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Research Article

STUDYING THE CYTOTOXIC AND ANTIOXIDANT ACTIVITIES OF CRUDE EXTRACTS FROM *ANACYCLUS NIGELLIFOLIUS* BOISS GROWN IN NORTH LEBANON

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ABSTRACT

Objective: The objective of this study is to evaluate the chemical contents (total phenolic and total flavonoid contents) of the crude ethanolic and aqueous extracts from leaves and stems of a Lebanese plant and to evaluate their cytotoxic and antioxidant activities.

Methods: Quantification of the total phenolic and total flavonoid contents of all extracts of *Anacyclus nigellifolius* from leaves and stems and the cytotoxic activity were evaluated through XTT assay, while antioxidant activities were evaluated using spectrophotometric analyses DPPH, H_2O_2, and chelating of ferrous ions tests.

Results: The ethanolic extract from leaves and stem of *A. nigellifolius* was better than aqueous extract and showed a higher content in total phenolic and total flavonoid which was 39.48±0.04 and 13±0.03, respectively. On the other hand, the best value of IC50 of DPPH, H_2O_2, and chelating of ferrous ions of ethanol extract from leaves was 0.1 mg/mL, 0.25 mg/mL, and 0.37 mg/mL, respectively, also, ethanol extract has significantly inhibited cells growth 293T and MCF7, a percentage of inhibition was in leaves, and it has reached the 70% and 54%, respectively.

Conclusions: Both ethanolic and aqueous extracts of both parts of *A. nigellifolius* have shown high efficiency and good antioxidant and antiproliferation power. Hence, this plant can be used in the prevention of a number of diseases related to oxidative stress as well as might be used for inhibition of cancer cell line growth.

Keywords: Total phenolic, Total flavonoid, Antioxidant, Cytotoxic potentially.

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INTRODUCTION

Oxidative stress is involved in a lot of an acute and chronic disease including cancer, neurodegenerative, and cardiovascular diseases. The balance between antioxidation and oxidation is believed to be critical in maintaining a healthy biological system [1,2]. The wild diversity in nature gives us a variety of useful sources of remedies to cure a number of diseases. Therefore, natural products have played a major role in drug discovery and development, especially agents active against cancer and infectious diseases [3].

Anacyclus nigellifolius Boiss. is an endemic plant to Lebanon their Arabic name: سطيحنق and English name: Nigella-leaved anacylus. It is perennial, procumbent herb, found in various region in Lebanon, especially in Arz tannourine, their Botanical descriptions scattered flat hairy plant 20–40 cm; divided, oblong, ovate leaves; white ligules are shorter than disk; outer achenes with large aricles and it belong to *Asteracea*e or *Composita*e family [4].

Anacyclus pyrethrum is considered a famous herb in *Anacyclus* genus many of article have been found their root that owned several activities in folk medicine in different countries is used in the treatment of various neuralgic and rheumatic affection of the head, face and the tooth in Algeria. It is used as a stimulant and tonic to the nervous system, and also, used in respiratory disease. The local ethnobotanical study carried out showed its possible antidiabetic activity [5,6]. Furthermore, a recent study shown that A. *pyrethrum* methanolic and aqueous extracts have a strong antibacterial activity against a wide range of bacteria species [7].

METHODS

The plant classification of *A. nigellifolius* Boiss. has been done by Professor George Tohme, president of C.N.R.S of Lebanon, this plant is considering as endemic in Lebanon, and it has been collected from west of Lebanon in 2011.

Preparation of crude extracts

10 g of milled stems and leaves of *A. nigellifolius* have been soaked by put inside a flask with half of liter of ethanol, afterward the blend being extracted by mixing for 5 h at 25°C. Then, the extracts have been left during overnight for 24 h, and later, the ethanolic part was taken by used the syringe. The extraction of residue part has been done through using 150 mL of ethanol, and the entirely extracts were filtered using filter paper sized 0.45 Millipore. Next step, the both parts of extracts were mixed together and then concentrated using a rotary evaporator at 40°C under reduced pressure, and finally, the extracts has been stored at −20°C until they are used in various trails, the extracts, and then are resolved in ethanol and distilled water.

The aqueous extract has been prepared using the same steps of the ethanolic extraction except for the temperature of the extraction should be 60°C [8].

Quantifications of chemical contents

Total phenolic content (TPC)

The Folin–Ciocalteau reagent method has been used for the estimation of total phenolic extracts quantities according to the study of Lister and Wilson [9] with slight modification. Five concentrations of all extracts of the used plants 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 mix kept in dark room for around quarter hour, and all blue-colored solution of samples absorbance has been measured at 765 nm. The results expressed in mg of gallic acid equivalent gram of dry weight of plant.

Total flavonoids content (TFC)

The approach was used according to the study of Quettier-Deleu *et al*. [10]. 1 mL of several concentrations of all crude extracts has been mixed with 1 mL of 2% methanolic aluminum chloride solution. Thereafter, left an incubation time around 15 min at dark room, and subsequently, absorbance of all samples has been taken at 430 nm. Methanol was used as blank, and the results were expressed in mg of rutin equivalent gram of dry weight of plant.

Evaluation of the antioxidant and cytotoxic activities

DPPH radical scavenging activity

The method of Chew *et al*. [11] has been used for the scavenging ability of DPPH antioxidant test with slight modification, this approach has been done through preparation of 1 mL of various concentrations of diluted extracts and added to 1 mL of DPPH, and also, 1 mL of DPPH with 1 mL of ethanol has been prepared as a control. All solutions were mixed by hand afterward left in the dark for half hour, and finally, the absorbance was taken at 517 nm. Ascorbic acid has been used as a positive control while ethanol used as a blank. DPPH scavenging ability of plant extracts was calculated using the following equation:

% Scavenging activity = [(Abs control − Abs sample)] / (Abs control)] ×100

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

Scavenging activity of hydrogen peroxide (H_2O_2) radical

The H_2O_2 scavenging of the extracts of leaves and stems of the different plants was determined according to the method of Ruch *et al*. [12]. A solution of H_2O_2 (40 mM) was prepared in PBS (pH 7.4), and concentration was determined spectrophotometrically (Gene Quant 1300 ultraviolet (UV)-visible) at 230 nm. Different concentrations of extracts from stems and leaves of these plants in distilled water were added to a H_2O_2 solution (0.6 mL, 40 mM), and the absorbance of H_2O_2 at 230 nm was determined after 10 min, a blank solution containing phosphate buffer without H_2O_2. Ascorbic acid was used as standard reference.

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

% Scavenged [H_2O_2] = [(Abs control – Abs sample) / Abs control] × 100

Chelating effects on ferrous ions

This method has been used to estimate the chelating effect on ferrous ions with some modifications [13]. 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 mixtures were allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured by Gene Quant 1300 UV-visible spectrophotometrically at 562 nm. Ultrapure water of sample solution was used as a control without extracts, and ultrapure water was used as a blank. EDTA-Na_2 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 ultrapure 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), and we were using the formula:

Ferrous ion - chelating ability (%) = [(Abs control − Abs sample) / Abs control] ×100

Cell culture preparation

MCF7 and 293T semi-adherent were cultured in DME. Medium DMEM was supplemented with 10% (v/v) fetal bovine serum and 2% penicillin-streptomycin solution. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cell numbers were counted by a hematocytometer, and the viability was always >95% in all experiments as assayed by the 0.4% trypan blue exclusion method. To ensure an exponential growth, cells were resuspended in fresh medium 24 h before each treatment. Furthermore, we used cell line MCF 10 as normal control, this cell line is cultured in DMEM F12 Ham supplemented with 10% (v/v) fetal bovine serum and 2% penicillin-streptomycin solution, and in addition, the medium requires 50 µl hydrocortisone, 25 µl insulin, and 5 µl cholera toxin.

XTT assay

The cytotoxicity of the six extracts was tested by the method of XTT-formazan dye formation by the method Weislow *et al*. [14] at various concentrations and with some modifications. Cultured MCF7, 293T, and MCF10 cells were seeded (25,000 cells/well) in a 96-well plate, and 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, 293T, and MCF10 cells were used alone without extracts treatment as a 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:

% Cytotoxicity = [(Abs C− Abs T) / Abs C] ×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. The result of scavenger activity, total phenolic compound, and total flavonoid compound was performed from the averages of all samples reading mean ± SD used Excel 2003 and other analytical methods. Furthermore, the results of anticancer and antioxidants have been done by SPSS version 16 using Student's t-test ($p<0.05$).

RESULTS AND DISCUSSION

Total phenolic and flavonoids content of *A. nigllifolius* have been estimated as shown in Table 1. All extracts of both leaves and stems contain high amounts of TPC and TFC.

Values are the average of triplicate experiments and values are expressed as mg ± SD

Table 1: Total phenolic and flavonoids content of A. *nigllifolius*

Estimation Test	Leaves		Stems	
	EtOH	Aqueous	Aqueous	EtOH
TPC	39.48±0.04	36.9±0.045	27.6±0.05	33.52±0.042
TFC	13±0.03	12.35±0.057	7.1±0.05	8.9±0.054

TPC: Total phenolic content, TFC: Total flavonoids content, *A. nigllifolius: Anacyclus nigllifolius*, GAE: Gallic acid equivalent

The obtained results showed that the contents in leaves were higher than in stems for both extracts and can be confirmed the result was estimated by the method Hijazi *et al*. [15] showed that the TPC and TFC of the ethanolic extract of *A. nigellifolius* were 86.7 mg/mL TPC and 50.2 µg/mL of TFC, respectively, as well as, the result was estimated by Sujith *et al*. [16] showed that the TPC and TFC of EtOH extracts of *A. pyrethrum* were 159.63 mg/g of TPC and 29.65 mg/g of TFC. Those results showed that the Anacyclus may have high contents of phenol and flavonoid contents in their parts.

Antioxidant activity

The obtained results showed that both EtOH and aqueous extracts from leaves of this plant have exerted high antioxidant activity through the three used *in vitro* assays. This activity was more than 80% for the DPPH and more than 70% for the H_2O_2 and iron chelating tests as shown in Figs. 1-3.

Figs. 4-6 showed that both EtOH and aqueous extracts from stems of the studied plant have exerted high antioxidant activity through the three used tests. This activity was higher for the EtOH extracts for DPPH and H_2O_2 than in aqueous once for iron chelating, and it has reached around 70% for the three tests of EtOH extracts.

The IC_{50} for each extract from both leaves and stems of *A. nigllifolius* was evaluated. Our results demonstrated that the IC_{50} of DPPH, H_2O_2, and iron chelating was different between leaves and stems and between extracts from the two parts as shown in Table 2.

This study showed the best value of IC50 of DPPH, H_2O_2, and chelating of ferrous ions of ethanol extract from leaves was 0.1 mg/mL, 0.25 mg/mL, and 0.37 mg/mL, respectively, that confirm the ethanolic extract result done by Hijazi *et al*. [15] which have been demonstrated that DPPH test presented a strong scavenging activity with an IC50 was 0.178 mg/mL.

There is closely correlation between the amounts of TPC and TFC and the scavenger activity of extracts. When the quantities of phenol are higher, the antioxidant activity of extracts is higher. The previous studies found that there was a direct relationship between antioxidant activity and TPC in selected herbs, vegetables, and fruits. Phenolic compounds had a major contribution to antioxidant activity [17-19], and there are several studies have been indicated that flavonoids and polyphenols possess antioxidant and free radical scavenging activity from various plants has been grown in Lebanon [20-22].

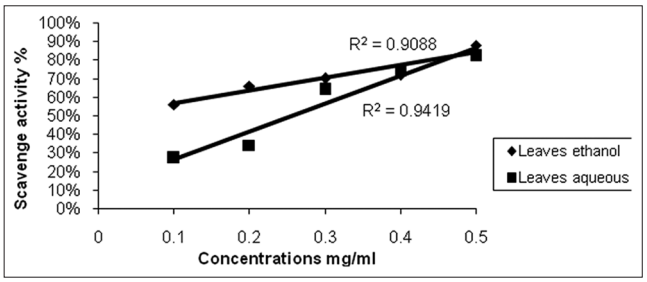

Fig. 1: The scavenging ability of EtOH and aqueous extracts of leaves on DPPH

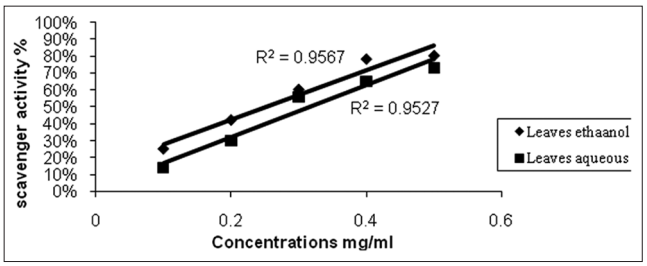

Fig. 2: The scavenging ability of EtOH and aqueous extract of leaves on H_2O_2

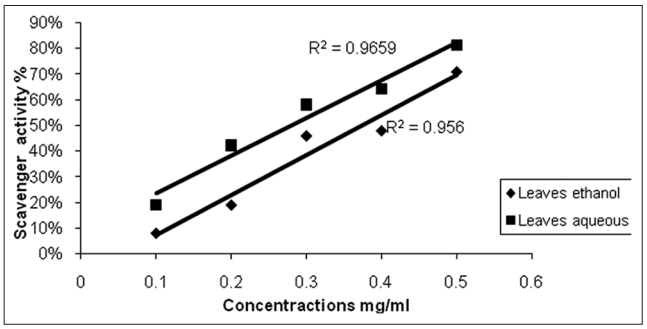

Fig. 3: The scavenging ability of EtOH and aqueous extract of leaves on iron chelating

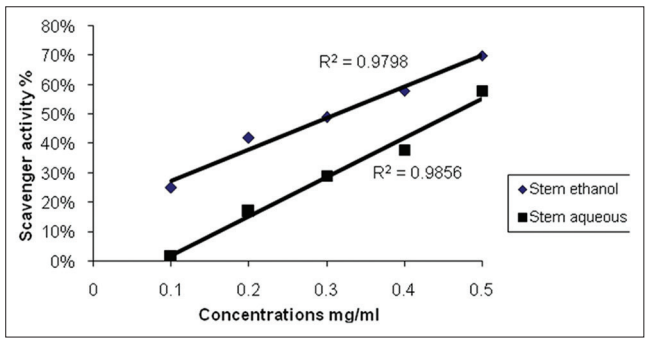

Fig. 4: The scavenging ability of EtOH and aqueous extracts of stem on DPPH

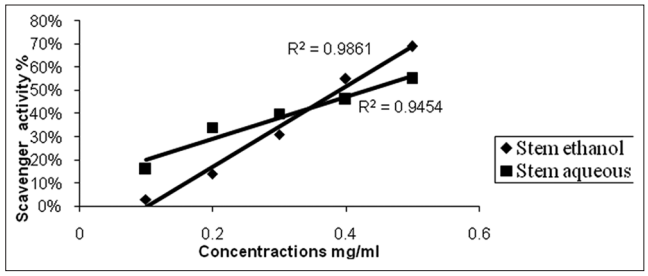

Fig. 5: The scavenging ability of EtOH and aqueous extracts of stems on H_2O_2

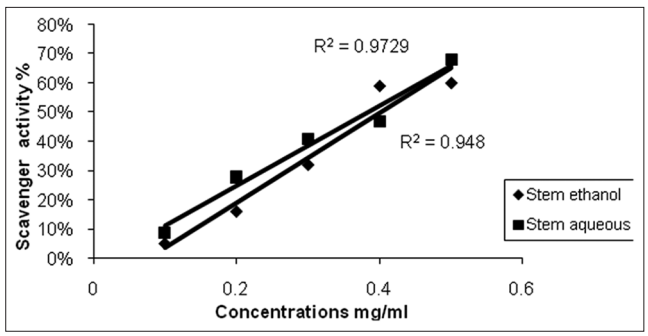

Fig. 6: The scavenging ability of EtOH and aqueous extract of stems on iron chelating

Table 2: IC_{50} values of stems and leaves extracts on DPPH, H_2O_2, and iron chelating tests

Extracts	IC_{50}		
	DPPH	H_2O_2	Fe
Ascorbic acid	2.5 µg/mL	10 µg/mL	-
EDTA	-	-	5 µg/mL
Leaves			
Aqueous	0.25 mg/mL	0.3 mg/mL	0.28 mg/mL
EtOH	0.1 mg/mL	0.25 mg/mL	0.37 mg/mL
Stems			
Aqueous	0.45 mg/mL	0.43 mg/mL	0.41 mg/mL
EtOH	0.31 mg/mL	0.39 mg/mL	0.38 mg/mL

Values are the average of triplicate experiments, and values are expressed as mg ± SD

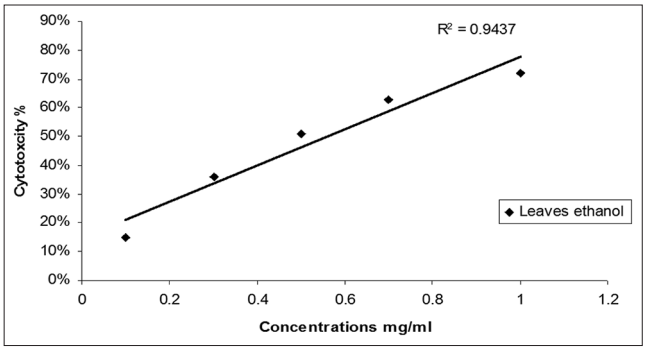

Fig. 7: Inhibition of 293T cell proliferation by the treatments of different concentrations of EtOH extracts from leaves of *Anacyclus nigllifolius*

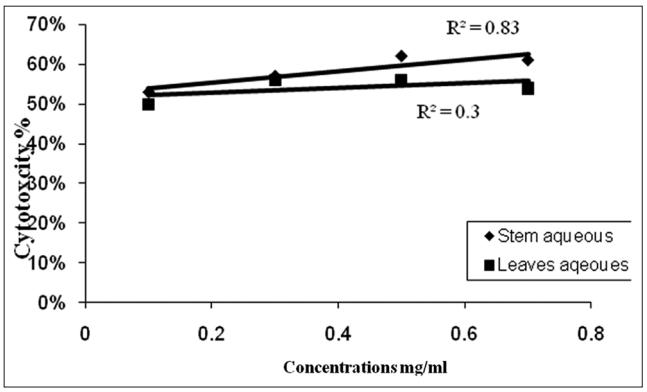

Fig. 8: Inhibition of 293T cell proliferation by the treatments of different concentrations of aqueous extracts from leaves and stems of *Anacyclus nigllifolius*

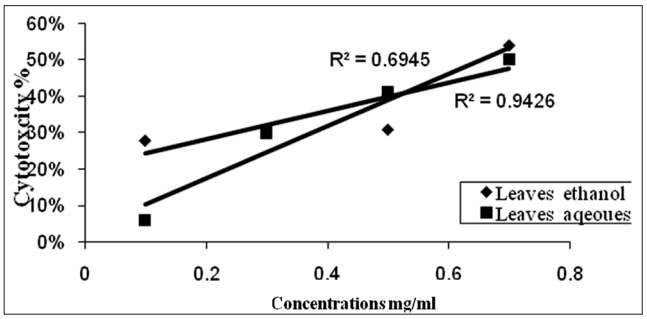

Fig. 9: Inhibition of MCF7 cell proliferation by the treatments of different concentrations of aqueous and EtOH extracts from leaves of *Anacyclus nigllifolius*

Cancer cell proliferation

The cytotoxicity of each part of *A. nigllifolius* on MCF7and 293T cells was examined by XTT assay. After 2 h of treatment by different concentrations of leaves and stems, it was shown that EtOH extracts have significantly inhibited cell growth 293T. The percentage of inhibition was in leaves, and it has reached the 70% as shown in Fig. 7. In addition, aqueous extracts of leaves and stems were found their ability to reduce and inhibit the cell lines around 54% and 61%, respectively, as shown in Fig. 8. Therefore, the aqueous and EtOH extracts from leaves have the ability to inhibit 54% of MCF7 as shown in Fig. 9. In the same purpose [23], tested hot water polysaccharide extracts of *A. pyrethrum* for their immunostimulating activity *in vitro* and *in vivo,* and they showed a markedly enhancing of the proliferation of the murine spleen cells *in vitro*. The results of the *in vivo* study at a dose of 50 mg/kg also showed that *A. pyrethrum* has better effects than obtained with the *in vitro* study at 50 µg/mL. Another research has been done by Krishnaraju *et al.* [24] who tested the toxicity of several medicinal plants on brine shrimp lethality bioassay which based on the ability to kill laboratory cultured brine shrimp. Our current study outcome has been proved a previous results that shown the effect of ethanolic extracts of leaves and stems of A. *nigellifolius* on human lung A549 adenocarcinoma cells.

CONCLUSION

Briefly, the obtained results demonstrated that the leaves and stems of extracts have a good amount of phenolics and flavonoids. The aqueous and ethanolic extracts showed potent radical-scavenging activities in DPPH, H_2O_2, and iron chelating, and likewise, it have antiproliferative activities in MCF7 and 293T cells. That can be lead us for an important potential role of *A. nigllifolius* extracts and their efficiency as antioxidant and anticancer from natural origin.

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