assays.[22-24] Plant polyphenols act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups,[25] so these polyphenols may be responsible for the observed antioxidant activity. More studies are recommended to determine the active antioxidant compounds of this plant.

CONCLUSION

This study showed that the extract and subfractions of the aerial parts of E. splendida have antioxidant activity. The antioxidant activity of the extract and fractions might be attributed to the presence of phenolic compounds. More studies are needed to determine the active antioxidant compounds of this plant.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Souri E, Amin G, Farsam H, Jalalizadeh H, Barezi S. Screening of thirteen medicinal plant extracts for antioxidant activity. Iran J Pharm Res 2008;7:149-54.
2. Gerber M, Boutron-Ruault MC, Hercberg S, Riboli E, Scalbert A, Siess MH. Food and cancer: State of the art about the protective effect of fruits and vegetables. Bull Cancer 2002;89:293-312.
3. Cao GH, Sofic E, Prior RL. Antioxidant capacity of tea and vegetables. J Agric Food Chem 1996;44:3426-31.
4. Bergman M, Varshavsky L, Gottlieb HE, Grossman S. The antioxidant activity of aqueous spinach extract: Chemical identification of active fractions. Phytochemistry 2001;58:143-52.
5. Jassbi AR. Chemistry and biological activity of secondary metabolites in *Euphorbia* from Iran. Phytochemistry 2006;67:1977-84.
6. Singla A, Pathak k. Phytoconstituents of *Euphorbia* species. Fitoterapia 1990;41:483-516.
7. Lanhers MC, Fleurentin J, Dorfman P, Mortier F, Pelt JM. Analgesic, antipyretic and anti-inflammatory properties of *Euphorbia hirta*. Planta Med 1991;57:225-31.
8. Ahmad VU, Hussain H, Bukhari IA, Hussain J, Jassbi AR, Dar A. Antinociceptive diterpene from *Euphorbia decipiens*. Fitoterapia 2005;76:230-2.
9. Duarte N, Gyémánt N, Abreu PM, Molnár J, Ferreira MJ. New macrocyclic lathyrane diterpenes, from *Euphorbia lagascae*, as inhibitors of multidrug resistance of tumour cells. Planta Med 2006;72:162-8.
10. Basma AA, Zakaria Z, Latha LY, Sasidharan S. Antioxidant activity and phytochemical screening of the methanol extracts of *Euphorbia hirta* L. Asian Pac J Trop Med 2011;4:386-90.
11. Maoulainine L, Jellasi A, Hassen I, Ould Boukhari O. Antioxidant proprieties of methanolic and ethanolic extracts of *Euphorbia helioscopia*, (L.) aerial parts. Int Food Res J 2012;19:1125-30.
12. Ghahraman A. Color Flor of Iran. A Joint Project by the Research Institute of Forests and Rangelands Tehran: Ministry of Reconstruction, Jahad Research Institute of Forests and Rangeland, Tehran, Iran. 2005;7:797.
13. Ayatollahi S, Shojaii A, Kobarfard F, Nori M, Fathi M, Choudhari MI. Terpens from aerial parts of *Euphorbia splendida*. J Med Plants Res 2009;3:660-5.
14. Noori M, Chehreghanib A, Kaveh M. Flavonoids of 17 species of *Euphorbia* (*Euphorbiaceae*) in Iran. Toxicol Environ Chem 2009;91:631-41.
15. Nickavar B, Kamalinejad M, Haj-Yahya M, Shafaghi B. Comparison of the free radical scavenging activity of six Iranian *Achillea* species. Pharm Biol. 2006;44:208-12.
16. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Method Enzymol. 1999;299:152-78.
17. Kaji H, Inukai Y, Maiguma T, Ono H, Teshima D, Hiramoto K, *et al*. Radical scavenging activity of bisbenzylisoquinoline alkaloids and traditional prophylactics against chemotherapy-induced oral mucositis. J Clin Pharm Ther 2009;34:197-205.
18. Oyaizu M. Studies on products of browning reactions: Antioxidative-17 activities of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet 1986;44:307-15.
19. Dinis TC, Madeira VM, Almeida LM. Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 1994;315:161-9.
20. Ul-Haq I, Ullah N, Bibi G, Kanwal S, Sheeraz Ahmad M, Mirza B. Antioxidant and cytotoxic activities and phytochemical analysis of *Euphorbia wallichii* root extract and its fractions. Iran J Pharm Res 2012;11:241-9.
21. Kandalkar A, Patel A, Darade S, Baviskar D. Free radical scavenging activity of *Euphorbia hirta* Linn. leaves and isolation of active flavonoid myricitrin. Asian J Pharm Clin Res 2010;3:234-7.
22. Zhang M, Swarts SG, Yin L, Liu C, Tian Y, Cao Y, *et al*. Antioxidant properties of quercetin. Adv Exp Med Biol 2011;701:283-9.
23. Das M, Ray PK. Lipid antioxidant properties of quercetin *in vitro*. Biochem Int 1988;17:203-9.
24. Yang JX, Guo J, Yuan JF. *In vitro* antioxidant properties of rutin. Lwt Food Sci Technol 2008;41:1060-6.
25. Aberoumand A, Deokule S. Comparison of phenolic compounds of some edible plants of Iran and India. Pak J Nutr 2008;7:582-5.