Chemical composition, *in vitro* cytotoxicity and anti-free radical properties of six extracts from Lebanese *Trigonella berythea* Boiss.

Hussein Farhan[1], Hassan Rammal[1,2]*, Akram Hijazi[1,2], Houssein Annan[1], Ahmad Daher[1], Mohamad Reda[3] and Bassam Badran[1]

[1]Doctoral School of Science and Technology, Research Platform for Environmental Science (PRASE), Lebanese University, Lebanon
[2]Lebanese University, Faculty of Agricultural and Veterinary Sciences, Lebanon
[3]Lebanese University, Faculty of Sciences -5-, Lebanon

Abstract: This study represents an original work aimed to recognize the main constitution of *Trigonella berythea.* The total phenolic content and total flavonoid content of leaves and stems of *T. berythea* have been estimated. An extraction and purification of phenolic mixture compounds from the leaves and stems of this plant have been performed and their antioxidant potential using the DPPH, H_2O_2 and chelating of ferrous ions tests has been evaluated. Then, their cytotoxicity on the MCF7 breast cancer cell line by the XTT Cell Viability technique has been studied. Our results demonstrated that leaves of *T. berythea* had higher total phenolic content and total flavonoid content than stems. On the other hand, the six extract from leaves and stems of this plant demonstrated a high antioxidant potential reaches more than 80%. Also, extracts from leaves of *T. berythea* had the highest inhibitory effect on MCF7 and U937 cell growth than that of stems. This inhibition was more than 60% for all extracts. These results provide new insight into the health functions of leaves and stems and demonstrate that *T. berythea* extracts can potentially have health benefits.

Keywords: *Trigonella berythea*, total phenolic content, total flavonoid content, antioxidant activity, cytotoxicity.

INTRODUCTION

Many ancient nations have awoken to the importance of herbal medicine (Ashur, 1986). Many countries of the world have traditional medicines as a source of first aid treatment. The existence and use of plants to treat human diseases, is as old as man. Lebanon is rich in plant species especially with medicinal properties. In fact, 2607 wild species of which 92 are endemics can be found in only 10452 km^2.

Widespread in nature and found in most classes of natural compounds, phenols are important constituents of some medicinal plants and in food industry. They are utilized as coloring agents, flavoring, aromatizens and antioxidants. These natural compounds are found ubiquitously in fruits, nuts, seeds, flowers, vegetables, barks and herbs (Kyle and Duthie, 2006).

Trigonella berythea belongs to family Fabaceae. It is considered as indigenous species and in English it is known as Helbat Beyrouth. This plant is used in traditional medicine as compress by decoction in association with *Brassica niger, Ficus carica* and vinegar. Kaviarasan *et al*., (2004) have evaluated fenugreek seeds for their potential to protect erythrocytes from oxidation induced by hydrogen peroxide (H_2O_2). Recent study in Palestine demonstrated that seeds of fenugreek have been used to treat diabetes mellitus (Ali-Shtayeh *et al*., 2012).

This study aimed first to investigate the chemical composition in order to evaluate the total phenolic content (TPC) and total flavonoid content (TFC). On the other hand, *in vitro* cytotoxicity using XTT assay and the anti-free radical properties of six extracts from Lebanese *Trigonella berythea* Boiss using different techniques such as DPPH, H_2O_2 and Iron chelating tests have been evaluated.

MATERIALS AND METHODS

Fresh plant was gathered from south of 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 of Lebanon. Stems and leaves of *T. berythea* 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.

Chemicals

Absolute ethanol, methanol, n-hexane and sodium hydroxide n-butanol, petroleum ether, ammonium hydroxide, acetic acid and sodium chloride were purchased from BDH England. Aluminium chloride and $FeSO_4.7H_20$ were purchased from Merck, Germany. Sodium carbonate and hydrogen peroxide were purchased from Unichem, India. 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino carbonyl)-2H-tetrazolium hydroxide (XTT), ascorbic acid, gallic acid and rutin, Folin-Ciocalteau reagent, ferrozine and DPPH were

*Corresponding author: e-mail: hasanrammal@hotmail.com

purchased from sigma Aldrich, USA. Phosphate buffer solution (PBS) was purchased from Gibco, UK.

Sample preparation

10 grams of powdered leaves and stems of *T. berythea* were putted into a flask with 500/mL of ethanol, and the mixture was then extracted by agitation for five hours at 25°C. Then, a maceration of the extracts was done overnight for 24 h. After, the ethanolic layer containing the extract was taken. The extraction was repeated on the remaining amount of the precipitate using 150/mL of ethanol and all extracts were filtered by using a 0.45 millipore filter paper. After that, the two fractions of EtOH extract were mixed together and then concentrated using a rotary evaporator at 40°C. Then, the extracts were stored at -20°C till their usage in the different tests.

Aqueous extract has been prepared using the same steps of the ethanolic extraction except for the temperature which was at 60°C (Harborne, 1973).

At the end, four extracts were obtained from leaves and stems, two EtOH and two aqueous extracts.

Extraction of phenolic mixture

The phenolic extraction was done using the method of (Djeridane *et al*., 2006) with some modifications. 1/g of dried stems and leaves was crushed and transferred by grinder to powder. Maceration during 48/h with 50/ml of ethanol (70%) at room temperature has been done. Then, 2/h in shaker into a water bath has been accomplished. After, a filtration step by Büchner funnel under reduced pressure has been finished and the removal of ethanol was done under reduced pressure in a rotary evaporator at 40ºC. The remaining solution was defatted separately twice with petroleum ether and n-hexane in order to remove the lipids and protein three times for each solvent and the lower phase was taken and discarded of upper phase.

Then, the obtained solution was extracted with ethyl acetate by separating funnel 10 minutes in the presence of aqueous solution formed by ammonium sulphate (20%), and meta-phosphoric acid (2%) in same amounts and left for 15 minutes to de separated and constants. The upper phase ethyl acetate fraction was taken and dried by adding a sufficient amount of anhydrous sodium sulphate and then evaporated to dryness using a rotary evaporator. The dried precipitate was then dissolved in 5/ml of absolute MeOH and kept at-20˚C till its use in the different tests.

At the end of this procedure, two phenolic extracts were obtained from leaves and stems.

Determination of total phenolic content

The Folin-Ciocalteau reagent method has been used for the estimation of total phenolic extracts quantities according to (Lister and Wilson, 2004) with slight modification. Five concentrations of all extracts of the used plant have been prepared and then 100/µl have been taken from each concentration and mixed with 0.5/ml of Folin-Ciocalteau reagent (1/10 dilution) and 1.5/ml of Na_2CO_3 2% (w/v). The blend was incubated in the dark at room temperature for 15/min. The absorbance of blue-colored solution of all samples was measured at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of gallic acid equivalent (GAE) per g of dry weight of plant powders.

Determination of total flavonoid content

The aluminum chloride method was used according to (Quettier-deleu *et al*., 2000) for the determination of TFC of the six plant extracts. 1 ml of various concentrations of all extracts was mixed with 1/ml of 2% methanolic aluminum chloride solution. After an incubation period for 15/min at room temperature in the dark, the absorbance of all samples was determined at 430 nm using a spectrophotometer the results were expressed in mg per g of rutin equivalent (RE) and methanol was used as blank.

DPPH radical scavenging activity

The method of (Chew *et al*., 2009) has been used for the scavenging ability of DPPH antioxidant test with slight modification. 1 ml of different concentrations of diluted extracts of the plant parts in ethanol was added to 1ml of DPPH (0.15 mM in ethanol). At the same time, a control consisting on 1ml DPPH with 1 ml ethanol was prepared. The reaction mixtures were mixed very well by hand and then incubated in the dark at room temperature for 30 min. Then, the absorbance was measured at 517 nm. The ascorbic acid (AA) was used as a positive control and the ethanol was used as blank. The DPPH scavenging ability of plant extracts was calculated using the following equation:

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

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

H_2O_2 radical scavenging activity

The H_2O_2 scavenging of the six extracts of *T. berythea* was determined according to the method of (Ruch *et al*., 1989). A solution of H_2O_2 (40 mM) was prepared in PBS (pH 7.4) and concentrations were determined spectro photo metrically at 230 nm. Different concentrations of the six extracts from stems and leaves of in distilled water were added to a H_2O_2 solution (0.6/ml, 40/mM) and the absorbance of H_2O_2 was determined at 230 nm after 10 min against a blank solution containing the plant extracts without H_2O_2. Ascorbic acid was used as standard reference.

The percentage scavenging of H_2O_2 was calculated using the following equation:

$$\%\ \text{Scavenging}\ (H_2O_2) = \frac{(\text{Abs control - Abs sample})}{(\text{Abs control})} \times 100$$

Chelating effects on ferrous ions

The method of (Dinis *et al*., 1994) has been used to estimate the chelating effect on ferrous ions with some modifications. 0.5/ml of various concentrations of all extracts 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 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 was used as reference standard. All measurements were performed in Triplicate. The ferrozine solution (3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4´-disulfonic acid Na-salt) (0.6/mM) was prepared in ultra-pure water and stored in the dark at room temperature. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and Ferrozine only) using the formula:

$$\text{Iron - chelating ability}\ (\%) = \frac{(\text{Abs control - Abs sample})}{(\text{Abs control})} \times 100$$

Cell culture

MCF7, adherent cells and U937, suspension cells, were both cultured in DMEM and RPMI 1640 medium respectively supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 2% penicillin-streptomycin solution at 37^{o}C in a humidified atmosphere containing 5% CO_2. The cell numbers were counted by a hematocytometer, and the viability was always greater than 95% in all experiments as assayed by the 0.025% trypan blue exclusion method. To ensure an exponential growth, cells were resuspended in fresh medium 24 h before each treatment.

The cytotoxicity assay (XTT assay)

The cytotoxicity of the six extracts was tested by the method of XTT–formazan dye formation by (Weislow *et al*., 1989) at various concentrations and with some modifications. Cultured MCF7 and U937 cells were seeded (25,000 cells/well) in a 96-well plate, then cells in each well were treated along with different extract dosages (0.1, 0.3, 0.5.0.7 and 1 mg/ml). MCF7 and U937 cells were used alone without extracts treatment as control. After treatment, the 96-well plate was incubated for 24/h maintaining the same conditions. After the incubation period, 25/µl of XTT reagent (0.6 mg/ml) containing 25/µM PMS has been added. The plate was further incubated for 2/h at the same conditions. Absorbance was measured at 490 nm with reference filter using in an ELX800 Microplate Reader. The percentage of cytotoxicity was calculated by the following formula:

$$\%\ \text{Cytotoxicity} = \frac{(\text{Abs C - Abs T})}{\text{Abs C}} \times 100$$

Where Abs C is the mean absorbance of the control wells and Abs T is the mean absorbance of tested wells with a particular extract dosage.

STATISTICAL ANALYSIS

All analyses were carried out in triplicates except for anticancer and total protein they were in duplicates. The results of scavenger activity, total phenolic and total flavonoid contents were performed from the averages of all samples reading mean ± SD using Excel 2003 and other analytical methods. Also the results of anticancer and antioxidants have been done by Spss version 16 using Student's t-test ($P<0.05$).

RESULTS

The total phenolic, total flavonoids contents

The TPC and TFC in leaves and stems of *T. berythea* have been evaluated. As shown in table 1, all extracts of both leaves and stems contain high amounts of TPC and TFC. This amount has increased with the concentration of the extracts and it was higher in leaves than in stems.

Antioxidant activity

Our obtained results showed that both leaves and stems of *T. berythea* have exerted high antioxidant activity at different concentrations. The DPPH test demonstrated that 0.4 mg/ml of the phenolic mixture (PM) extracts from leaves of *T. berythea* had highest antioxidant activity that reaches the 96 % than that of stems which was at 76 % as shown in fig. 1.

Figs. 2 and 3 show that both ethanolic and aqueous

Table 1: Total phenolic and total flavonoids contents in the leaves and stems of *T. berythea*

Extracts	Leaves			Stems		
	PM	Aqueous	EtOH	PM	Aqueous	EtOH
TPC	27.1±0.04	2±0.07	2.24±0.03	14.4±0.05	1.47±0.03	1.61±0.04
TFC	15.4±0.01	0.8±0.06	1.07±0.03	9.42±0.02	0.91±0.02	0.98±0.04

PM= Phenolic Mixture. Values are mean ± SD of triplicate analyses and are expressed as mg of GAE/g of dry weight. Values are calculated using Excel 2003.

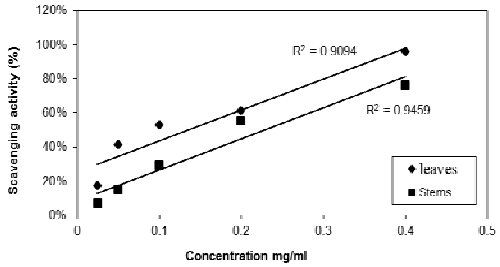

Fig. 1: DPPH scavenging activity of PM extracts from leaves and stems. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

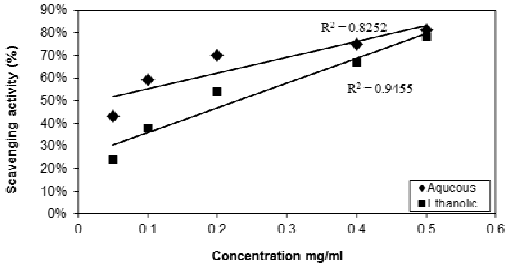

Fig. 2: DPPH scavenging activity of ethanolic and aqueous extracts from leaves. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

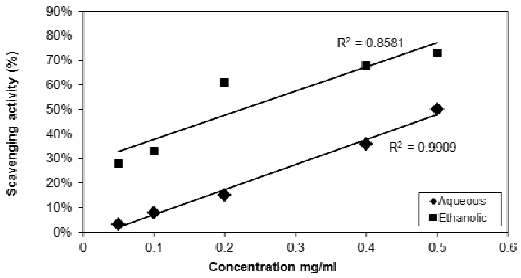

Fig. 3: DPPH scavenging activity of ethanolic and aqueous extracts from stems. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

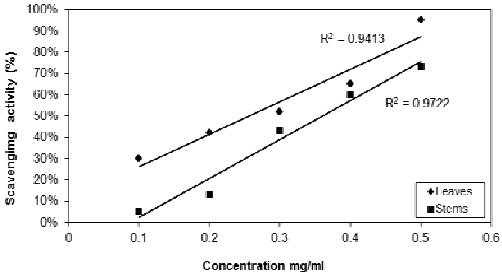

Fig. 4: H_2O_2 scavenging activity of PM extracts from leaves and stems. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

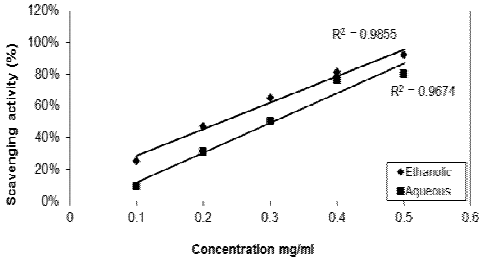

Fig. 5: H_2O_2 scavenging activity of ethanolic and aqueous extracts from leaves. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

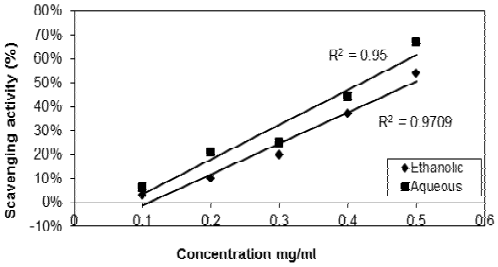

Fig. 6: H_2O_2 scavenging activity of ethanolic and aqueous extracts from stems. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

extracts from leaves and stems have exerted high scavenging activity. The % of this scavenging activity of leaves was higher than that of stems. This % has reached 81% for the aqueous extract from leaves and 73% for that of stems.

H_2O_2 is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell (Gulcin *et al*., 2003). In order to confirm the obtained antioxidant power of the studied plant, the H_2O_2 test has been used. Fig. 4 shows that PM extracts from *T. berythea* possess high antioxidant activity reaching the 95% and 73% for both leaves and stems respectively.

The aqueous and EtOH crude extracts from both leaves and stems were capable of H_2O_2 radical scavenging activity in a concentration dependent manner. The % of scavenging activity of 0.5 mg/ml of leaves of *T. berythea* was highest than that of stems as shown in fig. 5 and fig. 6. This percentage was 92% for the EtOH extract and 80% for the aqueous extract. Therefore, it was 67 % for the aqueous extract and 54% for the EtOH extract from the stems of *T. berythea*.

Table 2: IC_{50} values of stems and leaves of the plant for the DPPH, H_2O_2 and Iron chelating test.

	Extracts	IC_{50}		
		DPPH	H_2o_2	Fe
	Ascorbic acid	205 µg/ml	10 µg/ml	-
	EDTA	-	-	5µg/ml
Leaves	PM	0.10 mg/ml	0.23 mg/ml	0.17 mg/ml
	Aqueous	0.22 mg/ml	0.36 mg/ml	0.4 mg/ml
	Etoh	0.20 mg/ml	0.23 mg/ml	0.28 mg/ml
Stems	PM	0.09 mg/ml	0.30 mg/ml	0.18 mg/ml
	Aqueous	0.50 mg/ml	0.42 mg/ml	0.41 mg/ml
	EtOh	0.19 mg/ml	0.49 mg/ml	0.50 mg/ml

Values are mean of triplicate analyses and are calculated using Excel 2003.

The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion. PM extracts from leaves and stems of *T. berythea* induced 87% and 72% of iron chelating scavenging activity for both leaves and stems respectively as shown in fig. 7.

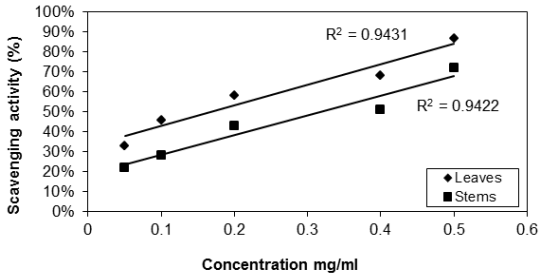

Fig. 7: Iron chelating scavenging activity of PM extracts from leaves and stems. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

Both aqueous and ethanolic extracts from leaves and stems of the studied plant exerted high iron chelating scavenging activity which has reached the 90% as shown in fig. 8 and fig. 9.

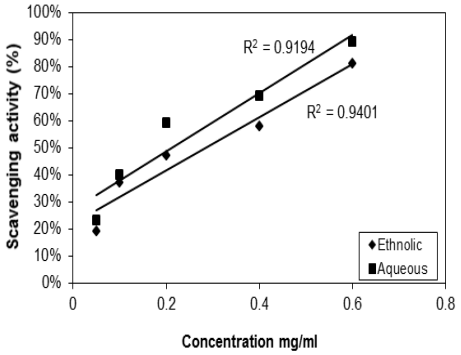

Fig. 8: Iron chelating scavenging activity of ethanolic and aqueous extracts from leaves. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using SPSS version 16.

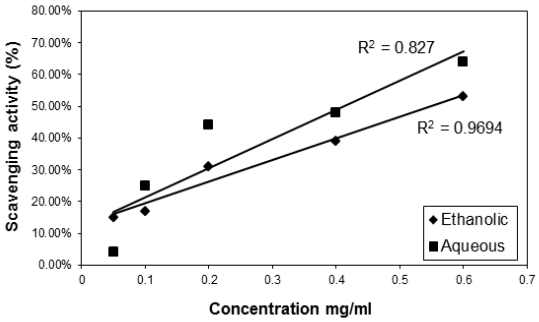

Fig. 9: Iron chelating scavenging activity of ethanolic and aqueous extracts from stems. Values are mean ± SD of triplicate analyses (Student's t-test) and are calculated using Spss version 16.

In order to determine the concentration of the extracts at which 50% of the initial DPPH, H_2O_2 and Iron were decreased, the IC_{50} for each extract from both leaves and stems of *T. berythea* was studied. Our results showed that the IC_{50} of DPPH, H_2O_2 and Iron was different between leaves and stems and between extracts from the two parts as shown in table 2.

All of these data showed that both parts of *T. berythea* have generally high antioxidant capacity.

Effect of Trigonella berythea extracts on cancer cell proliferation

The antiproliferative activity of each part of *T. berythea* on MCF7 and U937 cells was examined by XTT assay. After 2h of treatment by different concentrations of leaves and stems, EtOH extracts has significantly inhibited cell growth MCF7. The percentage of inhibition was higher in stems than in leaves and it has reached the 77% (table 3). Therefore, the aqueous extract from both parts of this plant has induced a non-significant inhibition of MCF7 as shown in table 3. On the other hand, the PM from leaves and stems has significantly inhibited the cell growth U937 by 58% and 46% respectively (fig. 10).

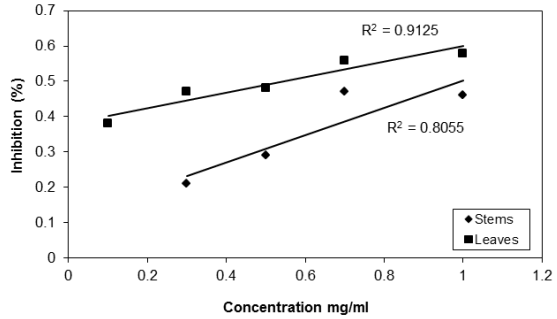

Fig. 10: Inhibition of U937 cell proliferation by the treatments of different concentrations of PM from leaves and stems of *T. berythea* for 2 h. Values are mean ± SD of duplicate analyses (Student's t-test) and are calculated using SPSS version 16.

Table 3: Inhibition of MCF7 cell proliferation by the treatments of extracts from of *T. berythea* for 2 h. Values are mean of duplicate analyses and are calculated using the mentioned formula.

Concentrations mg/ml	lnhibition of cell growth (%)			
	Aqueous extract		EtOH extract	
	Leaves	Stems	Leaves	Stems
0.1	17	0	20	15
0.3	12	0	5	30
0.5	24	0	28	77
0.7	32	0	48	75
1	33	21	62	77

DISCUSSION

Many reports indicated that flavonoids and polyphenols possess antioxidant and free radical scavenging activity (Farhan *et al*., 2012 a,b,c). These phytoconstituents may exert multiple biological effects against different pathologies due to their free radical scavenging activities.

Free radicals are known to be major factors in triggering biological damage such as DNA, RNA, protein, or lipid oxidation. Most antioxidants that exist in plants have radical-scavenging capacity. The health beneficial effects of polyphenols could result from either their antioxidant functions and/or independently from these properties e.g. by acting as modulators of cellular signaling processes (Rammal *et al*., 2012). Our obtained results are in agreement with those obtained in recent studies showed that Lebanese plants contain higher amounts of different phytochemical products mainly phenol, flavonoid and their antioxidant capacities (Farhan *et al*., 2012b, c).

Three in vitro tests have been used in order to evaluate the antioxidant power of this plant, DPPH, H_2O_2 and Iron chelating test. Scavenging of H_2O_2 by the plant extracts may be attributed to their phenolics, which donate electron to H_2O_2, thus reducing it to water.

On the other hand, the antiproliferative activity of the plant has been estimated. Thereby, the mechanisms of action of the antiproliferative activity of phenolics extracts are not yet clearly determined. Fan *et al*., (2011) have shown that phenolics extracts possess an antiproliferative activity. Possible mechanisms include interference with the metabolite activation of promutagen, as blocking agents and formation of adducts with ultimate mutagens, scavenging of free radicals and suppression of tumor cell invasiveness via the inhibition of matrix metal-oproteinase-2/-9 activity (Luo *et al*., 2011). In our study, we can estimate that the positive antioxidants effects of leaves and stems of *T. berythea* could contribute toward the antiproliferative activities of MCF7 and U937 cells.

CONCLUSION

In summary, the leaves and stems of *T. berythea* extracts were rich in phenolics, flavonoids. Both ethanol and aqueous extracts of *T. berythea* showed potent radical scavenging activities in DPPH, H_2O_2 and Iron chelating. Also, *T. berythea* has antiproliferative activities in MCF7 and U937 cells. This inhibition of cancer cell proliferation is at least partly due to the antioxidant activities of the extracts. Thus, *T. berythea* may be a potential source of natural composites.

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