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HPLC-UV profile of *Genista ulicina* Spach. (Fabaceae) extracts and *in vitro* antioxidant activity

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Abstract

This study was designed to perform a qualitative and quantitative analysis of the phenolic and flavonoid contents and evaluate the antioxidant activity of ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) extracts of the aerial parts of *Genista ulicina* Spach. from Algeria. The qualitative analysis of plant extracts was carried out by RP-HPLC using UV detector, whereas the quantification of total phenolic and flavonoid contents was completed according to the Folin-Ciocalteu procedure and aluminium chloride colorimetric method respectively. To evaluate the extract's antioxidant activity, two in vitro antioxidant tests were employed: DPPH and β-carotene bleaching assay. The HPLC/DAD chromatogram showed several peaks indicating the presence of phenolic acids, flavonoids and isoflavonoids in both extracts. The total phenolic content (TPC) ranged from 62.56 and 50.45 mgGAE/g extract, while the total flavonoids content (TFC) varied between 53.1 and 48.4 mgQE/g extract for EtOAC and *n*-BuOH respectively. The EtOAc extract showed a maximum inhibition value (78.15%) at 150µg/mL using DPPH test and highest antioxidative power (82.42%) using β-carotene bleaching assay comparing with standards. The HPLC-UV analysis showed the richeness of both extracts in phenolic and flavonoid contents. The EtOAc extract exhibited good antioxidant activities comparing to the *n*-BuOH extract. Thus *Genista ulicina* could be indicated as a plant of phytopharmaceutical importance.

Keywords : HPLC-DAD, Phenolic compounds, Antioxidant activity, *Genista ulicina, Fabaceae.*

Introduction

The use of natural products and search for drugs derived from plants with therapeutic properties is as ancient as human civilization [1]. These therapeutic properties, such as antioxidant, antibacterial, antiviral, analgesic, antispasmodic, neuroprotective, cytostatic, and anti-inflammatory activity [2–7], are induced by many substances, including some vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, etc. [8]. Among the natural products present in *Genista* species (Fabaceae), are isoflavonoids which reflect interesting biological activities, such as hypoglycemic [9], antioxidant [10], antiulcer [11], anti-inflammatory [12], estrogenic and cytotoxic activity against different human cancer cell lines [13]. As a part of our continuing study of *Genista* species (Fabaceae), occurring in Algeria [14-19], we have examined *Genista ulicina* Spach., which is endemic to North Africa [20]. This medicinal herb has been used for traditional healing by many people in the east region of Algeria. To the best of our knowledge, reports on the phytochemical screening of *G. ulicina* are scanty and there is no report on its biological evaluation in the literature. Thus, the main objective of the present work is the detection by HPLC-UV then the quantification of phenolic and flavonoid contents in EtOAc and *n*-BuOH extracts of the aerial parts of *G. ulicina*. The later extracts were subjected to DPPH radical scavenging activity and β-Carotene/linoleic acid bleaching assay to evaluate the antioxidant activities for the first time.

Materials and methods

Plant material

Aerial parts of *Genista ulicina* Spach.were collected during the flowering phase in June 2009, in the East of Algeria, and was authenticated by Dr. D. Sarri (Biology Department, University of

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M'Sila, Algeria). A voucher specimen (LGU06/09) has been deposited in the Herbarium of the VARENBIOMOL Unit Research, University Frères Mentouri Constantine, Algeria.

Extraction

The dried aerial parts of *G. ulicina* Spach. (495 g) were macerated with EtOH: H_2O (70:30 v/v) three times for 72 hours. The crude extract was concentrated and diluted with 250 ml H_2O. The obtained aqueous solution was extracted with petroleum ether aimed to remove chlorophyll. The remaining aqueous solution was extracted successively with $CHCl_3$, EtOAc and *n*-BuOH giving 2.04 g, 1.31g and 21.76 g of extracts respectively. The EtOAc and *n*-BuOH extracts were submitted to HPLC/UV analysis and quantification of phenolic and flavonoid contents was performed by spectrophotometry as well as the evaluation of the antioxidant activity.

Determination of total phenolic and total flavonoid contents

Determination of total phenolic content

To determine the total phenolic content (TPC) in both extracts, the method reported by Singleton et al. (1999) [21] was used and slightly modified. Gallic acid was used as a standard (the concentration range was 10 to 250µg/ml) and the total phenolic content was expressed as GAE in mg/g of extract.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined using the method described by Quettier et al. [22]. This method is based on the formation of flavonoid-ferric complex providing an absorbance maximum at 415 nm. The total flavonoid content was determined using a standard curve with quercetin (the concentration range was 10 to 250µg/ml). Values were expressed as QE in mg/g of extract.

Detection of phenolic and flavonoid compounds by HPLC

The HPLC/UV analysis of phenolic and flavonoid compounds in both extracts was carried out using a HPLC system [15]. Samples of 20 µl (0.32 mg/ml of EtOAc extract and 0.16 mg/ml of *n*-BuOH extract) were injected. The eluting system: Water (0.11% phosphoric acid conc.), Acetonitrile (ACN) were used as solvents A and B respectively, with the flow rate of 1.0 ml/min. The eluting gradient was: 5 % of B at 0 min, 30 % of B at 40 min, 90 % of B at 70 min and 5 % of B at 90 min. A wavelength of 340 nm was used for the detection of flavonoids. The tentative identification of the class of each compound was carried out comparing the retention time and UV spectra of the peaks with the literature data.

Antioxidant activity

DPPH radical scavenging activity

In order to evaluate the free radical scavenging activity of both extracts, the DPPH test was used. DPPH solution (0.2mM) was prepared in methanol. Each extract was mixed with methanol to prepare the solution (1mg/ml). Freshly prepared 2 ml of DPPH solution was added to 2 ml of various concentrations (10-250µg/ml) of each extract. After 30 min at room temperature in darkness, absorbance was measured at 517 nm. Ascorbic acid was used as a reference compound [23] and the percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with the extract) using the following formula :

$$\text{Radical scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control}] 100,$$

where: $A_{control}$ is the absorbance of blank, A_{sample} is the absorbance of the sample.

β-Carotene/linoleic acid bleaching assay

The antioxidant activity using β-Carotene/linoleic acid bleaching assay was determined by the method described by Miraliakbari and Shahidi [24] with slight modifications. A stock solution of *β*-carotene and linoleic acid was prepared with 0.5 mg of *β*-carotene in 1 ml chloroform, 20 mg of linoleic acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of oxygenated distilled water was then added to the residue. A quantity of 350 µl of each sample solution (2g/l) prepared in DMSO was added to 2.5 ml of the above mixture in test tubes which were incubated at 50 °C for 2 h. BHT and BHA were used as positive controls. The absorbances were measured at 470 nm each 20 min during 2 h. Antioxidant activities (inhibition percentage) of the samples were calculated using the following equation:

Inhibition % = (Absorbance of β-carotene at 2 h /Initial absorbance of β-carotene) 100.

Statistical analysis

All assays were carried in triplicates and results expressed as means ± standard deviation. Statistical comparisons were done with the Student's test. Differences were considered to be highly significant at $P< 0.01$ and significant at $P< 0.05$.

Results

Estimation of total phenolic (TPC) and total flavonoid contents (TFC) in *G. ulicina* Spach

Table 1: TPC and TFC of *G. Ulicina* extracts

Extracts	**TPC** (mgGAE/g extract)	**TFC** (mgQE/g extract)
EtOAc	62.84±1.66	53.06±0.98
***n*-BuOH**	50.45±0.83	48.39±1.56

TPC and TFC of both EtOAc and *n*-BuOH extracts of *G. ulicina* Spach are given in Table 1. Results from this table showed that The TPC level was higher in the EtOAc (62.56±1.66 mgGAE/g extract) compared to the *n*-BuOH (50.45±0.84 mgGAE/g extract). The EtOAc fraction was also richer in flavonoids (53.06±0.98 mgQE/g extract) than the *n*-BuOH extract (48.39±1.56 mgQE/g extract).

HPLC chromatogram with UV detection

Both extract EtOAc and *n*-BuOH of the aerial parts of *G. ulicina* Spach. were analysed by HPLC-UV method. The resulting chromatogram obtained at 340 nm and the UV spectra of selected peaks are shown in figure 1 (1a and 1b). Peak numbers were consistent with those shown in table 1 (1a and 1b).

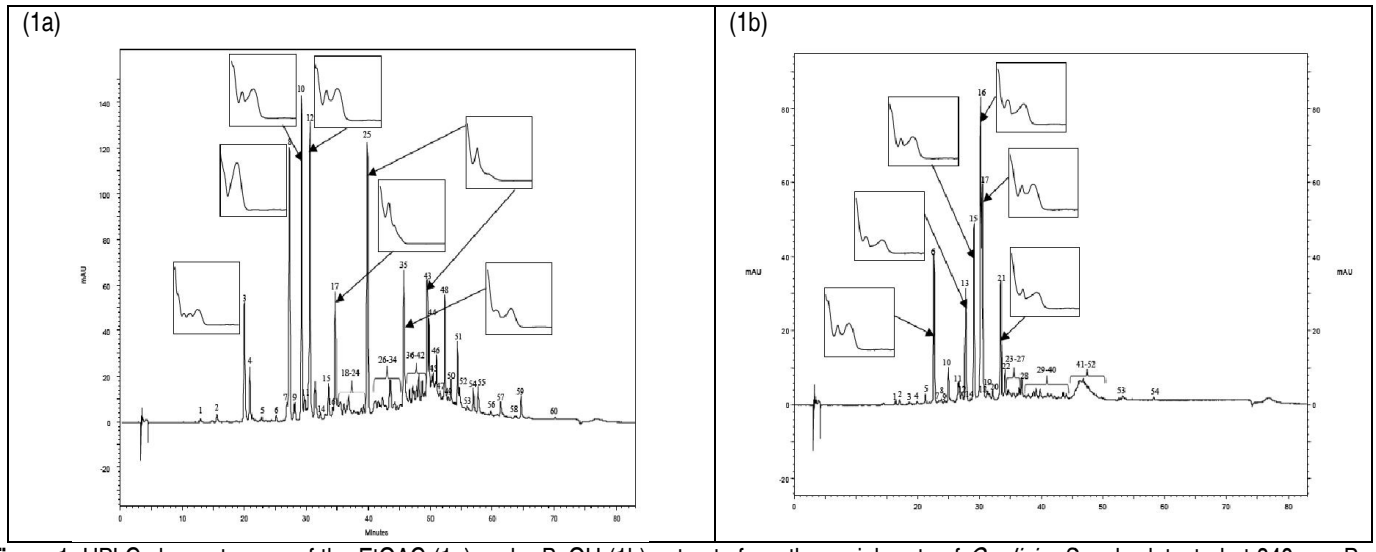

Figure 1: HPLC chromatogram of the EtOAC (1a) and *n*-BuOH (1b) extracts from the aerial parts of *G. ulicina* Spach. detected at 340 nm. Peak numbers were consistent with those shown in table 1.

As shown in table 1 (1a and 1b), tentative identifications have been made by examination and comparison of their retention time (t_R) and UV spectra with literature data.

Table 2: Tentative identification of phenolic and flavonoid contents in EtOAc and *n*-BuOH Extracts from the aerial parts of *G. ulicina* Spach. by HPLC-DAD.

Table 1a : EtOAc extract				**Table 1b: *n*-BuOH extract**			
Peak no	R_T (min)	λ_{max} (nm)	Tentative identification	Peak no	R_T (min)	λ_{max} (nm)	Tentative identification
3	19.80	258, 295, 344	Flavonoid	6	22.66	272, 335	isovetexin
8	27.10	300, 320	Rosemarinic Acid	13	27.66	258, 303, 352	isoquercetrin
10	29.12	267, 333	Flavonoid	15	29.09	258, 336	vetexin
12	30.44	270, 335	Flavonoid	16	30.22	260, 303, 352	isoquercetrin
17	34.55	254, 284 sh, 316 sh	Isoflavonoid	17	30.45	270, 338	Flavonoid
25	39.71	254, 316 sh	Isoflavonoid	21	33.46	265, 348	vetexin
35	45.55	255, 346	Flavonoid				
43	49.35	260, 325 sh	Isoflavonoid				

Antioxidant activities

DPPH assay

In the DPPH test, the ability of a compound to act as donor for hydrogen atoms or electrons was measured spectrophotometrically. Figure 3a shows the free radical scavenging ability of EtOAc and *n*-BuOH extracts.

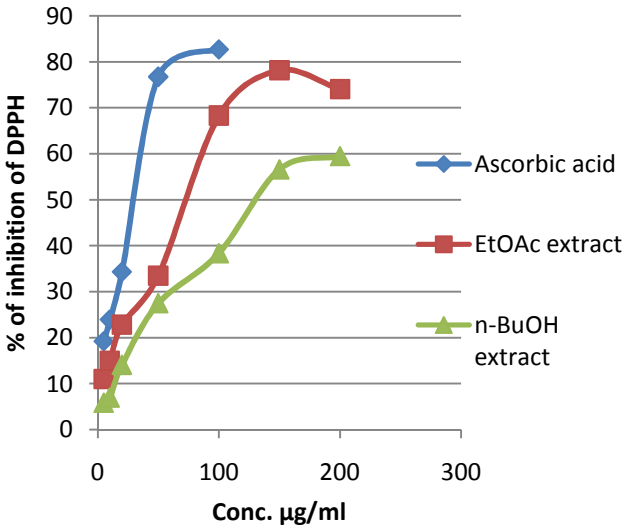

Figure 2: DPPH radical scavenging activities of *G. ulicina* extracts and standard. Each value represents a mean ± SD (n=3), P<0.05.

Figure 2 shows the free radical scavenging ability of both EtOAc and *n*-BuOH extracts indicating a gradual increase in DPPH radical scavenging activity in a dose dependent manner. At 100 µg/mL, the EtOAc extract (68,33%) showed the highest DPPH radical scavenging activity, although *n*-BuOH extract (38,39%) exhibited weaker antioxidant effects than EtOAc extract,which was about twice lower than that expressed by standard reference (82,70%). EtOAc extract showed a maximum inhibition value (78.15%) at 150µg/mL, although this maximum effect needed 200µg/mL for *n*-BuOH extract.

B-carotene

Both EtOAc and *n*-BuOH extracts of *G. ulicina* Spach. were examined for their β-carotene/linoleic acid bleaching assay which was based on the linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules.

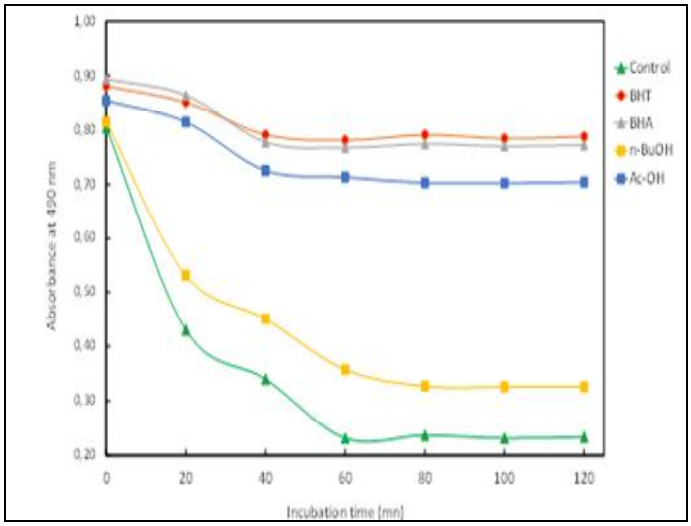

Figure 3: Effect of *G. ulicina* antioxidant on oxidation of β-Carotene/linoleic acid.SD (n=3), P<0.05.

As a result, β-carotene molecules lose their double bonds by oxidation in this model system (Fig 3). At the concentration of 1 mg/mL, both extracts inhibited peroxidation of linoleic acid and subsequent bleaching of β-carotene to various degrees. The high absorbance noticed from EtOAc recorded the highest antioxidative power of 82.42 %. This value was comparably near those of the synthetic antioxidants BHA and BHT (86.34 and 89.39%) respectively. Apparently, the *n*-BuOH extract is the less effective which recorded the lower antioxidative effect of 40.04% (Figure 3).

Discussion

The chromatogram presented in figure 1a showed that the EtOAc extract contained several main peaks which had intense absorption at the selected λ (340 nm). According to their UV spectra profiles, these compounds are phenolic and flavonic types, especially, compounds corresponding to peaks 10, 12, 17 and 25 seem to be the main important. Peaks 10 (t_R= 29.12 mn) and 12 (t_R= 30.44 mn) presented spectral characteristics of flavone skeleton with UV λ_{max} at 267, 333 nm and 270, 335 nm respectively. The same chromatogram showed peaks 17 (t_R= 34.55 mn) and 25 (t_R= 39.71 mn) which presented spectral characteristics of an isoflavone skeleton with UV λ_{max} at 254, 284 sh, 316 sh nm and 254, 316 sh nm respectively.

However, on the chromatogram of the *n*-BuOH extract (figure 1b), the peak 16 (t_R= 30.22 nm) is strongly prominent. This compound presented spectral characteristics of a flavonol skeleton with UV λ_{max} at 260, 303 and 352 nm. Generally, most of the constituents of the EtOAc extract had similar UV spectra with a maximum of absorbance of band II varying between 254 and 268 nm and a maximum of band I as shoulder varying between 316 and 326 nm

indicating isoflavonoid like character of the compounds [25]. Isoflavones may be distinguished from flavones and flavonols by their UV spectra which typically exhibit intense band II absorption with a shoulder peak representing band I [26]. Furthermore, the main constituents of the *n*-BuOH extract showed a similar UV spectrum to flavone type. These results clearly indicate the richness of this species in flavones, flavonols and isoflavones compounds.

Several studies showed a correlation between antioxidant effect and phenolic content, this effect increased proportionally to the polyphenol content [22]. The highest activities of *G. ulicina* Spach. appeared in EtOAc fraction; the free radical scavenging ability (DPPH) and its potential to inhibit lipid peroxidation (*β*-carotene assay) may most likely be attributed to their phenolic and flavonoid contents. Besides the presence of appreciable amount of antioxidant compounds such as flavonoid and Isoflavonoid plus phenolic compounds (Rosemarinic Acid) indicated by HPLC analysis have been reported. These polyphenols could also contribute to chain radical reactions termination; by donating hydrogen atoms to the peroxy radical. The antioxidant activity of EtOAc compounds was significantly heigher than those of *n*-BuOH as mentioned by HPLC data (isovetexin, isoquercetrin, vetexin) which were probably less active than those found in EtOAc fraction. The ability of phenolic compounds scavaging radicals' ability depends on the availability of properly oriented functional groups [27]. Therefore, the low activity of the *n*-BuOH extract may be due to the presence of phenolic glycoside groups or not having suitable groups that could act as antioxidant.

Conclusion

The phytochemical screening of this investigation attested the presence of several secondary metabolites in *G. ulicina.* The fractionation of MeOH extract into two different phases using EtOAc and *n*-BuOH solvents resulted into an interesting distribution of TPC and TFC. The EtOAc extract exhibited good antioxidant activities (DPPH scavinging, β-bleaching) comparing to the *n*-BuOH extract. In addition, we noticed the presence of more active substances in EtOAc extract than those found in *n*-BuOH extract reflects the different redox properties. Thus *G. ulicina* could be indicated as a plant of phytopharmaceutical importance.

Author's contribution

MR is supervisor of this study participated in the design conception of this study and involved in drafting the manuscript.
CK prepared the plant extracts and phytochemical studies.
ME contributed in carrying HPLC-DAD analysis
BO and SR collected the plant.
SD identified the plant material
MA contributed in carrying antioxidant activity and statistical analysis, and helping in the drafting the manuscript.
BF and BS have general supervision of the research group.

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Conflict of Interest

The authors declare no conflict of interest.

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