INTERNATIONAL RESEARCH JOURNAL OF PHARMACY

www.irjponline.com ISSN 2230 – 8407

Research Article

ANTIOXIDANT, CYTOTOXIC PROPERTIES AND PHYTOCHEMICAL SCREENING OF TWO LEBANESE MEDICINAL PLANTS

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Article Received on: 14/03/13 Revised on: 09/04/13 Approved for publication: 17/05/13

DOI: 10.7897/2230-8407.04527

IRJP is an official publication of Moksha Publishing House. Website: www.mokshaph.com

ABSTRACT

Nowadays herbal medicine presents a significant adjuvant tool for hard treatment, especially in the case of cancer where modern medicine has access to traditional medicine to deprive the patient of the side effects of therapeutic approaches such as surgery and chemotherapy. Thus, Lebanese 10452 km^2 are so rich in medicinal plants such as *Eryngium creticum* L. and *Euphorbia macroclada* Boiss that are traditionally used in the treatment of various diseases (leukemia, asthma, skin diseases, antidote to snake venom, tumors, etc.). To fully realize the importance of these two Lebanese plants, we tried at first to study the phytochemistry of three extracts (aqueous, methanolic and ethyl acetate) from fresh leaves and stems of both plants. In a second step, the antioxidant capacity of these three extracts from the two parts of the fresh plants using spectrophotometric analysis has been evaluated and their cytotoxicity on the MCF7 breast cancer cell line by the XTT Cell viability technique has been studied. Our results showed that both leaves and stems of these two plants contain alkaloid, tannin, coumarin, saponin, flavonoid, polyphenol and reducing sugars in different concentrations. Moreover, both leaves and stems have exerted an antioxidant activity that may be due to their phenolic content and they have also inhibited the growth of cancer cell line from 68 % to 72 %. These results showed that both plants can be considered as a good source of natural products that can be used in the prevention of several diseases.

Keywords: *Eryngium creticum* L., *Euphorbia macroclada* Boiss, phytochemistry, antioxidant activity, cytotoxicity.

INTRODUCTION

For millennia, disease processes remained the major area of detailed understanding to humanity. Cancer, in all forms, thought to be a heterogeneous group of related disorders. This disease was often diagnosed in the past on the basis of macroscopic features such as mass, relentless growth and metastatic spread. Cancer is a growing public problem whose estimated worldwide new incidence is about 6 million cases per year. It is the second major cause of deaths after cardiovascular diseases. It is a disease characterized by unregulated proliferation of cells[1]. And carcinogenesis is a multistage process consisting of initiation, promotion and progression phases. Thus, the multistage sequence of events has many phases for prevention and intervention[2]. Based on the activities, three major types of chemopreventive agents from plant origin have been identified, namely inhibitors of carcinogen formation, blocking agents to prevent carcinogens from reaching or reacting to target sites and suppressing agents or anti-progression agents. In the other hand, nature has been a source of medical treatments for thousands of years and even today plants based systems continue to play an essential role in the primary health care of 80 % of the world's population. Therefore, plants continue to be a major source of medicines. Nowadays, drug discovery from plants involves multidisciplinary approach combining ethnobotanical, phytochemical and biological techniques to provide us new natural compounds for the development of drugs against various pharmacological targets, including cancer, diabetes and their secondary complications[3]. Medicinal plants are a source for a wide variety of natural products, such as phenolic acids and flavonoids which are very interesting for their antioxidant properties[4,5]. Epidemiological studies have shown that many of these antioxidants compounds possess anti-inflammatory, antitumor, antimutagenic, anticarcinogenic or antibacterial activities to a greater or lesser extent[6,7].

Euphorbia macroclada Boiss belongs to family Euphorbiacae. It is a large family of flowering plants including 300 genera and over 5000 species, widely present in various Lebanese regions. Species of Euphorbia has been used in the treatment of different diseases such as warts, asthma, leukemia and cancer. Also, this plant has been used as laxative and diuretic in different countries[8].

Eryngium creticum, perennial plants belongs to family Umbellifereae, is commonly known as Field Eryngo. It is found only in Lebanon, Palestine, Jordan and in Syria. It is cultivated for use as vegetable mainly in salad. *E. creticum* is traditionally used as diuretic, laxative. Submerged roots and seeds in water have been drunk to treat kidney stone, infections, skin diseases and tumors. It is an antidote used in the treatment of the snakebite. It also showed an anti-inflammation property and an anti-microbial activity[9]. It was also used in the treatment of liver diseases, poisoning, anemia and infertility[10]. *E. creticum* has showed an antioxidant property by inhibiting the lipid peroxidase in the liver of the rat[11]. In a recent study, the aqueous extract from both leaves and stems of *E. creticum* has exerted an antioxidant activity using the ABTS[12].

The purposes of this study were first, to determine the phytochemical screening of three extracts (aqueous, methanolic and ethyl acetate) from fresh leaves and stems of both *E. creticum* and *E. macroclada* grown in Lebanon and to evaluate their antioxidant power using two in vitro tests, the DPPH and iron chelating. Second, we aimed to study for the first time their cytotoxicity on the MCF7 breast cancer cell line by the XTT Cell Viability technique.

MATERIALS AND METHODS

Plant material and chemicals

Fresh plants were gathered from different regions in Lebanon on spring season between March and May in 2011 and the biological authentication was carried out by Professor George

Tohme, president of C.N.R.S. Lebanon. Stems and leaves of these plants were left on air at room temperature for two weeks to be very well dried. After that, they were crushed up and ground to get homogeneous fine powder by a grinder and then kept in a dark place at room temperature till their use in the different studies. All the chemicals used were of analytical grade. Methanol, sodium hydroxide, ethyl acetate, dichloromethane were purchased from BDH, England. Aluminum chloride, $FeSO_4.7H_2O$ and silica gel were purchased from Merck, Germany. Ferrozine and DPPH were purchased from sigma Aldrich, USA. Phosphate buffer solution (PBS) was purchased from Gibco, UK.

Preparation of crude extracts

10 grams of powdered leaves and stems of the two studied plants were putting into a flask with 500 mL of methanol, and the mixture has been extracted by agitation for 5 hours at 25 °C. Then, a maceration of the extracts was done overnight for 24 hours. After, the methanolic layer containing the extract was taken. The extraction was repeated on the remaining amount of the precipitate using 150 mL of methanol and all extracts were filtered by using a 0.45 Millipore filter paper. After that, the two fractions of extracts were mixed together and then concentrated using a rotary evaporator at 40 °C under reduced pressure. After that, the extracts were stored at -20 °C till their usage in different tests. The extracts resolved in ethanol and distilled water.

The aqueous extract has been prepared using the same steps of methanolic extraction except the temperature of extraction should be 60 °C.

Phytochemical screening

The different steps of the phytochemical screening have been made according to Muanda[13].

DPPH radical scavenging activity

The method of Farhan et al.[14] has been used for the scavenging ability of DPPH antioxidant test. 1 mL of different concentrations of diluted extracts of each plant parts in methanol was added to 1 mL of DPPH (0.15 mM in methanol) and at the same time, a control consisting of 1 mL DPPH with 1 mL methanol was prepared. The mixture was shaked very well by hand and then incubated in the dark at room temperature for 30 min and then the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The ascorbic acid was used as a positive control and the methanol was used as blank. The DPPH scavenging ability of plant extracts was calculated using the following equation:

$$\% \text{ Scavenging activity} = [(\text{Abs control} - \text{Abs sample})]/ (\text{Abs control})] \times 100$$

The Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample. Also, three controls have been prepared.

Chelating effects on ferrous ion

The method of Farhan et al. [14] has been used for the scavenging ability. 0.5 mL of various concentrations of all extracts of each plant was mixed with 0.5 mL of $FeSO_4$ (0.12 mM), and with 0.5 mL of ferrozine (0.6 mM). The mixture was allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured by Gene Quant 1300 UV- Vis spectrophotometer at 562 nm. Ultra-pure water of sample solution was used as a control without extracts. Ultra-pure water instead of ferrozine solution was used as a blank. EDTA-Na_2 was used as reference standard. All measurements were performed in triplicate. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the formula:

$$\text{Ferrous ion - chelating ability (\%)} = [(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100$$

Cytotoxicity

Cell Culture and treatment

MCF7 Breast cancer cells were counted using Trypan blue then plated in a 96 wells microplates with complete DMEM medium (DMEM, 10 % of fetal bovin serum and 1 % penicillin) (8000 cells/well in 100 µL of medium). After 24 hours of pre-incubation in a humidified air under 5 % CO_2 and at 37 ˚C, we added 20 µL of the plants extracts to the wells after dilution with the medium culture DMEM to five concentrations (0.5, 1, 1.5, 2 and 2.5 mg/mL). Every well is duplicated. The plates were incubated for further 48 hours in the same conditions.

Cytotoxicity assay

Cytotoxicity of our three extracts was estimated by the XTT cell viability assay where XTT, a yellow tetrazolium salt, is cleaved to a soluble orange formazan dye, which can be measured by absorbance at 490 nm with a reference wavelength of 630 nm in a microplate reader. Cleavage of the tetrazolium salt to formazan occurs via the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells.

After 48 hours of treatment 50 µL of XTT solution were added to each well. Then, the microplate was incubated in the same conditions for 2 hours and finally the absorbance was measured.

Statistical Analysis

All analyses were carried out in triplicate. The results of the scavenger activity were performed from the averages of all samples reading Mean ± SD used Excel 2003.

RESULTS AND DISCUSSION

Phytochemical screening

***Eryngium creticum* L.**

The obtained results of the phytochemical screening presented in Table 1 show that the two studied parts (stems and leaves) of *E. creticum* are rich in various secondary metabolites at different concentrations depending on the used solvent. Indeed, we note the presence of saponins and coumarins in the aqueous extract while the methanolic extract is richer in phenols, terpenoids, alkaloids and flavonoids. Moreover, the ethyl acetate extract is rich in flavonoids and alkaloids. Thus, there is a difference in the presence of metabolites between leaves and stems of this plant using the same solvent in favor of the leaves that contain more secondary metabolites than the stems, which gives them a greater bioavailability.

***Euphorbia macroclada* Boiss**

The phytochemical screening showed that *E. macroclada* contains saponin and tannin in the aqueous extract from leaves and stems while methanolic extracts and ethyl acetate once are the richest in alkaloids, flavonoids, terpenoids and phenols as showed in Table 2.

Table 1: Chemical Composition of Leaves and Stems of *E. creticum*

	Aqueous extract		Methanolic extract		Ethyl acetate extract	
	Stems	Leaves	Stems	Leaves	Stems	Leaves
Tanin	+	++	+	+	-	-
Resin	-	-	+	++	+	+
Coumarin	+	++	-	-	-	-
Saponin	++	++	-	-	-	-
Alkaloid	+	+	++	++	++	+++
Flavonoid	-	-	++	++	++	++
Phenol	+	++	++	+++	+	+++
Terpenoid	++	++	++	+++	+	+
Volatil oil	-	-	-	-	-	-

+++ = high amount after added of reagent immediately; ++ = moderate amount after 5 minutes of reagent added; + = low amount after 10 minutes of reagent added and - = absent of active compound after 20 minutes

Table 2: Chemical Composition of Leaves and Stems of *E. macroclada*

	Aqueous extract		Methanolic extract		Ethyl acetate extract	
	Stems	Leaves	Stems	Leaves	Stems	Leaves
Tanin	+	++	-	+	-	-
Resin	-	-	+++	++	+++	+++
Coumarin	-	-	-	-	-	-
Saponin	++	+	+	-	-	-
Alkaloid	++	++	++	++	+++	+++
Flavonoid	++	++	+	+	++	+++
Phenol	++	+	+	+++	+	+++
Terpenoid	++	++	+++	+++	++	++
Volatil oil	-	-	-	-	-	-

+++ = high amount after added of reagent immediately; ++ = moderate amount after 5 minutes of reagent added; + = low amount after 10 minutes of reagent added and - = absent of active compound after 20 minutes

Table 3: Anticancer Activity of Aqueous and Methanolic Extracts of *E. macroclada*

	Inhibition of cell growth %			
	Aqueous extract		Methanolic extract	
Concentrations mg/ml	**Leaves**	**Stems**	**Leaves**	**Stems**
0.5	82	78	80	52
1	83	78	83	57
1.5	83	86	84	59
2	84	86	85	66
2.5	84	86	86	67

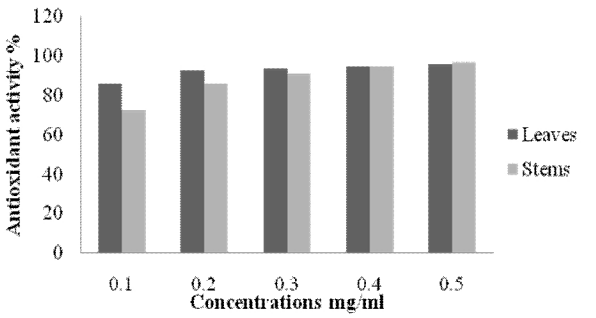

Figure 1: Antioxidant activity of methanolic extract of *E. creticum*

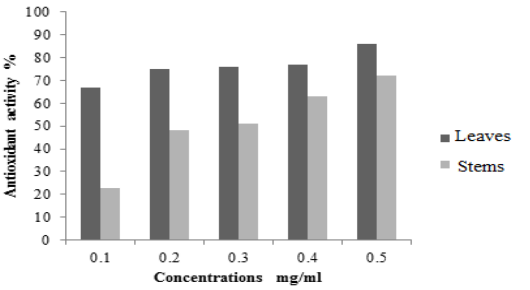

Figure 2: Antioxidant activity of aqueous extract of *E. creticum*

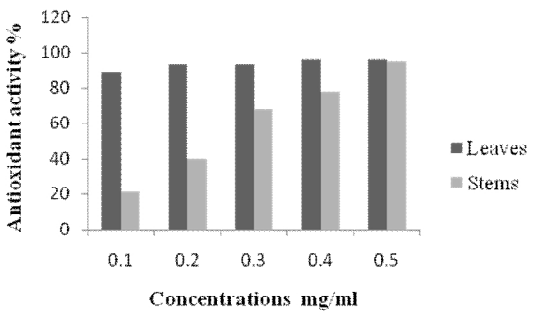

Figure 3: Antioxidant activity of methanolic extract of *E. macroclada*

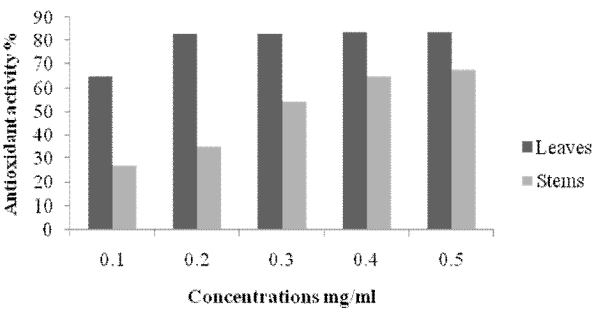

Figure 4: Antioxidant activity of aqueous extract of *E. macroclada*

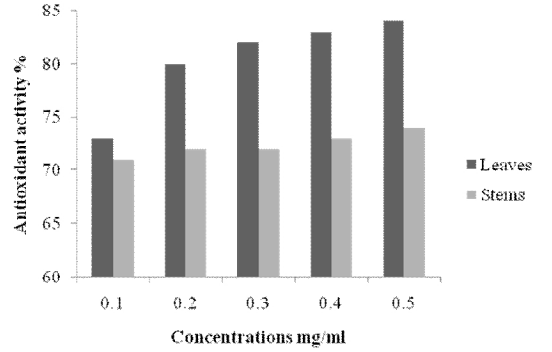

Figure 5: Antioxidant activity of methanolic extract of *E. creticum*

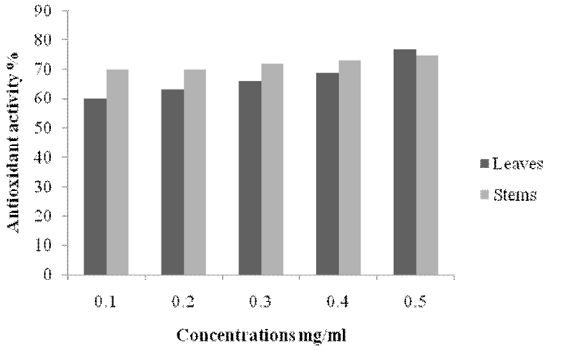

Figure 6: Antioxidant activity of aqueous extracts of *E. creticum*

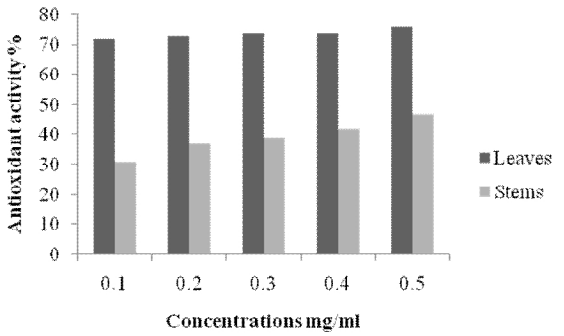

Figure 7: Antioxidant activity of methanolic extract of *E. macroclada*

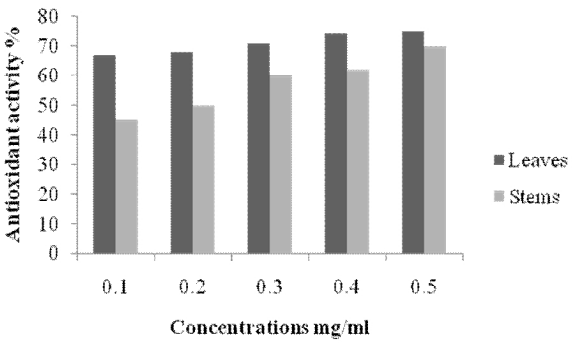

Figure 1: Antioxidant activity of aqueous extract of *E. macroclada*

Several epidemiological studies showed a positive correlation between the phytochemical composition of medicinal plants and their importance and their medical use[15]. However, phenolic compounds are related to the antioxidant activity and play an important role in stabilizing lipid peroxidation[15]. In addition, flavonoids may be responsible for many medical activities such anti-carcinogenic, allergenic, pest control etc.[16].

Antioxidant activity

DPPH radical scavenging activity

***Eryngium creticum* L.**

Figure 1 showed that methanolic extract from both leaves and stems of *E. creticum* has exerted an antioxidant power depending on the used concentration. In fact, 0.5 mg/mL of both parts of this plant had shown 95 % of scavenger activity. On the other hand, the aqueous extracts from both leaves and stems of this same plant have exerted higher antioxidant power depending on the used concentration. The maximal scavenger activity was at the concentration 0.5 mg/mL and it was at 90 % as shown in Figure 2.

***Euphorbia macroclada* Boiss**

Figure 3 and Figure 4 showed that aqueous and methanolic extracts from both leaves and stems of *E. macroclada* have exerted higher antioxidant activity depending on the used concentration. The percentage of scavenger activity of both parts varies between 20 % and 90 % for the doses 0.1 mg/mL and 0.5 mg/mL respectively. This antioxidant capacity was higher in the leaves than in stems due to the richness of this part in various active compounds mainly polyphenols as revealed by the phytochemical screening.

Thus, the obtained results demonstrate the antioxidant activity of the two Lebanese plants which are in agreement with the richness of the different studied extracts in phenolic compounds as obtained from the phytochemical screening. Indeed, recent studies have shown that these compounds possess antioxidant activity and may help in the prevention of certain diseases associated with oxidative stress such as cardiovascular diseases and diseases of aging by neutralizing free radicals in the body[17].

Ferrous ion chelating activity

To confirm the obtained results of antioxidant activity by DPPH method, we used the method Iron-Ferrosine.

Figure 5 shows that the antioxidant activity of leaves of *E. creticum* is higher than that of stems at all used concentration. The methanolic extracts from both parts showed a scavenger activity varying between 85 % and 73 % respectively in favor of leaves as they contain more phenolic compounds than stems.

On the other hand, aqueous extracts from both parts of *E. creticum* showed an antioxidant activity ranging between 60 % and 77 % for the different tested concentrations (Figure 6).

The evaluation of the antioxidant activity by DPPH method showed the importance of this activity especially for the methanolic extract from leaves of *E. macroclada.* This activity was higher at all concentrations mainly at 0.5 mg/mL that reaches a value of 75 % (Figure 7).

According to the results showed in the Figure 8, the aqueous extract from leaves and stems of *E. macroclada* has a remarkable antioxidant capacity in all concentrations reaching a value of 72 % and 70 % respectively at the concentration of 0.5 mg/mL.

The results of the evaluation of antioxidant activity by the method iron-ferrosine have confirmed the presence and the importance of the antioxidant properties of leaves and stems of both plants, especially the importance of this activity in leaves as in stems.

E. creticum and *E. macroclada* are traditionally used in the treatment of various diseases such leukemia, asthma, skin diseases, antidote to snake venom, tumors, etc.. Despite its remarkable array of medical applications, to our knowledge, no research has been carried out on the antioxidant and antiproliferative properties of aqueous, methanolic and ethyl acetate extracts from leaves and stems of these Lebanese plants. The phytochemical screening of two studied parts of these plants indicated that the main components of the three extracts were alkaloid, terpenoid, flavonoid and phenol.

Radical scavenging activity of three extracts of leaves and stems of the two plants was analyzed using two in vitro methods, DPPH and ferrous ion chelating. All of these extracts showed potent radical scavenging activity which is mainly due to the presence of high amounts of phenolic compounds that can play an important role in neutralizing free radicals. This antioxidant activity of the different extracts from the two studied parts was concentration dependent and it has reached the 90 % for the aqueous extracts from leaves of both plants.

Antiproliferative activity

The results of XTT technique showed that aqueous and ethyl acetate extracts of both leaves and stems of *E. creticum* didn't show any cytotoxicity on this cell line while the methanolic extract of both studied parts has inhibited the growth of MCF7 by 72 % and 68 % respectively. On the other hand, the obtained results showed that ethyl acetate extract from both leaves and stems of *E. macroclada* didn't inhibit the growth of MCF7 cells. Therefore, aqueous and methanolic extracts have presented cytotoxicity for this type of cell line as shown in Table 3.

The cytotoxicity of the genus Euphorbia has been the subject of several studies that attributed an anti-tumor activity to various species of this plant with the potential for cytotoxicity against different cell lines. Sadeghi et al.[18] showed that the methanolic extract of leaves of *E. macroclada* doesn't possess cytotoxicity on the cell line MDA-MB-468 while the ethyl acetate extract showed cytotoxicity on this cell line. In the same way, another study showed an inhibition by 66.3 % and 56.1 % of growth of cell lines K562 and U937 respectively after their treatment with methanolic extract prepared from the leaves of *E. herbecta*[19]. These results are consistent with our results showing the cytotoxicity of methanolic extract from leaves and stems on breast cancer cells MCF7. This difference in cytotoxicity of extracts of this plant against different cell lines may be due to the difference in the sensitivity of cancer cell lines.

CONCLUSION

The results of this study demonstrate the pharmacological importance of these two Lebanese plants highlighting the possibility of their medical use especially their antioxidant activity and their wealth in various secondary metabolites and thus the possibility of being used in the prevention of several diseases related to oxidative stress.

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Cite this article as:

Rammal Hassan, Farhan Hussein, Mohsen Hawraa, Hijazi Akram, Kobeissy Ahmad, Daher Ahmad, Badran Bassam. Antioxidant, cytotoxic properties and phytochemical screening of two Lebanese medicinal plants. Int. Res. J. Pharm. 2013; 4(5):132-136

Source of support: Nil, Conflict of interest: None Declared