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HPLC Analysis and Antioxidant Properties of Algerian *Lepidium draba* Ethyl acetate Extract

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Abstract: The aim of this study was to identify and quantify the phenolic compounds of the Ethyl acetate extract from *Lepidium draba* using HPLC-TOF/MS; and to investigate the antioxidant potencies. In this work, we have found that the Ethyl acetate soluble fraction of *L. draba* is a good source of antioxidant, which is the result of its phenolic acids and flavonoids. Five methods were conducted, to determine the antioxidant components of the Ethyl acetate extract: DPPH, ABTS, CUPRAC, Reducing power assay and Metal chelating assay. Nineteen compounds from which 13 phenolic acids and 6 flavonoids known for their pharmacological properties were identified by using HPLC-TOF/MS technique: Gallic acid, Gentisic acid, Chlorogenic acid, 4-Hydroxy benzoic acid, Protocatechuic acid, Caffeic acid, Vanillic acid, 4-hydroxybenzaldehyde, p-coumaric acid, Chicoric acid, Ferulic acid, Rosmarinic acid, Salicylic acid, Catechin, Rutin, Hesperidin, Apigenin-7-glucoside, Quercetin, and Kaempferol. All These compounds were identified and quantified in *Lepidium draba* for the first time. The IC_{50} and $A_{0.50}$ of the Ethyl acetate extract were 305.69±1.22, 39.42±0.45, 152.27±0.38 and 183.83±2.47 µg/mL in DPPH, ABTS, Metal chelating assay, and CUPRAC. However, the results indicate that *L. draba* is a good source of natural antioxidants.

Key words: *Lepidium draba*, HPLC-TOF/MS, phenolic acids, antioxidant activity.

Introduction

Oxidative stress is a new concept that has been widely implicated in biomedical sciences during the last 20 years. It causes different diseases such as cancer, hypertension and Diabetes etc. Antioxidants are class of molecules, which are considered as an essential part of optimal health [1], Phenolic compounds as secondary metabolites have the potential to act as natural antioxidants [2].

The genus *Lepidium*, which belongs to the

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Brassicaceae family contains about 250 species [3], it mainly consists of alkaloids, saponins, anthracene glycosides, carbohydrates, proteins, amino acids, flavanoids, and sterols [4-5]. *Lepidium draba* L. (Brassicaceae; syn. *Cardaria draba*) called Haref machriki, which is a perennial herb with a stout, deep tap-root and branched woody rootstock. Flowering stems somewhat angular, 20-90 cm high, erect, rigid, with short adpressed, simple hairs, branched above with numerous leaves [6]. It is indigenous to Southwestern, Central Asia [7], Europe [8], North America [9] and North Africa including Algeria [10].

Very little data on compositional studies of *Lepidium draba* exists [11-17]. In previous studies we reported some flavonoids from aerial parts of this plant and we investigated the antioxidant activity of *n*-butanol extract using only free radical-scavenging assay DPPH [18]. The purpose of the present work was the identification and quantification of phenolic compounds present in the ethyl acetate extract using HPLC-TOF/MS; and it was subjected to DPPH radical scavenging activity, ABTS, CUPRAC, reducing power; and metal chelate tests to evaluate the antioxidant activities.

Materials and methods

Plant material

Aerial parts of *Lepidium draba* L. were collected from M'sila, located in the Northeastern of Algeria in May 2014 and authenticated by the botanist Dr. Khellaf Rebbas of M'Sila University. The voucher specimen (SB/KR, 002) is maintained in the botanical laboratory of the department.

Preparation of extracts

Air-dried and powdered aerial parts (1200 g) of *Lepidium draba* L. were exhaustively extracted from maceration with methanol /water (80/20) at room temperature. After filtration, evaporation of the solvent under reduced pressure and temperature. The residue was dissolved in water and extracted with chloroform, ethyl acetate and *n*-butanol. The organic solutions were concentrated in vacuum (up to 35°C) to obtain the following extracts $CHCl_3$ (1.96 g), EtOAc (8.46 g) and *n*-butanol (3.18 g).

Quantitative analysis by HPLC-TOF/MS analysis

The quantification of flavonoids, phenolic acids in the ethyl acetate extract was carried out using 1260 Infinity HPLC system (Agilent technology) coupled with TOF (6210 Time of Flight) LC/MS detector and ZORBAX SB-C18 (4.6 x 100 mm, 3.5 µm) column. The mobile phase comprised of solvent mixtures (A) water (ultra-pure) with 0.1 % formic acid and (B) acetonitrile, respectively. The flow rate was 0.6 mL min^{-1}. The column temperature was maintained at 35°C. The solvent program was as follows: 0-1 min 10 % B; 1-20 min 50 % B; 20-23 min 80 % B; 23-30 min 10 % B. The injection volume was 10 µL. Ionization mode of HPLC-TOF/MS instrument was negative operating with nitrogen gas at 325°C, and the gas flow of 10.0 L/min, nebulizer of 40 psi, capillary voltage of 3500 V; finally, fragmentor voltage of 175 V. The EtOAc extract (200 ppm) was dissolved in methanol at room temperature. The particulates of the samples were removed using a PVDF (0.45 µm) filter [19]. The limits of detection were found to be between 25 and 2500 ppb using HPLC-TOF/MS.

Antioxidant activity

Antioxidant activity by DPPH assay

The antioxidant activity was established according to method described by Blois [20]. In the radical form, DPPH has a deep violet color and it pale yellow when presence of antioxidant product followed by decreases in its absorbance. Briefly, in a 96-well plate, 160 µl of $DPPH^{\cdot}$ solution at 0.1 mM was added with 40 µl of sample solutions in methanol at different concentrations. The plate was kept in the dark at room temperature. The absorbance was measured at 517 nm after 30 mn of incubation. BHA, BHT, ascorbic acid and tannic acid were used as antioxidant standards for comparison of the activity. The scavenging activity of the extract was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ and A_{sample} are the absorbances of the reference and sample obtained from the UV-visible spectrophotometer, respectively.

Antioxidant activity by ABTS assay

The $ABTS^{\bullet+}$ scavenging activity was evaluated according to the method of Apak *et al*.,[21]. The $ABTS^{\bullet+}$ solution was prepared by the mixture of 7 mM ABTS with 2.45 mM potassium persulfate using the water as solvent. The $ABTS^{\bullet+}$ solution was diluted in ethanol or water to get an absorbance of 0.708±0.025 at 734 nm. In each well the reaction mixture containing 40 µL of sample solution in methanol at different concentrations and 160 µL of $ABTS^{\bullet+}$ solution. After incubation is complete, the absorbance was measured at 734 nm using a 96-well microplate reader. Each assay for all samples was carried out in triplicate. BHA, BHT, ascorbic acid and tannic acid were used as antioxidant standards for comparison of the activity. Percentage inhibition of all samples was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = [A_{control} - A_{sample} / A_{control}] \times 100$$

where $A_{control}$ and A_{sample} are the absorbances of the reference and sample obtained from the UV-visible spectrophotometer, respectively.

Cupric reducing antioxidant capacity (CUPRAC)

The CUPRAC was determined according to the method described previously [22-23]. 40 µl of the Ethyl acetate solution at different concentrations and 50 µl of $CuCl_2$ solution (10 mM) were added into a 96 well round-bottomed plate. Following this, 50 µl of neocuproine solution (7.5 mM) and 60 µl of NH_4Ac buffer (1 M, pH 7.0) solution were given to each well. After 60 min the absorbance was measured at 450 nm. Results were given as $A_{0.5}$ corresponding to the concentration indicating 50 % absorbance intensity compared with the absorbance of BHA, BHT, ascorbic acid and tannic acid, which were used as antioxidant standards.

Reducing power assay

The reducing power of ethyl acetate extract was determined according to the method of Oyaizu (1986) [23]. 10 µl of sample solutions at different amounts were mixed with 40 µl of 0.2 M phosphate buffer (pH 6.6) and 50 µl of potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 min, 50 µl of trichloroacetic acid (10 %) were added. The solutions obtained was mixed with distilled water (40 µl) and 10 µl of ferric chloride (0.1 %), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates greater reducing power. BHT, BHA, ascorbic and tannic acid were used as antioxidant standards for comparison of the activity. The increase of reducing power by the extract and standard was calculated as follows:

$$\text{\% Reducing power} = [(A_{test}/A_{blank})-1] \times 100$$

Where, A_{test} and A_{blank} are absorbance of sample and blank solutions, respectively.

Metal chelating activity assay

The ferrous ion chelating potential of ethyl acetate extract of *L. draba* was measured according to the method of (Decker and Welch with slight modifications [24]. 40 µL of $FeCl_2$ (0.2 mM) was mixed at ethyl acetate extract of different concentrations. The reaction was initiated by the addition of ferene (0.5 mM). The mixture was shaken well then incubated for 10 min at room temperature. Absorbance was determined at 593 nm. EDTA was used as antioxidant standard and percent chelation was calculated using the following equation. The results were given as IC_{50} value (mg/mL) (50 % inhibition):

$$FE^{+2} \text{ chelating effect (\%)} = [A_{control} - A_{sample} / A_{control}] \times 100$$

Statistical analyses

All the experimental results are mentioned as a mean ± standard deviation of three trials.

Results

Identification and quantification of compounds by HPLC-TOF/MS analysis

The ethyl acetate extract was obtained from the aerial parts of *L. draba* and analyzed by HPLC-TOF/MS. The identification of individual compounds were performed on the basis of their retention times and mass spectrometry by comparison with those of different standards.

Nineteen compounds were identified and listed

Table 1. HPLC-TOF/MS analysis of ethyl acetate extract of *Lepidium draba* L.

No.	Compounds	Contents (mg/kg plant)	RT (min)
1	Gallic acid	1.47	2.40
2	Gentisic acid	176.10	4.50
3	Chlorogenic acid	2.50	5.50
4	Catechin	0.50	5.80
5	4-Hydroxy benzoic acid	3880.50	7.00
6	Protocatechuic acid	92.10	7.10
7	Caffeic acid	819.90	7.60
8	Vanillic acid	4.20	7.90
9	Rutin	10.80	9.20
10	4-hydroxybenzaldehyde	1.10	9.40
11	Ferulic acid	61.80	10.60
12	Hesperidin	7.11	10.80
13	Apigenin-7-glucoside	130.80	11.40
14	Rosmarinic acid	4040.00	12.00
15	p-Coumaric acid	0.90	12.10
16	Salisylic acid	24.20	13.10
17	Quercetin	1.82	14.00
18	Kaempferol	0.92	15.70
19	Chicoric acid	35.30	18.00

in table 1. As it can be seen, the analyzed extract comprises, phenolic acids and flavonoids. These phenolic acids were identified as gallic acid, gentisic acid, chlorogenic acid, protocatechuic acid, vanillic acid, 4-hydroxybenzaldehyde, p-coumaric acid, chicoric acid, ferulic acid, salisylic acid. rosmarinic acid have the highest concentration 4040 mg/kg followed by 4-hydroxybenzoic acid 3880.5 mg/kg and caffeic acid 819.9 mg/kg. While, flavonoids were identified as catechin, rutin, hesperdin, apigenin-7-glucoside, quercetin and kaempferol.

Determination of anti-oxidant activity

In this study the antioxidant activity of ethyl acetate extract from *L. draba.* was evaluated by five different methods: DPPH, ABTS, reducing power, CUPRAC and metal chelating tests. The results, except for CUPRAC and reducing power assay, were recorded as IC_{50}. As shown in table 2 the antioxidant activity of the tested extract was compared to those of standards BHA, BHT, ascorbic acid, tannic acid and EDTA. The extract displayed a good activity in ABTS ($A_{0.50}$ = 39.42± 0.45 µg/mL), in the metal chelating (IC_{50}=152.27± 0.38 µg/mL) and in CUPRAC (IC_{50}= 183.83±2.47 µg/mL). In DPPH the ethyl acetate extract exhibited a fairly well activity (305.69±1.22 µg/mL). It was observed that in reducing power, the antioxidant activity was lower than those found for antioxidant standards ($A_{0.50}$>800 µg/mL)

Discussion

The analysis of the ethyl acetate extract from *Lepidium draba* using HPLC-TOF/MS reveals the presence of the phenolic acids and flavonoids. These compounds showing relevant antioxidant properties [26]. Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants. Rosmarinic acid, 4-Hydroxy benzoic acid and caffeic acid were the main components of ethyl acetate extract. Rosmarinic acid has beneficial properties, including anti-inflammatory and antibacterial effects [27]. 4-Hydroxy benzoic acid was reported to have antibacterial, antifungal, antialgal, antimutagenic, antisickling and estrogenic activities [28]. Caffeic acid exhibits cytoprotective

Table 2. IC_{50} and $A_{0.5}$ values of antioxidant activity of ethyl acetate extract by the DPPH, CUPRAC, ABTS, reducing power and metal chelating assays

	Antioxidant activity				
	DPPH assay	**ABTS assay**	**Metal chelating assay**	**CUPRAC assay**	**Reducing power assay**
		IC_{50} (µg/mL)		**$A_{0.50}$ (µg/mL)**	
Ethyl acetate extract	305.69±1.22	39.42±0.45	152.27±0.38	183.83±2.47	>800
BHA[b]	6.14±0.41	1.81±0.10	NT	5.35±0.71	7.99±1.87
BHT [b]	12.99±0.41	1.29±0.30	NT	8.97±3.94	>800
Ascorbic acid [b]	13.94±2.81	1.74±0.10	NT	12.43±0.09	6.77±1.15
Tannic acid [b]	7.74±0.19	1.01±0.16	NT	3.76±0.73	41.07±2.36
EDTA[b]	NT	NT	12.11±0.32	NT	NT

[a]IC_{50} and $A_{0.50}$ values expressed are means ± SD of three parallel measurements ($p < 0.05$)
[b] Reference compounds: BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene; EDTA: Ethylenediaminetetraacetic acid,; NT: Not tested

effect on endothelial cells against oxidized low density lipoprotein and inhibits the oxidation of lipoprotein [29], whereas flavonoids such as rutin and others are well known to have special activities such as antioxidant [30], antimicrobial [31], anti-inflammatory [32], antiviral [33], cardiovascular protection and antitumor effects [34]. Antioxidant capacity of *L. draba* was performed using five methods in parallel because different methods could give different results. The results in table 2 reveal that the ethyl acetate extract of *L. draba* possesses a good antioxidant activity. The antioxidant effect is because of the presence of flavonoids such as rutin, kaempferol, caffeic acid and rosmarininc acid [35]. All these results are in agreement with other studies about other species of *Lepidium* genus [4-5,18].

Conclusion

The present study evaluates the antioxidant activity of ethyl acetate extract from aerial part of *L. draba*. The extract possesses good antioxidative properties *in vitro*. The activity is confirmed by HPLC-TOF/MS analysis that reveals the presence of phenolic acids: gallic acid, gentisic acid, chlorogenic acid, 4-hydroxy benzoic acid, protocatechuic acid, caffeic acid, vanilic acid, 4-hydroxybenzaldehyde, p-coumaric acid, chicoric acid, ferulic acid, rosmarinic acid, salisilic acid, and flavonoids: catechin, rutin, hesperidin, apigenin-7-glucoside, quercetin and kaempferol. *L. draba* demonstrates an important antioxidant activity; It serves as well as a source of various bioactive products, including phenolic ompounds and Flavonoids.

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