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Phenolic Compounds and Antiradical Scavenging Activity Changes During *Borago officinalis* Stalk Leaf Development

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Borage (*Borago officinalis*) stalk leaves were sampled in the region of Amdoun (north of Tunisia) during their vegetative stage in order to analyze their phenolic composition and to ascertain their antiradical scavenging activity. The harvesting time effect on some physical properties of borage seed were significant. Total phenolic contents ranged from 3.6 mg gallic acid equivalent (GAE) g^{-1} DW in young leaves to 4.80 mg gallic acid equivalent (GAE) g^{-1} DW in adult leaves. RP-HPLC analysis identified nine phenolic acids during stalk leaf development with the predominance of syringic acid. Total phenolic contents and IC_{50} values in leaves during their development, allowed to conclude that antioxidant activity does not depend on the high content of total phenolics but on the phenolic composition.

Key Words: Borage, Stalk leaves, Free radical activity, Syringic acid, Variation.

INTRODUCTION

In the recent years there has been increasing interest in antimutagenes[1] and antioxidant activity of plant origin compounds. Such compounds may be useful in preventing cancer and other mutation related diseases, by fortifying physiological defence mechanism, or by favouring the intake of protective factors[2]. Therefore, researchers have made numerous efforts to find antioxidants. The development of antioxidants that scavenge ROS, would support biological resistance to free radicals, retard the process of ageing and decrease the risk of age associated degenerative diseases, such as cancer, cardiovascular disease, immune system decline and brain dysfunction[3].

For Borage, an annual plant belonging to Boraginaceae family and native to some parts of the Mediterranean region, an extensive investigation on antioxidant properties of borage meal was reported. Wettahsinghe and Shahidi[4] reported on the antioxidant properties of borage meal and identified the dominant antioxidative compounds as rosmarinic. This latter was also present in borage leaves[5] and seeds[6,7].

There are several uses of borage culinary. Its leaves may be used in salads. In addition, leaves and stems enhance cheese, poultry, most vegetables and salad dressing. Many researchers have reported on the fatty acid composition of borage leaves or the whole plant[8]. In fact, fatty acid profile of leaves revealed the prevalence of two polyunsaturated fatty acids (PUFA): α-linolenic followed by stearidonic acid[9,10].

The phenolic composition and the antioxidant activity during leaf development have not yet been studied. This study was undertaken to investigate changes in the phenolic components and antiradical scavenging activity at different stages of leaf development in order to determine the optimal accumulation period of desirable compounds and to try to valorize this borage leaf as source of bioactive molecules. We also aimed at studying whether a correlation exists between phenolic composition and the antioxidant activity at different leaf development stages.

EXPERIMENTAL

Butylated hydroxytoluene (BHT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma. Folin-Ciocalteu reagent and sodium azide were purchased from Aldrich. Authentic standards of phenolic compounds were purchased from Sigma and Fluka. Stock solutions of these compounds were prepared in HPLC-grade methanol. These solutions were wrapped in aluminium foil and stored at 4 °C. All other chemicals used were of analytical grade.

Borage stalk leaves were randomly collected during their development from spontaneous plant in Amdoun area (North Western of Tunisia, latitude: 36°45'55.02" (N), longitude: 9°5'22.5344 (E), altitude: 40 m). Amdoun region is characterized by high annual rainfall of 1600 mm and annual temperature of 16 °C. To avoid variability, collection always took place in the same geographic area from 20-30 plants having the same development stage. The plant material was botanically characterized by Prof. Abderrazek Smaoui (Botanist, Biotechnology Center, Borj Cedria Technopark, Tunisia).

Extraction and determination of total polyphenols: Harvested stalk leaves were dried at room temperature for 1 week. Leafs extracts were obtained by strirring 1 g of dry leaf powder with 10 mL of pure methanol for 0.5 h. The extracts were then kept for 24 h at 4 °C, filtred through a Whatman no. 4 filter paper and evaporated under vacuum to dryness and stored at 4 °C until analyzed[11]. The amount of total phenolic of methanolic extract of *Borago officinalis* was determined using the Folin-Ciocalteu (F-C) reagent according to the method described by Dewanto *et al.*[12] using gallic acid as standard. The absorbance of solution was then measured at 760 nm against a blank. Total phenolic amount was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight through the calibration curve of gallic acid. The sample was analyzed in three replications.

Hydrolysis, quantification and identification of phenolic compounds using HPLC: Dried samples from seeds were hydrolyzed according to the method of Proestos *et al.*[13], slightly modified. 20 mL of methanol containing BHT (1 g L^{-1}) were added to 0.5 g of a dried sample. Then 10 mL of 1 M HCl were added. The mixture was stirred carefully and sonicated for 15 min and refluxed in a water bath at 90 °C for 2 h. The obtained mixture was injected to HPLC. The phenolic compounds' analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC, Palo Alto, CA) coupled with an UV-Vis multiwavelength

detector. The separation was carried out on a 250 × 4.6 mm, 4 µm Hypersil ODS C_{18} reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2 % sulphuric acid (solvent B). The flow rate was kept at 0.5 mL min^{-1}. The gradient programme[14] was as follows: 15 % A/85% B 0-12 min, 40% A/60% B 12-14 min, 60% A/40% B 14-18 min, 80% A/20% B 18-20 min, 90% A/10% B 20-24 min, 100% A 24-28 min. The injection volume was 20 µl and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Peaks were identified by congruent retention times compared with standards. Analyses were performed by triplicate

Statistical analysis: All data were reported as means ± standard deviation of three samples. Statistical analysis was performed by the "STATISTICA v 5.1" software[15]. Differences were tested for significance by using the ANOVA procedure, using a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Total phenolic content changes during stalk leaf development: Polyphenolic amounts showed an irregular variation during different stages of leaf development and reaching a maximum value at adult stage (105 DACA) with 4.80 mg GAE g^{-1} DW (Table-1).

TABLE-1
POLYPHENOLIC AMOUNTS (DETERMINED BY FOLIN-CIOCALTEU) DURING *Borago officinalis* STALK LEAF DEVELOPMENT

Days after cotyledon appearing (DACA)	60	66	78	91	105
Polyphenolic amounts (mgGAE/g DW)	3.60 ± 0.8^{b}	3.17 ± 0.95^{c}	2.85 ± 0.74^{d}	3.20 ± 0.45^{c}	4.80 ± 1.1^{a}

Values followed by the same small letter did not share significant differences at 5% (Duncan test).

Higher polyphenol contents determined by the Folin-Ciocalteu method, compared to those determined by HPLC method. Probably the main cause of the difference obtained by the two methods is the fact that the Folin-Ciocalteu method does not provide a specific assay for phenolic compounds as it reacts positively with many easily oxidizable non-phenolic compounds[16,17].

As can be seen in Fig. 1, the increase of total polyphenols amount at last stages of leaves development (from 78 DACA to 105 DACA) coincides with the increase of the temperature in Amdoun (north of Tunisia) region. In fact, Toor *et al.*[18] showed that high temperature had a positive effect on the accumulation of major antioxidant components of tomato. So, the accumulation of phenolic compounds was considered as a protective mechanism of plant against environmental conditions.

Phenolic acid contents variations during stalk leaf development: In this study, HPLC successfully identified nine phenolic acids during different stages of stalk leaves development including gallic acid, sinapic acid, chlorogenic acid, syringic acid, vanillic acid, rosmarinic acid, *p*-coumaric acid, ferrulic acid and *trans*-cinnamic acid.

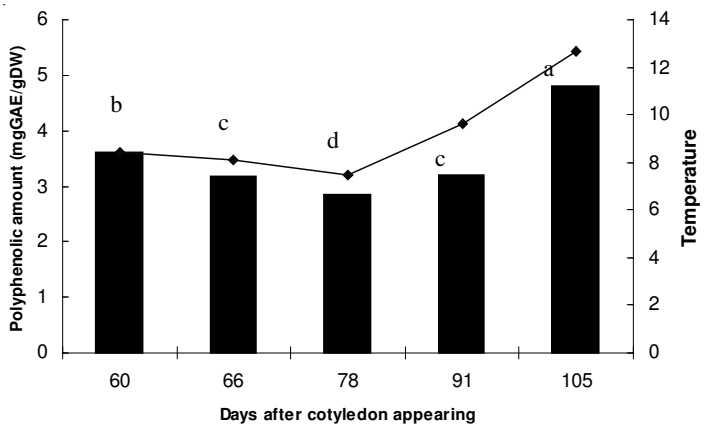

Fig. 1. Variations of polyphenol contents (mg GAE/gDW) and temperature values (°C) during borage leaf development

Table-2 presented the amount of the individual phenolic acids. In young stalk leaves, syringic acid was the predominant phenolic acid and the highest percentage was detected at 66 days after cotyledon appearing (DACA) with 932.18 mgGAE/ gDW. However, rosmarinic acid and sinapic acid presented low content.

TABLE-2
PHENOLIC COMPOUND CONCENTRATIONS* (μg g^{-1} DW)
DURING BORAGE STALK LEAF DEVELOPMENT

Phenolic compound	Days after cotyledon appearing (DACA)				
	60	66	78	91	105
Gallic acid	36.36±4.5^{b}	34.55±7.5^{b}	5.44±0.9^{c}	57.6±^{a}	4.03±^{d}
Sinapic acid	101.21±15.36^{d}	205.67±20.6^{c}	229.67±80.5^{b}	109.01±40.2^{d}	243.26±50.4^{a}
Chlorogenic acid	56.32±11.2^{b}	65.52±18.7^{a}	49.09±12.3^{c}	51.62±9.8^{bc}	16.91±2.6^{d}
Syringic acid	418.5±30.6^{b}	932.18±40.9^{a}	129.47±20.6^{c}	97.6±11.3^{d}	65.37±11.5^{e}
Vanilic acid	74.77±23.8^{b}	108.1±41.3^{a}	12.78±3.6^{d}	46.47±12.5^{c}	41.31±12.3^{c}
Rosmarinic acid	123.17±20.6^{d}	206.21±50.3^{c}	251±45.6^{a}	85.95±14.6^{e}	240.74±35.8^{b}
Ferulic acid	16.49±2.3^{d}	53.46±11.3^{a}	36.43±8.6^{b}	21.49±5.6^{c}	9.26±2.1^{e}
p-Coumaric acid	18.29±3.2^{b}	42.77±8.5^{a}	11.7±2.3^{c}	2.3±0.5^{e}	5.06±1.1^{d}
trans-Cinnamic acid	22.93±4.1^{bc}	23.71±6.3^{b}	20.11±3.6^{c}	15.52±3.2^{d}	30.73±7.6^{a}

Values followed by the same small letter did not share significant differences at 5 % (Duncan test).

During development, the percentage of syringic acid decrease whereas those of rosmarinic acid and sinapic acid presented increase. Wettasinghe *et al.*[6] showed the presence of these three phenolic acids in borage seed.

Antiradical activity: The DPPH radical scavenging is a commonly used method to evaluate the ability of plant extracts to scavenge free radicals generated from DPPH reagent[19]. The methanolic extract of borage leaves at 66 days after cotyledon appearing (DACA) (IC_{50} equal at 120 μg mL^{-1}) showed very high hydrogen-donating capacity towards the DPPH radical (Table-3).

TABLE-3
ANTIRADICAL ACTIVITY (TEST DPPH) EXPRESSED BY IC_{50} VALUES (µg/mL) OF DIFFERENT BORAGE LEAF EXTRACTS

Days after cotyledon appearing (DACA)	60	66	78	91	105
IC_{50} µg/mL (DPPH)	160 ± 0.80^{c}	120 ± 1.89^{d}	275 ± 1.12^{ab}	255 ± 0.75^{b}	280 ± 2.11^{a}

Values followed by the same small letter did not share significant differences at 5% (Duncan test).

The comparison of total phenolic amounts and IC_{50} values in stalk leaves during their growth, allowed us to conclude that antioxidant activity does not depend on the amount of total phenolics. Indeed, at 66 days after cotyledon appearing where we detected the best antioxidant capacity, the amount of total phenolics was weak (3.17 mg EAG g^{-1} DW) in comparison to other stages such as at 105 days after cotyledon appearing (4.80 mg GAE g^{-1} DW and IC_{50} value of was 280 µg mL^{-1}). This idea was also reported by Mhamdi *et al.*[7] which showed this result in borage seed and by Elzaawely *et al.*[8] that showed that although seeds extract contained lower amount of total phenolics compared to flowers, it showed a high DPPH radical-scavenging efficiency similar to that of flower extract.

Ma olepsza and Urbanek[20] showed that the ability of plant material to scavenge free radicals is due to an overall reaction of its active constituents and depends both on their structure and concentration.

Hence, it appears that antioxidant activity could be related to the nature of the compound and not necessarily to the amount. For example, at 66 days after cotyledon appearing we reported the highest antioxidant activity corresponding to the highest amounts of syringic acid. This acid is known by its antioxidant activity. In fact, Que *et al.*[21] noticed that catechine and syringic acid were the dominant phenolic compounds in rice wines and highly correlated with the antioxidant activities in all rice wines tested. Hee *et al.*[22] showed that syringic acid was the one of many strongest antioxidants in wheat bran extracts. So the antiradical activity could be content correlated with phenolic components present in the extract and not with total phenolics.

So, the phenolic composition indicated that borage leaves are good source of bioactive compounds such as syringic acid which are known by their antimicrobial and antioxidant activities.

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