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Research Article

Phytochemical Analysis, Quantitative Estimations of Total Phenols and Free Radical Scavenging Activity of *Bupleurum subovatum* from Jerusalem

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ABSTRACT

Background: The crude extracts from *Bupleurum subovatum* plant were used to screen the presence of secondary metabolic products, to estimate the total phenol content and free radical scavenging activity for the plant extracts. ***Methods:*** Antioxidant activity was evaluated by using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, while total phenol content was determined by using Folin Ciocalteu' s method. ***Result:*** The phytochemical analysis confirmed the presence of phenol, proteins, starch, reducing sugars, tannins, volatile oils, cardiac glycosides, steroid, and huge amounts of saponins. Total phenolic content in the methanolic extract was 9.05 mg/g Gallic acid. At the same time, methanolic extract showed a mild potential oxygen free radical scavenging ability as well as the IC_{50} for the plant was 18.60 ± 0.36 µg/ml, which justified its uses in the folkloric medicine and could be further subjected for the isolation of their therapeutic active compounds. ***Conclusion:*** The results of this study revealed the antioxidant activity and confirmed the therapeutic usage of *Bupleurum subovatum* in the traditional medicine.

Introduction

Crude herbal products and their isolated compounds have been traditionally used for the treatment of various diseases; they are still the richest source for novel medications to elevate the human health care systems all over the word.[1,2] Moreover, it estimated that about 60% of the dosage forms in the pharmaceutical markets were derived from natural products.[3]

In the recent years, there is a growing interest in the utilizations of herbal products. Among medications prescribed by physicians worldwide, about 25% were from herbal origin and 11% are exclusively from pure herbal origin as digoxin, theophylline and physostigmine. Large number of the prescribed drugs are semi-synthetic medications synthesized from natural precursors combined with synthetic chemicals as neostigmine from physostigmine, aminophylline from theophylline and others.[4]

A genus *Bupleurum* is wildly distributed in the nature with 200 species especially in the northern regions of the hemisphere, North Africa and Eurasia. It belongs to the Apiaceae (Umbelliferae) family, only 150 species of them screened phytochemically and biologically. However, our studied species in this research are not yet mentioned in any literature.[5]

Bupleurum subovatum English common names are lance leaf and thorow-wax while the synonym Latin scientific names are *Bupleurum aegyptiacum* Wild. Ex Steud, *Bupleurum granulatum* Gaudin, *Bupleurum intermedium* (Loisel.) Steud., *Bupleurum lancifolium* subsp. *subovatum* (Link ex Spreng.) O. Bolos and Vigo, *Bupleurum panacifolium* Hornem. ex Steud., *Bupleurum perfoliatum* var. *longifolium* Desf., *Bupleurum protractum* Hoffmanns. & Link, *Bupleurum rotundifolium* subsp. *intermedium* (Loisel.) DC., *Bupleurum savignonii* De Not. It is annual herbaceous plant, about 50 cm tall with hermaphrodite and compound umbel borne on a peduncle entire, smooth or very finely granulose yellow flowers, the stems are glabrous, waxy, hairless and erect, the leaves are dark green, simple, apiculate, perfoliate, nearly lanceted to oval shaped, narrow, alternates with a smooth and entire cartilaginous margins, the fruits are strongly tuberculate and have schizocarp form.[6-8]

B. subovatum plant grows wildly and widely in Palestinian territory, especially in the Semi-steppe, deserts, woodlands, fields and scrublands areas.[9-11]

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Early 19th century studies on the *B. subovatum* plant focused only on the folk uses. The plant leaves were used for treatment of various infectious diseases and inflammations,[12-13] while the roots extracts were used to treat hemorrhoids, brain and gastrointestinal disorders.[14,15-17] The leaves were also used for female reproductive problems,[18] improving the liver functions and the whole plant is traditionally used for prevention and treatment of cancers.[19]

Recently, published studies proved the presence of several compounds in *Bupleurum* plant which have relevant physio-biological activities. These ingredients are triterpenoid sapogenin (saikosaponins),[20,21] volatile oils (germacrene D, pinene, heptanal, spathulenol, undecane),[22,23] complex polysaccharides,[24] caffeic acid derivatives,[25] flavonoids,[26] and lignans [14]. The physio-biological activity of these compounds include antiviral,[27] immune-modulator,[28] anti-ulcer [29] and anti-inflammatory activities[30] and antiproliferative activity.[31]

Saikosaponins-D and saikosaponons-A in particular and other saikosaponins saponins have been proved to have many pharmacological effects including hepatoprotective,[14] anthelmintic,[32] anti-inflammatory,[33] and analgesic activities.[26]

Materials and Methods

Collection and preparing plant materials

The *B. subovatum* plant was collected in June, 2014 from the mountains of Jerusalem/Palestine. The plant was botanically identified by Dr. Nidal Jaradat from the Pharmacy Department at An-Najah National University. Voucher specimen was deposited in the Herbarium of the Pharmaceutical Chemistry and Technology Division (Laboratory of Pharmacognosy) and its voucher specimen code is (Pharm-PCT-453).

All plant parts were dried at controlled temperature (25 ± 2 °C) and humidity (55 ± 5 RH). The plant materials were then powdered using mechanical grinder and placed into a well closed glass containers for future use.

Instrumentation

Shaker device (Memmert shaking incubator, Germany), rotary evaporator (Heidolph OB2000 Heidolph VV2000, Germany), spectrophotometer (Jenway 7135, UK), freeze dryer (Mill rock technology, model BT85, Danfoss, china), grinder (Moulinex model, Uno, China), balance (Rad wag, AS 220/c/2, Poland), filter paper (Machrery-Nagel, MN 617 and Whatman no.1, USA).

Chemical Reagents

For phytochemical screening the following reagents were used: Millon's reagent (Gadot, USA), Ninhydrain solution (Alfa Agar, England), Benedict's reagent (Gadot, USA), Molish's reagent, H_2SO_4 and iodine solution (Alfa aesar, England), NaOH (Gadot, USA), chloroform and HCl (Sigma Aldrich, Germany) magnesium ribbon and acetic acid (frutarom LTD, Haifa), $FeCl_3$ (Riedeldehan, Germany).

Total phenol content: Folin-Ciocalteu reagent was purchased from Sigma Aldrich, Germany.

For evaluation of antioxidant activity the following reagents were used: Methanol (Lobachemie, India), n-hexane (Frutarom LTD, Haifa), Trolox ((s)-(-)-6 hydroxy -2, 5, 7, 8-tetramethychroman- 2-carboxylic acid) (Sigma Aldrich, Germany), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany).

Preparation of plant extracts for phytochemical screenings

The phytochemical extraction was performed using organic solvent which was performed by Soxhlet apparatus taking 20 gm of dried powdered plant with 250 ml of methanol. The extract was then heated using water bath at 35 °C until all the solvent evaporated. The dried plant crude extract was kept in refrigerator at 2-8 °C for further use.

The aqueous extraction was performed by taking five grams of the powdered plant and mixed with 200 ml of deionized water in a beaker. The mixture was heated to 30-40 °C and mixed with continuous stirring for 20 minutes. The mixture was filtered and the filtrate was used for the further phytochemical analysis.

Preparation of plant extracts for antioxidant evaluation

The grounded plant powder (10g) was soaked in 1 liter of methanol (99%) and placed in a shaker device at 100 rounds per minute for 72 hours at room temperature. Then it was stored in refrigerator for 4 days. The extract was then filtered using whatman filter paper no.1 and concentrated under vacuum on a rotator evaporator. Finlay, the crude extract was stored at 4 °C in the refrigerator for further use.

Antioxidant activity

Trolox standard and plant working solutions

A stock solution of a concentration of 1 mg/ml in methanol was prepared for the plant extract and trolox. The working solutions of the following concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/ml) were prepared by serial dilution with methanol from the stock solution.

Spectrophotometric measurements

DPPH was freshly prepared at a concentration of 0.002 % w/v. The DPPH solution was mixed with methanol and the above prepared working concentration in a ration of 1:1:1 respectively. The solutions were incubated in dark for 30 minute at room temperature before the absorbance readings were recorded at 517 nm.

Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of the plant and the Trolox standard were calculated using the following

formula:
Percentage of inhibition of DPPH activity (%):
(A-B)/A ×100% Eq.(1)
Where: A, is the optical density of the blank and B, is the optical density of the sample.
The antioxidant half maximal inhibitory concentration (IC50) for the plant samples and the standard were calculated using BioDataFit edition 1.02 (data fit for biologist).

Phytochemical qualitative analysis

The plant aqueous and methanolic extracts were screened for the presence of the phytochemical classes using the standard following methods.[34] Testing for proteins was performed using Millon's test by mixing 2 ml of Millon's reagent to test the presence of protein in the plant. The Ninhydrin test was also carried out by boiling 2 ml of 0.2% Ninhydrin solution to test the presence of proteins and amino acids. The presences of carbohydrates were performed using different test. These include Fehling's test to test the presence of reducing sugars. Benedict's reagent test was also performed to test the presence of carbohydrates. Moreover, Molisch's solution test carried out to confirm the presence of the carbohydrate. Testing for phenols and tannins was performed by mixing $FeCl_3$ solution with the crude extract to test for the presence of tannins and phenols.
The presence of flavonoids was tested using Shinoda test. Testing for saponins was performed by taking five milliliter of distilled water then added to crude plant extract in a test tube and it was shaken vigorously. The foam formation indicated the presence of saponins.
Testing for glycosides was done using Liebermann's test to test for the presence of aglycone steroidal part of glycosides and Salkowski's test was performed to examine the presence for steroidal aglycone part of the glycoside. Keller-kilani test was performed to examine for the entity of cardiac steroidal glycosides. Test for steroid was performed by taking two milliliter of chloroform and concentrated H_2SO_4 and were then mixed with the crude plant extract in the lower chloroform layer to produce red color that indicates the presence of steroids. Another test was performed by mixing 2 ml of each of acetic acid with H_2SO_4 concentrated and crude extract with 2 ml of chloroform. Green color indicated the entity of steroids.
Testing for terpenoids was performed by mixing two milliliter of chloroform with the plant extract and evaporated on the water path then boiled with 2 ml of H_2SO_4 concentrated. A grey color produced indicates the entity of terpenoids.

Determination of total phenol content

Total phenol content in the plant was determined using methanolic extracts by spectrophotometric method with some modifications.[35] 1 mg/ml aqueous solutions for both methanolic extracts were prepared in the analysis. The reaction mixture was prepared by mixing 0.5 ml of plant extract solution, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$ aqueous solution. The samples were thereafter incubated in a thermostat at 45 ºC for 45 minutes. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained using spectrophotometer at wave length 765 nm. The same procedure was repeated for the standard solution of Gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of Gallic acid equivalent expressed in terms of (mg of GA/g of extract).[36]

Data analysis

The antioxidant activity was reported as percentage of inhibition. The inhibition of *B. subovatum* plant and Trolox standard at different concentrations were plotted and tabulated. The IC_{50} for each of them was calculated using the BioDataFit fitting program in which the sigmoidal fitting model was the adapted model.

Results and Discussion

In the recent time discovering and exploring a pharmacological potential drugs for treatment and prevention of cancer and Alzheimer's disease (antioxidant agents) from natural plant products still the main scope for many of the pharmaceutical research centers and scientists all over the world.[37,38]
The Phytochemical screening tests results for *B. subovatum* extract showed that the plant contain various phytochemical compounds such as cardiac glycosides, proteins, starch, reducing sugars, phenols, volatile oil, tannins and steroids as well as the aqueous plant extract had very high contents of saponins as presented in Table 1.

Table 1. Phytochemical tests for the aqueous and methanolic extracts of *B. subovatum*.

Phytochemical compound	Aqueous extract	Methanolic extract
Cardiac glycosides	+	+
Alkaloids	-	-
Proteins and amino acids	++	+
Saponin	++++	++
Starch	++	-
Reducing sugars	+	++
Phenols	+	+++
Volatile oils	++	++
Tannins	++	++
Flavonoids	-	-
Steroids	+	+

Where; (-) means the absence of the content, (+) means low content, (++) means mild content and (+++) means high content.

The estimation of total phenol in the extracts was determined using Folin Ciocalteu's method and the standard compound (Gallic acid).
The total phenols results were expressed as mg/g Gallic acid equivalent using the standard curve equation:

$Y = 0.011X + 0.0898$, R^2=0.9968 Eq.(2)

Where Y is Absorbance at 765nm and X is total phenol in the plant extract.

The total phenolic content of the plant extract was found to 9.05 mg Gallic acid equivalent/g of extract. It is relatively not high content of phenolic compounds.

The free radical scavenging activity of the methanolic extract of *B. subovatum* has been tested by DPPH radical method using Trolox as a reference standard. The concentration ranged from 1–100 µg/ml. The zero inhibition was considered for the solution which contained only DPPH without any plant extract. The results are showed in Table 2, and the table readings are explained in Figure 1.

Table 2. Percentage inhibition activity for Trolox and *B. subovatum*.

Concentration µg/ml	% of inhibition by Trolox ±SD	% of inhibition by *B. subovatum* ±SD
1	40.6 ± 0.91	19.29 ± 0.64
2	48.7 ± 1.32	23.16 ± 0.74
3	56.09 ± 0.83	23.59 ± 1.24
5	60.12 ± 1.98	24.21 ± 1.02
7	80.12 ± 1.06	24.21 ± 0.96
10	87.65 ± 1.66	28.42 ± 1.14
20	88.21 ± 1.47	35.79 ± 1.23
30	91.05 ± 2.71	39.29 ± 1.04
40	91.46 ± 1.93	53.68 ± 1.11
50	99.05 ± 2.79	65.96 ± 1.43
80	99.05 ± 1.87	95.79 ± 2.81
100	99.05 ± 2.64	95.79 ± 1.77
IC_{50}	2.75 ± 0.21	18.60 ± 0.36

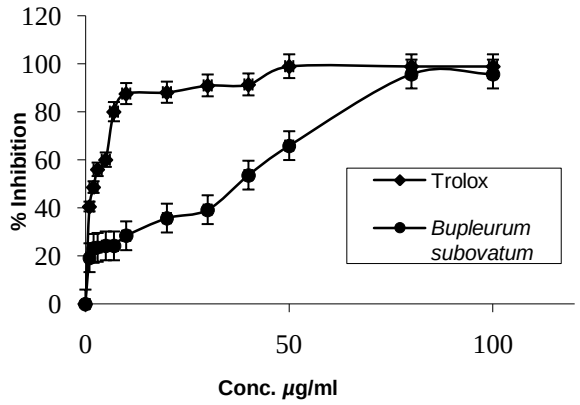

Figure 1. Antioxidant inhibition activity of Trolox standard and *B. subovatum*.

The half maximal inhibitory concentration (IC_{50}) was 18.60 µg/ml for *B. subovatum* and 2.75 µg/ml for Trolox. The antioxidant activity of *B. subovatum* was comparatively lower than Trolox reference standard which is known to have high antioxidant activity.

Conclusion

In conclusion, the phytochemical screening of *B. subovatum* proves that it consists of several secondary metabolic products (phenols, proteins, starch, reducing sugars, tannins, volatile oils, cardiac glycosides, steroids and saponins). In addition, this species showed mild antioxidant activity; therefore, this finding supports the traditional usage of *B. subovatum* in the folk medicine. The reported activities are worthy for further phytochemical and pharmacological studies and possible isolation of active constituents responsible for the activities demonstrated.

Conflict of interests

The author claims that there is no conflict of interest.

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