REPORT

Comparison of qualitative, quantitative analysis and antioxidant potential between wild and cultivated *Borago officinalis* leaves from palestine

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Abstract: *Borago officinalis* plant is an important plant of high medicinal and nutritional values. This study designed to evaluate antioxidant activity, screen the existence of phytogenic chemical compounds and to determine the total flavonoid and phenol contents of wild and cultivated *Borago officinalis*. Total flavonoid contents of the wild and cultivated *Borago officinalis* were determined by using rutin reference standard method and total phenols determined by using Folin Ciocalteu's method while antioxidant activity evaluated by using 2, 2-diphenyl-1-picryl-hydrazyl-hydrate assay. Phytochemical analyses indicated the presence of carbohydrate, phenols, flavonoids, phytosteroids tannins and volatile oil. The total flavonoid content of the methanolic extract from the wild borage plant was 22.4mg RU/g this value was reduced to 13.1mg RU/g for the cultivated methanolic extract as well as the total phenols contents was dropped from 5.21mg GA/g to 2.37mg GA/g methanolic extracts. Total tannins content of the wild growing borage plant was 13.7mg GA/g methanolic extract. This value was higher in the cultivated borage with 21.33mg GA/g methanolic extract. The wild leaves extract had IC_{50} =6.3µg/mL for wild leaves extract was closer to IC_{50} value of Trolox (standard reference with high antioxidant activity), while the cultivated leaves extract had higher IC_{50}= 8.7µg/ml which mean lower antioxidant activity than the wild growing one. The data of this study showed that the extracts of *Borago officinalis* possess antioxidant and free radical scavenging activities. Variation was clear between wild and cultivated species, these findings propose that such plant extract could have a wide range of applications in both food and pharmaceutical industries. Therefore, more research is necessary to investigate different cultural practices on the efficiency of borage plant.

Keywords: *Borago officinalis,* phenols, flavonoids, tannins, antioxidants.

INTRODUCTION

Borago officinalis L., belong to Boraginaceae family, is an important plant of high medicinal and nutritional values. It has different usages in pharmaceutical, industrial and forage fields (Gilani *et al*., 2007; Komaki *et al*., 2015). Borage extract has a high level of the gamma-linolenic acid (GLA) which is used as dietary or food with high resistance to oxidation, the oil of borage seeds, contains up to 25% γ-linolenic acid (Berti *et al*., 2010; De Haro-Bailón and Del Rio, 1998; Navaey *et al*., 2014), the oil in these seeds resists oxidation (Zadernowski *et al*., 2002), in addition, the oil was used successfully in curing many diseases such as; multiple sclerosis, diabetes, heart disease, arthritis and eczema (Asadi-Samani *et al*., 2014). Moreover, The leaves of borage are reportedly used as diuretic, demulcent, emollient, expectorant (Navaey *et al*., 2014), De Ciriano *et al*., (2009) reported that lyophilized water extract of borage leaves (340ppm) showed high antioxidant activity similar to 200 ppm concentration of both butylhydroxyanisol (BHA) and butylhydroxytoluene (BHT) mixture.

Natural antioxidants have been shown to be beneficial in a variety of complications such as cancer (Shirzad *et al*., 2011), burn (Asadi *et al*., 2013), diabetes (Farokhi *et al*., 2013), hyper-lipidemia and amnesia (Nasri *et al*., 2013). These compounds are able to prevent or cure the side effects of other compounds (Asadi-Samani *et al*., 2014). Nowadays, researches focus on the antioxidant properties of borage extracts, these extracts showed strong antioxidant properties. In addition, borage plant has been utilized as a protective measure against gastric infections. (Mhamdi *et al*., 2010). For these reasons its cultivation has been carried out recently. Borage originates in the Mediterranean region, it has been spread to Asia Minor, Europe, North Africa, and South America. Initially it was cultivated for culinary and medicinal utilization (Farhadi *et al*., 2012). In many areas, borage plant grows wildly, it can grow in different type of soil, it grows better in land

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exposed to moderate sun. Intense sun can change plant to rosette state (Yazdani *et al*., 2004), in addition, cultural practices such as time of planting and nitrogen fertilizer composition were found to affect the GLA and oil content of borage plants (Gupta and Singh, 2010), therefore, cultivation conditions may affect the chemical contents of the plant compared to natural wild growing conditions, up to our knowledge, most of the studies were focused on the seed extracts of borage plant, limited studies were published on leaf extract, therefore, the aim of this research is to study the antioxidant properties and phytogenic chemical compounds of both wild and cultivated *Borago officinalis* L. plant.

MATERIALS AND METHODS

Collection and preparing plant materials

Wild and cultivated *Borago officinalis* leaves were collected in February 2015 from both cultivated and wild growing area from Jenin area, West Bank/ Palestine. The leaves were washed and then dried in the oven at controlled temperature (25±2°C) and humidity (55±5 RH) until all the plant leaves became well dried. After that, the dried leaves were well powdered using a mechanical grinder and placed into a well closed light proof glass containers for further use.

Instrumentation

Orbital Shaker (Memmert shaking incubator, Germany), rotary evaporator (Heidolph OB2000, VV2000, Germany), spectrophotometer (Jenway 7135, UK), grinder (Moulinex model, Uno, China), balance (Rad wag, AS 220/c/2, Poland), automatic Deionizer Unit, Mime water inc. Haifa, and 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, Israel), Ninhydrain solution (Alfa Agar, England), Benedict's reagent (Gadot, Israel), Molish's reagent, H_2SO_4 and iodine solution (Alfa aesar, England), NaOH (Gadot, Israel), chloroform and HCl (Sigma Aldrich, Germany) magnesium ribbon and acetic acid (frutarom LTD, Israel), $FeCl_3$ (Riedeldehan, Germany). For evaluation of antioxidant activity, the following chemicals were used: methanol (Lobachemie, India), n-hexane (Frutarom LTD, Israel), Trolox ((s)-(-)-6 hydroxy -2, 5, 7, 8-tetramethychroman- 2-carboxylic acid) and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany).

For evaluation of total phenol contents, Folin-Ciocalteu's reagent (Sigma Aldrich, Germany) was used. For evaluation of total flavonoid contents, rutin hydrate was purchased from MP-Biomedical USA, $AlCl_3$ and potassium acetate (Sigma Aldrich, Germany). For evaluation of total tannins contents, tannic acid from Sigma Aldrich, Germany, and sodium carbonate was used from Merck (Darmstadt, Germany). The organic solvents used in this study were of HPLC grade, however, methanol was used as solvent for extraction of technical quality.

Preparation of plant extracts for phytochemical screenings

The plant leaves extracts were obtained employing water and various organic solvents to screen polar and non polar constituents. Organic extraction was obtained using Soxhlet extraction method. In which, 20 g of dried plants powders were placed into a glass thimble then extracted with 250ml of different organic solvents separately (ethanol, acetone and methanol). The extraction processes were carried on until the solvent in siphon tube of the Soxhlet apparatus become colorless. Then the extract was 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 future use. These procedures repeated for both *Trigonella* species (Wang and Weller, 2006).

The aqueous extractions for both *Trigonella* species were performed by taking 5 g of the plant powder and mixing it with 200ml of deionized water in a beaker. The mixture was heated on a hot plate at 30-40°C and mixed with continuous stirring for 20 minutes. Whatman filter papers no.1, were used to filter the mixture and the filtrate was then used for further phytochemical analysis (Aiyegoro and Okoh, 2010)

Preparation of plants extracts for antioxidant evaluation

About 10g of the grounded plant leaves were soaked in 1 L of methanol (99%) and placed in a orbital shaker at 100 revolutions per minute (rpm) for 72 h at room temperature and then stored in refrigerator for 4 days. After that, the extract was filtered using what man filter paper no.1 and then concentrated under vacuum on a rotary evaporator. The crude extracts for two species were stored at 4 °C in the refrigerator for further use (Theo *et al*., 2009).

Primarily Phytochemical qualitative analysis

Both plants extracts were screened for the presence of the phytochemicals by using the following phyto-analytical standard methods (Harborne, 1998).

Tests for proteins

Millon's test: 2ml of Millon's reagent mixed with the plants crude extracts, the white precipitate appeared which upon gentle heating turned into red color indicated the presence of proteins in the plant.

Ninhydrin test: 2ml of 0.2% Ninhydrin solution was boiled with the plants crude extracts, the violet color appeared indicate the presence of proteins and amino acids.

Tests for carbohydrates

Fehling's solutions test: a mixture of Fehling solutions A and B was boiled with and equal volumes were added to both crude plants extracts. The presence of reducing sugars was indicated by the appearance of a red color precipitate.

Benedict's reagent test: 2 ml of Benedict's reagent was boiled with crude extracts; the presence of the carbohydrates was indicated by the appearance of a reddish brown color.

Molisch's solution test: 2ml of Molisch's solution was shaken with crude plants extracts then 2ml of H_2SO_4 concentrated solution was added and poured carefully along the side of the test tube. A violet ring appeared at the inter phase of the test tube indicated the presence of carbohydrate.

Iodine test: 2ml of iodine solution was mixed with both crude plants extracts. Purple or dark blue colors indicated the presence of the carbohydrate.

Test for phenols and tannins

Crude extracts were mixed with 2 ml of 2% solution of $FeCl_3$. Black or blue-green color indicated the presence of tannins and phenols.

Tests for flavonoids

Shinoda test: pieces of magnesium ribbon and HCl concentrated were mixed with crude plants extracts after few minutes pink colored scarlet appeared that indicated the presence of flavonoids.

Alkaline reagent test: 2ml of 2% NaOH solution was mixed with plants crude extracts, intensive yellow color was formed, which turned into colorless when added 2 drops of diluted acid to solution, this result indicated the presence of flavonoids.

Test for saponins

Five ml of distilled water were added to crude plants extracts in a test tube and shaken vigorously. The presence of saponins was indicated by foam formation.

Tests for glycosides

Liebermann's test: 2ml of acetic acid and 2ml of chloroform were mixed with plants crude extracts. The mixtures were then cooled and added H_2SO_4 concentrated, green color indicated the entity of aglycone steroidal part of glycosides.

Salkowski's test: H_2SO_4 concentrated (about 2ml) was added to the plants crude extracts. A reddish brown color produced indicated the entity of steroidal aglycone part of the glycoside.

Keller-kilani test

A mixture of Acetic acid glacial (2ml) with 2 drops of 2% $FeCl_3$ solution was added to the plants extracts and H_2SO_4 concentrated. A brown ring produced between the layers which indicated the entity of cardiac steroidal glycosides.

Test for steroid

2 ml of chloroform and concentrated H_2SO_4 were mixed with the plants crude extracts. In the lower chloroform layer produced red color that indicated the presence of steroids.

Another test was performed by mixing 2ml of each of acetic acid with H_2SO_4 concentrated and crude extracts with 2ml of chloroform. Green color indicated the entity of steroids.

Test for terpenoids

2 ml of chloroform was mixed with the plants extracts and evaporated on the water path then boiled with 2ml of H_2SO_4 concentrated. A grey color produced indicated the entity of terpenoids.

Antioxidant activity

Trolox standard and plant working solutions

A stock solution of a concentration of 1mg/ml in methanol was firstly 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, 100µg/ml) were prepared by serial dilution with methanol from the stock solution (Rice-evans *et al*., 1995).

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. Solutions were incubated for 30 min at room temperature in darkness. Methanol was used as negative blank while DPPH /methanol mixtures were used as positive blank. All absorbance readings were recorded at 517nm

Percentage of inhibition of DPPH activity

The percentage of antioxidant activity of both plants species and the Trolox standard were calculated using the following formula:

Percentage of inhibition of DPPH activity (%) = (A-B)/A ×100%

Where: A = absorbance of the positive blank,

B = absorbance of the sample.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the plants samples and the standard were calculated using BioDataFit edition 1.02 (data fit for biologist).

Determination of total tannin content

The total tannin content was determined by method of Broad hurst *et al*., 1978 with slight modification (Broadhurst and Jones, 1978), using catechin as a reference compound. 100µg/mL of stock aqueous solutions for methanolic extract was prepared then several

serial dilutions were prepared from this stock solution. 1 mL from each dilute was added to 3mL vanillin (4% in methanol) solution and 1.5mL of concentrated hydrochloric acid. After 15 minutes of incubation, the absorbance was read at 500 nm. Total tannin content was expressed as (mg CA/1g plant extract).

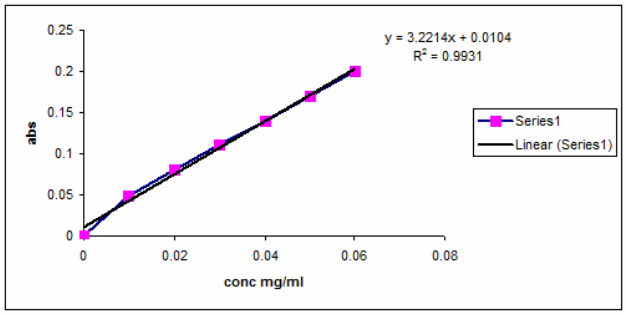

Fig. 1: Standard calibration curve of different concentrations (mg/ml) of rutin and their respective optical density at 415nm**.**

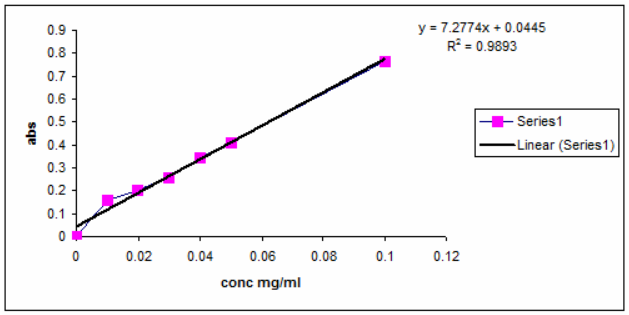

Fig. 2: Standard calibration curve of different concentrations (mg/ml) of Gallic acid and their respective optical density at 765nm.

Determination of total phenol content in the methanolic extract

Total phenols content in the plant methanolic extracts was determined using spectrophotometric method with some modifications (Petretto *et al*., 2015). 100µg/ml of stock aqueous solutions for methanolic extract was prepared. The reaction mixture was prepared by mixing 0.5ml of both plants extracts solutions, 2.5ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5ml of 7.5% of $NaHCO_3$ aqueous solution. The samples were incubated in a thermostat at 45°C for 45min. Spectrophotometer at wave length of 765nm was used to measure the absorbance. Triple samples were prepared for each analysis and the mean value of absorbance was obtained. The same procedure was used for the standard solution of Gallic acid and the calibration line. The concentration of expressed in terms of Gallic acid equivalent (mg of GA/g of both extracts)

Determination of total flavonoids contents in the methanolic extract

The total flavonoid content was determined from the calibration curve of Rutin and expressed as milligram of Rutin Equivalent per gram of extract (mg RU/g extract). Total flavonoid content was determined according to the procedure of Chang *et al*., validated by Nugroho (Chang *et al*., 2002; Nugroho *et al*., 2011), with some modifications using rutin as reference standard. Rutin (100mg) was dissolved in 10ml distilled water and diluted to 100ml. Subsequently, the stock solution was used to provide a series of diluted concentrations. From each solution (0.5 ml) was mixed with 3 ml methanol, 0.2ml of 10% $AlCl_3$, 0.2ml potassium acetate 1M and 5 ml distilled water, and then incubated at room temperature for 30 min. Furthermore, absorbance was measured at 415nm wavelength, and distilled water with methanol, 10% $AlCl_3$ and potassium acetate was used as a blank. Total flavonoid in methanolic extract was expressed in terms of Rutin equivalents (mg of RU/g of both *Trigonella* species extracts) (Singh *et al*., 2015).

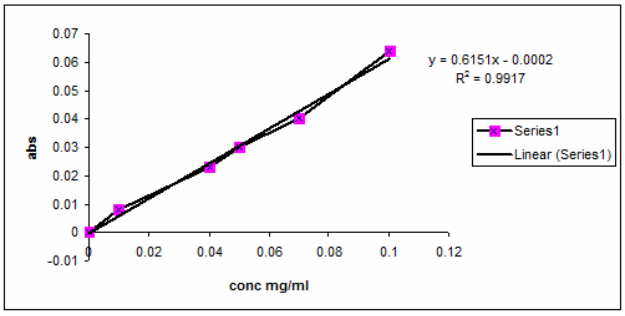

Fig. 3: Standard calibration curve of different concentrations (mg/ml) of Catechin and their respective optical density at 500 nm

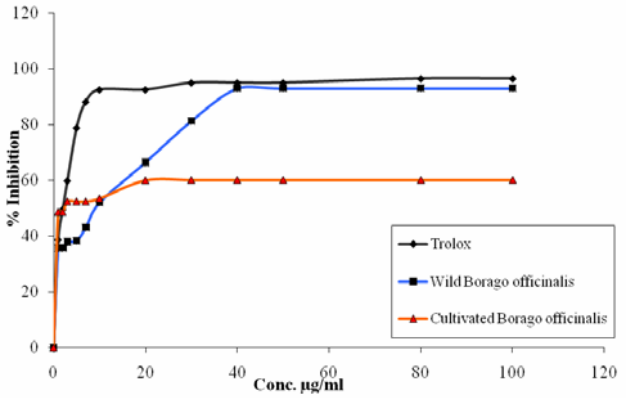

Fig. 4: Inhibition activity of Trolox standard, wild and cultivated methanolic plant extracts.

RESULTS

Phytochemical screening

Phytochemical screening tests for leaves extracts showed the presence of carbohydrate, phenols, flavonoids, phytosteroids tannins and volatile oil as presented in table 1.

Table 1: Phytochemical screening tests results for the methanolic extracts for both types of leaves

Phytochemical Compound	Test name	Wild leaves	Cultivated leaves
Protein & amino acids	Millon test	-	-
	Ninhydrin test	-	-
Carbohydrate and reducing Sugars	Bendicts test	+	+
	Molisch test	+	+
	Iodine test for starch	+	+
Glycosides	Liebermans test	-	-
	Keller-kilani test	-	-
Flavonoid	Alkaline reagent test	+	+
Saponins	Frothing test	-	-
Phytosteroids	Libermann Burchard's test	+	+
Volatile oil	50% KOH test	+	+
Tannin compounds	Ferric chloride test	+	+
Phenolic compounds	Folin test	+	+
Alkaloids	Wagner's test	-	-

Table 2: Total Flavonoid, Total Phenols and total Tannin for the methanolic extracts

Leaves methanolic extracts	Total flavonoid content (mg RU/g) ± SD	Total phenolic content (mg GA/g) ± SD	Total Tannin content (mg CA/g) ± SD
Wild growing Borage	22.4±0.54 ***	5.21±1.11 ***	13.7±0.59 **
Cultivated Borage	13.1±1.2***	2.37±1.33***	21.33±0.59**

***, $p \leq 0.0001$, **, $p \leq 0.001$

Total flavonoids, phenols and tannins contents results

Standard calibration curves used for the determination of total flavonoids, phenols and tannins contents which were prepared using different concentrations of Rutin equivalent (mg of RU/g for both leaves extracts), Gallic acid equivalent (mg of GA/g for both species extracts), and catechin (mg of TA/g for both species extracts) and their respective optical density as shown in figs. 1, 2 and 3 respectively.

Variation in the total flavonoid content of both wild and cultivated borago plants were detected from methanolic extract (22.4 mg and 13.1 mg RU/g), respectively. Similar trend was observed with total phenols contents, it was dropped from 5.21mg GA/g to 2.37mg GA/g methanolic extracts, while the total tannins contents in the cultivated leaves methanolic extract exceeded that in wild as it was 21.33mg CA/g. as following in table 2.

Antioxidant activity using trolox as standard equivalent

DPPH assay was used to test the free radical scavenging activity of the methanolic (polar protic solvents) extract of both wild and cultivated leaves. Trolox was used as a reference standard. The concentration ranged from 1–100 µg/ml. the baseline was:

IC_{50}= 6.3µg/ml for wild leaves extract was closer to IC_{50} value of Trolox (standard reference with high antioxidant activity) which was 2.17µg/ml. while the cultivated leaves extract had higher IC_{50}=8.7µg/ml which mean lower antioxidant activity than wild growing one.

DISCUSSION

In our study, both wild and cultivated Borago plants showed variation on screened phytochemicals. Differences in the total phenolic content and antioxidant compounds among different varieties and cultivars were reported in other studies. Augusto *et al*., 2014 reported variation in the total antioxidant activity and polyphenols between both wild and cultivated dried murtilla extracts, with higher content expressed by the cultivated plant extract.

One reason that could explain the difference in phytochemicals between wild and cultivation is the cultural practices of the cultivated plant, for instance, fertilizers and water, highly, affect plant growth. It was reported that irrigation and nitrogen fertilizer increases length of the plant, twigs and production efficiency of borage plant (Asadi-Samani *et al.*, 2014). Also, spraying plants of calcium nitrate had significant (p<0.01) effect on antioxidant activity and phenolic content(Shams *et al*., 2012). In contrary, it was found that no effect of nitrogen addition on oil yield and content; palmitic acid, stearic acid, oleic acid, and linoleic acid of borage extract (Navaey *et al*., 2014). The antioxidant activity of plant extracts is usually related to their phenolic content, for that reason, several studies have evaluated the relationships between the antioxidant activity of plant products and their phenolic content. In other studies, a correlation between them was found. In this study, a

relationship between the antioxidant activity and total phenolic content was obvious.

Different results might obtained from other researchers, extraction and bioassay procedures may differ, the antioxidant potential of 18 fruits, 13 vegetables, and 19 beverages was evaluated using both ABTS and DPPH methods, the result indicated significant higher activity with ABTS assay (Floegel, 2011). Other results showed that Phenolic content and antioxidant activity (IC_{50}) in the various stages of growth of borage plant was significantly different ($p<0.05$). (Shams *et al*., 2012), in another plant species, it was found that the cultivation of Salvia tomentosa resulted in an increase in the total phenolic content, except for flavonoid content (Dincer *et al*., 2013; Shams *et al*., 2012). Haroa *et al*., (2002) reported high variation in oleic, linoleic and erucic acids among two hundred and six Borago officinalis L. accessions of both cultivated and wild germplasm collections. This is in agreement with the idea that the phenolic compounds have a key role in free radical scavenging and/or reducing systems (Villano *et al*., 2007). The antioxidant effect of borage extracts was attributed to their phenolic constituents (Mhamdi *et al*., 2010). The bioactivity of phenolic compounds be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals (Robards *et al*., 1999). Oxidative stress is highly related to adverse health effects of several diseases, including cardiovascular, respiratory and neurological as well as for aging process. The free radical damage to lipids, proteins and DNA resulted in such adverse effect. Protection from damage occurs through the action of multiple antioxidants resulted in the protection from these damages (Ranjbar *et al*., 2006).

CONCLUSION

The extract of *Borago officinalis* leaves exhibits antioxidant and free radical scavenging activities. Therefore, the leaf extracts of borage could also exert *in vivo* protective effects against oxidative and free radical injuries of various disease conditions. Variation was clear between wild and cultivated plants, therefore, more research are necessary to investigate different cultural practices on the efficiency of borage plant.

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