https://doi.org/10.31925/farmacia.2023.5.5 **ORIGINAL ARTICLE**

POLYPHENOL QUANTIFICATION ON *DIPSACUS LACINIATUS* AND *ARMORACIA RUSTICANA* FROM ROMANIAN FLORA

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Abstract

In recent years, the polyphenolic compounds profile of plants has been intensively studied. These compounds protect the body against oxidative stress and certain diseases such as diabetes and cancer, where the antioxidant efficiency of the defence system is low. The plant material (harvested from Romania) was analysed for polyphenols and flavonoids using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) coupled with photo diode array and mass spectrometer. The polyphenols/flavonoids (caffeic acid, chlorogenic acid, *p*-coumaric acid, protocatechuic acid, rutin and quercetin) were identified and quantified in different part of the plants (flower, leaf, stem and roots for *Dipsacus laciniatus* and both roots and leaves for *Armoracia rusticana*). The least amount of polyphenols were found to be as expected in the root part of *Dipsacus laciniatus.* This was also sustained by the further UHPLC-MS analysis. The results revealed that the flower extract has the highest radical scavenging activity with 87.43% followed by the leaf extract with 86.63%, the stem extract with 83.54% and the root extract with 78.17%. Higher concentrations of p-Coumaric acid and ferulic acid were identified in the *Armoracia rusticana* samples whereas the *Dipsacus laciniatus* samples contained higher concentrations of both protocatechuic acid and chlorogenic acid. The results of the present study revealed that the methanolic extracts of both *Dipsacus laciniatus* and *Armoracia rusticana*, can be considered an effective source of antioxidant compounds.

Rezumat

Profilul polifenolic al plantelor a fost intens studiat în ultimii ani. Acești compuși protejează organismul de stresul oxidativ și anumite afecțiuni precum diabet și cancer când eficacitatea antioxidantă a sistemului imunitar este scăzută. Plantele (recoltate din Romania) au fost analizate în vederea determinării polifenolilor și a flavonoidelor prin cromatografie în strat subțire și cromatografie de lichide de înaltă performantă cuplată cu spectrometrie de masă. Flavonoidele/polifenolii (acid cafeic, acid clorogenic, acid p-cumaric, acid protocatecuic, rutina și quercetina) au fost identificate și cuantificate în diferite părți ale plantei (flori, frunze, tulpina și rădăcina pentru *Dipsacus laciniatus* și respectiv rădăcină și frunze pentru *Armoracia rusticana*). Cantitatea cea mai mică de polifenoli a fost identificată conform așteptărilor în rădăcina plantei *Dipsacus laciniatus*, ceea ce a fost confirmat și prin analiza UHPLC-MS. Rezultatele arată ca extractul din flori are cea mai mare capacitate de captare a radicalilor liberi respectiv 87,43%, urmată de extractul din frunze cu 86,63%, extractul din tulpină cu 83,54% și extractul din rădăcină cu 78,17%. Concentrații ridicate de acid p-cumaric și acid ferulic au fost identificate în probele de *Armoracia rusticana* în timp ce în probele de *Dipsacus lacinatus* s-au determinat concentrații mai mari de acid protocatecuic și acid clorogenic. Rezultatele acestui studiu arată că atât extractul metanolic de *Dipsacus lacinatus* cât și cel de *Armoracia rusticana* pot fi considerate o sursă eficientă de compuși antioxidanți.

Keywords: thin layer chromatography; polyphenols; flavonoids; liquid chromatography; mass spectrometry

Introduction

The genus *Dipsacus* includes 12 species of plants, annual or perennial, which are found in the Romanian flora, being spread over various areas [1].

Due to their diverse composition, *Dipsacus* species have a multiple spectrum of pharmacological actions. In practice, preparations from *Dipsacus* species have been used for the following actions: antiosteoporotic, anti-Alzheimer's, antiaging, hepatoprotective, anti-inflammatory, antioxidant [2, 3].

Having an especially varied composition, *Dipsacus* species are also notable for the presence of polyphenols. The generally mentioned polyphenolic compounds are chlorogenic acid together with its isomers, neochlorogenic acid, isochlorogenic acid and cryptochlorogenic acid, caffeic acid, syringic acid, ferulic acid, 3,5-dicafeoylchinic acid, as studies have shown [4, 5].

The polyphenols concentration in the composition is closely related to the antioxidant activity of the plant.

Therefore, *Dipsacus* extracts have been studied in this regard by different methods [6, 7].

Armoracia rusticana is a perennial crop used as a spice in both Europe and Asia. It belongs to the *Brassicaceae* family. Furthermore, both horseradish roots and leaves have been used in ethno-medicine [8, 9].

Horseradish contains a number of phenolic compounds namely rutin, quercitin, p coumaric acid which have been reported to show antioxidant activity. In addition, phenolic compounds are highly studied for their protective properties against cancer. In a study by Calabrone *et al*., *Armoracia rusticana* was evaluated for its potential use in the treatment of obesity. Furthermore, two different studies determined that the horseradish root extracts show antibacterial and anti-inflammatory effects [8-11].

Therefore, in this study we aim to determine and compare the polyphenol composition of both *Dipsacus laciniatus* and *Armoracia rusticana* from the south-western region of Romania.

Materials and Methods

Plant material

The plant material was harvested in July 2021, from *Dipsacus laciniatus*, in the blooming period, spontaneous in the environs of Tismana city, Gorj County (south-western Romania). Sample washing and cleansing were accomplished by deionized water for further processing. Plant parts (leaves, flowers, stem and roots) were separated, dried at room temperature, and milled into powder and sifted through a 160/350 μm sieve.

Plant material was harvested between 2019 and 2022, at the end of October, starting with the second year after planting from an *Armoracia rusticana* culture in Lipovu, Dolj county (southwestern Romania). The roots were harvested from depths of 20 - 30 cm of the soil. Mature leaves and roots (approx. 20 cm long) were chosen for the study. The pant products (leaves and roots) were dried at room temperature, powdered and sifted through a 160/350 μm sieve.

Extraction

A stock solution of 0.1 mg/mL from all reference compounds was prepared by dissolving 10 mg of each reference in 100 mL methanol. This stock solution was kept refrigerated at 4°C and used when needed. To obtain the concentrations for the calibration curve the stock solution was diluted with the first gradient line of the mobile phase.

The extracts were obtained by weighing 1 g of plant product to 10 mL solvent. The solvent used to extract the polyphenols was 70% methanol. The extraction procedure consisted in ultrasounding for 15 minutes and then centrifugation for another 15 minutes at 10 000 RPM. The supernatant was filtered through 0.2 μm syringe filters and deposited at 4°C until analysis.

Abbreviations: *Armoracia rusticana* roots methanolic extract (*Arm*R), *Armoracia rusticana* leaves methanolic extract (*Arm*L), *Dipsacus laciniatus* roots methanolic extract (*Dip*R), *Dipsacus laciniatus* leaves methanolic extract (*Dip*L), *Dipsacus laciniatus* stems methanolic extract (*Dip*S), *Dipsacus laciniatus* flowers methanolic extract (*Dip*F).

Chemicals and solvents

The solvents used for the sample preparation and mobile phase were acetonitrile, methanol water and formic acid and were purchased from Merck (Burlington, Massachusetts, USA). Reference compounds such as protocatechuic acid, *p*-coumaric acid, caffeic acid were obtained from Merck, while rutin and chlorogenic acid were obtained from Alfa-Aesar (Ward Hill, Massachusetts, USA). The HPTLC Si 60 F_{254} 20 cm × 10 cm glass plates were also obtained from Merck.

Equipment

For the preparation of samples, an Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany) and a Bandelin Sonorex DL102H ultrasound bath (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) were used. A CAMAG (Muttenz, Switzerland) Linomat 5 was used for sample application on the plates. The instrument was controlled through the CAMAG visionCATS v2.5 software package. The derivatization was performed using the the CAMAG Chromatogram Immersion Device. The plates were developed in twin-trough glass chambers (10 cm × 10 cm). The plates were documented using a Canon 700D digital camera (Tokyo, Japan).

HPTLC Method

The HPTLC Si 60 F254 20 cm × 10 cm glass plates were cut in half to minimise the cost of the assay. The samples were applied on the glass plates using the Linomat 5 as follows: band length 8 mm, distance from the lower edge 8 mm, dosage speed 150 nL/s, application volumes 2 μL/band. The development was performed with ethyl acetate, toluene, formic acid, water (13:4:3:2, *v*,*v*,*v*,*v*). Documentation was performed at 254 nm (UV) and 366 nm (FLD).

UHPLC-MS analysis

The separation of polyphenols was carried out on a Waters (Milford, Massachusetts, USA) Arc System coupled with a Waters 2998 PDA detector and a Waters QDa mass detector. The column used was a Waters Cortecs C18 (4.6 × 50 mm, 2.7 μm) eluting with solvent A (0.1% formic acid in water), solvent B (0.1% formic acid in methanol) and solvent C (0.1% formic acid in acetonitrile). Solvent B was set at 1% during the entire separation. The gradient was as follows: 0 - 4 min 3% o 14% C, 4 - 9 min 14% to 39% C, 9 - 11 min 29% to 3% C. The flow rate of the mobile phase was set at 1.0 mL/min. The column temperature was equilibrated to 35°C. The injection volume was 5 μL. All samples were kept at 20°C during the entire analysis.

Eluted compounds were analysed using a QDa mass detector equipped with an electrospray ionization (ESI) source. Capillary voltage was maintained at 0.8 kV, cone voltage was kept at 20 V and the mass spectra were recorded in negative ion mode in the range 100 - 800 m/z. Quantification was established in SIR mode for each compound (as shown in Table I) using external calibration curves prepared for each standard. The equipment was controlled using the EmPower 3 software package.

Results and Discussion

Polyphenolic compounds from Dipsacus laciniatus

The effect-directed profiling was performed for all the four samples in parallel, and it was estimated to take approximately 3 - 5 minutes *per* sample. The least polyphenols were found to be as expected in the root part of the plant. This was also sustained by the further UHPLC-MS analysis. In the 254 nm UV light all the three reference compounds that were applied on the plate can be seen, while under the 366 nm fluorescence light only the chlorogenic and the *p*-coumaric acid can be observed (Figures 1 and 2). After derivatization with the DPPH reagent yellow zones appear on a purple background (Figure 3). The UHPLC-MS analysis was fast and we managed to obtain a good separation on all reference compounds.

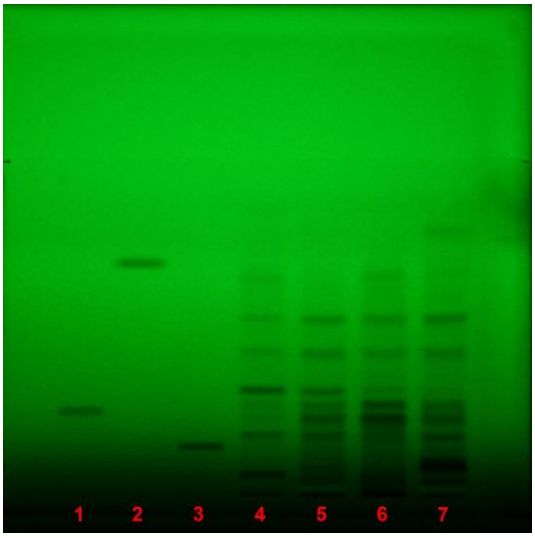

Figure 1.
254 nm (UV) chromatogram; (1) chlorogenic acid; (2) *p*-coumaric acid; (3) rutin; (4) *Dip*R; (5) *Dip*S; (6) *Dip*L; (7) *Dip*F

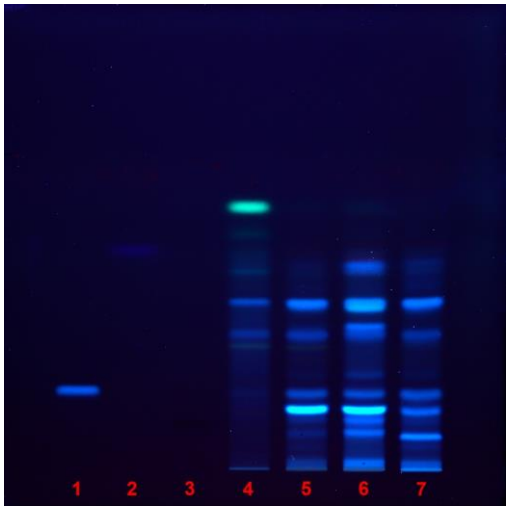

Figure 2.
366 nm (FLD) chromatogram; (1) chlorogenic acid; (2) *p*-coumaric acid; (3) rutin; (4) *Dip*R; (5) *Dip*S; (6) *Dip*L; (7) *Dip*F

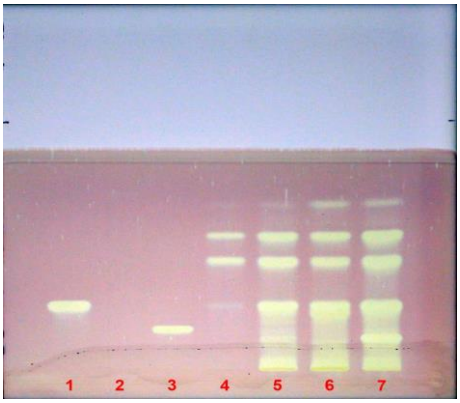

Figure 3.
DPPH derivatization in white light chromatogram; (1) chlorogenic acid; (2) *p*-coumaric acid; (3) rutin; (4) *Dip*R; (5) *Dip*S; (6) *Dip*L; (7) *Dip*F

Dipsacus laciniatus

The detection was conducted in SIR mode at the specific retention times for each reference compound to gain higher sensitivity (Table I).

The calibration curves were all linear in the specified range. We used two calibration curves for chlorogenic acid since we encountered high differences between plant parts.

Good linearity was obtained for all reference compounds with regression coefficients above 0.995.

Table I

Characteristics regarding the separation of reference compounds

Peak no.	Reference compound	m/z	RT (min)
1	Protocatechuic acid	153	1.647
2	Chlorogenic acid	353	3.068
3	Caffeic acid	179	3.311
4	*p*-Coumaric acid	163	4.440
5	Rutin	609	5.568

A Waters UHPLC Arc System coupled with a Waters QDa mass detector was used to identify and quantify the polyphenols from all the plant parts. The separation for the reference compounds was excellent. We obtained high concentrations of chlorogenic acid in all *Dipsacus laciniatus* plant organs. Most surely, this finding is responsible for the significant antioxidant effect of the plant extracts. But as observed from the DPPH HPTLC-EDA (Figure 3) plate there are also other bands at higher Rf that exert scavenging activity. Apart from chlorogenic acid, *Dipsacus* plant samples also contained higher concentrations of protocatechuic acid when compared to the horseradish extracts (Figure 4) [12].

It was reported that in addition to an antioxidant and anti-inflammatory effect, chlorogenic acid plays an important role in the lipid and glucose metabolism regulation. Furthermore, it is considered to have a beneficial effect in several disorders such as obesity, diabetes, cancer, hepatic steatosis and cardiovascular disease [13].

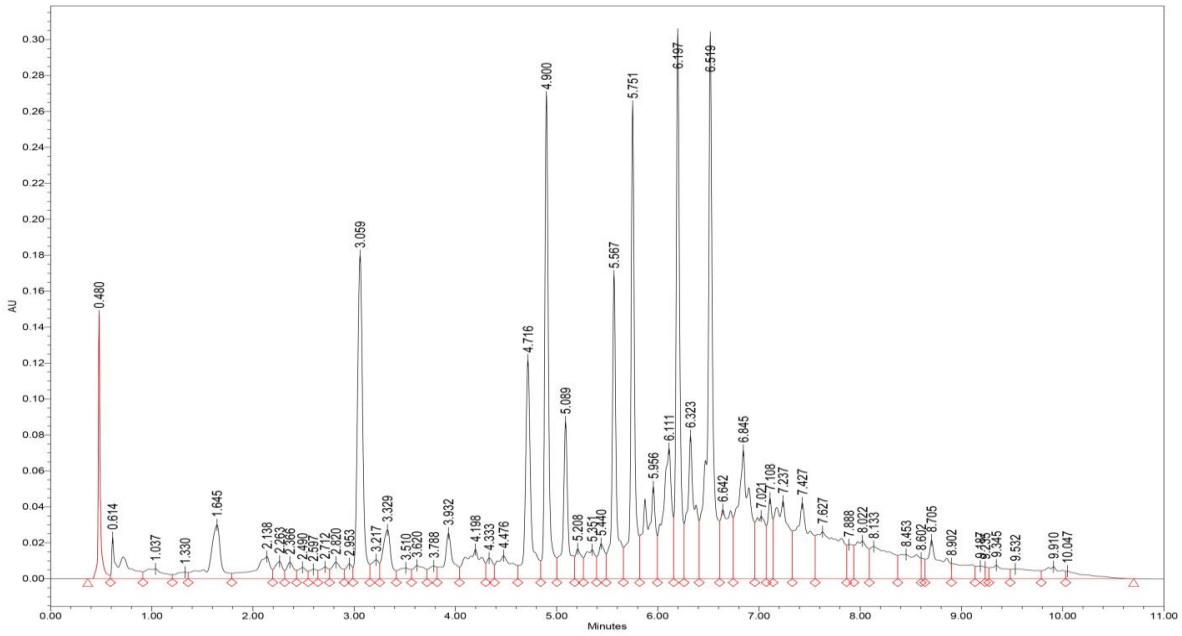

Figure 4.
UV (280 nm) chromatogram of *DipL*

Polyphenolic compounds from Armoracia rusticana

Experimental data on the UHPLC - UV - MS analysis of polyphenolic compounds from *Armoracia rusticana* are presented in Table II, Figure 5.

MS-TIC and UV (280 nm) chromatogram of *ArmF* are shown in Figures 6 and 7. A very good linearity was obtained for all the polyphenolic compounds determined in *Armoracia rusticana*.

The polyphenols detected in the analysed *Armoracia rusticana* samples are presented in Table III. In the leaves extract we identified the polyphenols in the following order rutin > p-coumaric acid > chlorogenic acid > protocatechuic acid > quercetin (Figure 7). In the root extract we identified p-coumaric acid > ferulic acid > caffeic acid > chlorogenic acid > rutin > protocatchuic acid > quercetin. Both roots and leaves of *Armoracia rusticana* show low concentrations of quercetin which is proven to have an anti-inflammatory effect.

Table II

Linearity range and detection characteristics of the reference polyphenolic compounds

Compound name	Main ion (*m/z*)	Retention time t_R (min)	Linearity range (ng)	R^2
Protocatechuic acid	153	1.639	0.5 - 5	0.998
Chlorogenic acid	353	3.063	100 - 400	0.998
Caffeic acid	179	3.306	0.5 - 5	0.999
p-Coumaric acid	163	4.435	0.5 - 5	0.999
Ferulic acid	193	5.130	1 - 10	0.998
Rutin	609	5.561	0.5 - 5	0.998
Quercetin	301	7.528	1 - 10	0.994

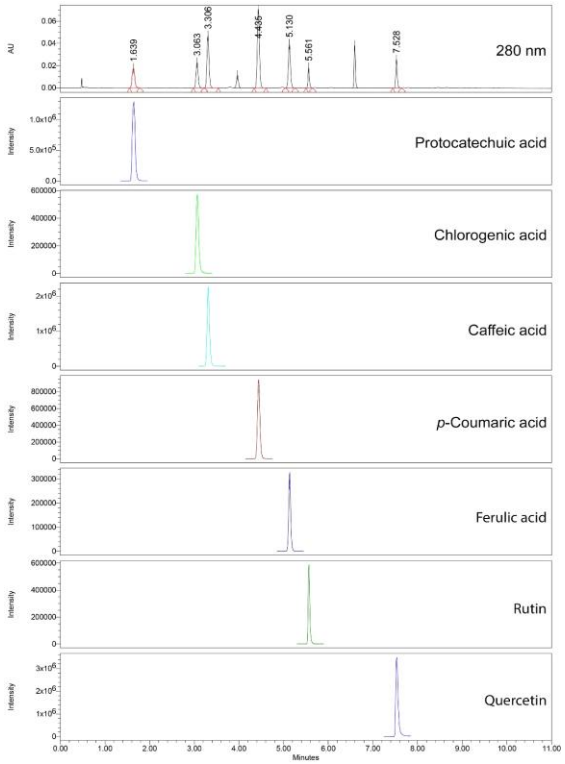

Figure 5.
UHPLC chromatograms obtained on the basis of 280 nm wavelength and selective ion recording mass spectra (MS - SIR) for the reference polyphenolic compounds in *Armoracia rusticana*

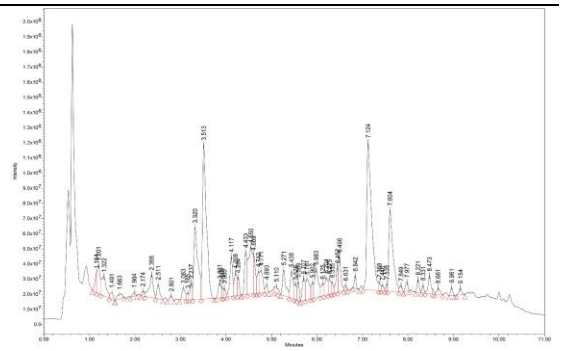

Figure 6.
MS-TIC chromatogram of *ArmF*

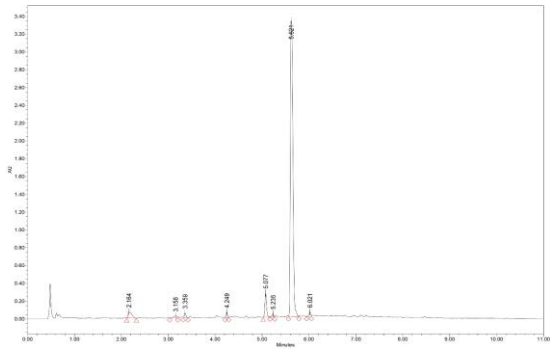

Figure 7.
UV (280 nm) chromatogram of *Arm*F

Comparative study
The concentrations for each compound in every plant part are shown in Table III.

Table III
Concentration of polyphenols found in each plant part

Plant part	Compound	Concentration found in *Dipsacus lacinatus* (µg/g)	Concentration found in *Armoracia rusticana* (µg/g)
Radix	Protocatechuic acid	9.53	0.76
	Chlorogenic acid	6.036	1.02
	Caffeic acid	1.62	3.66
	p-Coumaric acid	0.31	12.01
	Rutin	-	1.00
	Quercetin	-	0.15
	Ferulic acid	-	8.88
Stipes	Protocatechuic acid	0.92	-
	Chlorogenic acid	585.29	-
	Caffeic acid	03.17	-
	p-Coumaric acid	0.48	-
	Rutin	8.06	-
Folium	Protocatechuic acid	11.07	1.98
	Chlorogenic acid	588.19	4.36
	Caffeic acid	5.97	-
	p-Coumaric acid	0.35	6.27
	Rutin	2.54	19.74
	Quercetin	-	0.34
	Ferulic acid	-	-
Flos	Protocatechuic acid	3.88	-
	Chlorogenic acid	858.41	-
	Caffeic acid	7.10	-
	p-Coumaric acid	1.68	-
	Rutin	3.01	-

The analysed root samples of *Armoracia rusticana* showed higher concentrations of ferulic acid, caffeic acid and p-coumaric acid when compared to *Dipsacus laciniatus*. Furthermore, a higher concentration of rutin was identified in the leaves of *Armoracia rusticana*. p-Coumaric acid is a phenolic compound associated with several pharmacological effects that include anti-oxidant, antimicrobial, antiviral, anticancer, antidiabetic, anti-inflammatory. Furthermore, it was reported by Shen *et al*. that p-Coumaric acid reduces the steatosis of liver cells in mice [14]. Among the notable bio-activities of phenolic compounds, the antioxidant activities have been widely studied, including scavenging of free radicals, inhibition of lipid oxidation, reduction of hydroperoxide formation, etc [15, 16].

A detailed phenolic profile was obtained by Benabderrahim *et al*. who identified and quantified 19 compounds in both *Datura inoxia* and *Datura laciniatus*. Furthermore, the study also shows high concentrations of chlorogenic acid, caffeic acid and protocatechuic acid in the methanol extracts of *D. laciniatus* [4]. Moreover, chlorogenic acid was identified as the most abundant component in different parts of the *Dipsacus* species such as *D.asper/ D. asperoides* roots and *D. fullonum* leaves and roots [17].

The analysed root samples of *Armoracia rusticana* showed higher concentrations of ferulic acid, caffeic acid and p-coumaric acid when compared to *Dipsacus laciniatus*. Furthermore, a higher concentration of rutin was identified in the leaves of *Armoracia rusticana*. p-Coumaric acid is a phenolic compound associated with several pharmacological effects that include anti-oxidant, antimicrobial, antiviral, anticancer, antidiabetic, anti-inflammatory. Furthermore, it was reported by Shen *et al*. that p-Coumaric acid reduces the steatosis of liver cells in mice [14]. Among the notable bio-activities of phenolic compounds, the antioxidant activities have been widely studied, including scavenging of free radicals, inhibition of lipid oxidation, reduction of hydroperoxide formation, etc. [15, 16].

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Ferulic, caffeic, chlorogenic, *p*-coumaric acid and protocatechuic acid, were identified to contribute to the antioxidant potential of different natural products. Because of strong antioxidant and anti-inflammatory properties ferulic acid was also reported to be beneficial in Alzheimer's disease, polyphenols in increased quantities in plants having a neuroprotective activity [25]. Protocatechuic acid and rutin showed potential inhibitory activity against *Pseudomonas aeruginosa*. Also, the combination of sulfamethoxazole and protocatechuic acid showed synergistic mode of interaction. In nutrient medium, the combinations of gallic and protocatechuic acids, gallic and caffeic acids, and rutin and quercetin were the best antibacterial agents, with synergistic effects, and were selected to test their activity in a meat model system [18-22]. Both rutin and quercetin were identified in the *Armoracia rusticana* root and leaves samples. It was reported that quercetin has a protective role against coronary disease [23]. A study conducted by Gafrikova found kaempferol and quercetin as the main flavonoid component of an *Armoracia rusticana* aqueous extract. Furthermore, the two components may help prevent DNA damage [9]. Several factors such as growing conditions, climate, developmental stages and extraction solvents may alter the phenolic content [24].

Conclusions

The results of the present study revealed that the methanolic extracts of both *Dipsacus laciniatus* and *Armoracia rusticana*, can be considered as an effective source of antioxidant compounds. The antioxidant activities of the extracts were confirmed by TLC-DPPH method, and they were compared with the standard phenolic acids and flavonoids. The presence of phenolic acids determined by TLC-DPPH method was also confirmed by LC-MS analysis.

Conflict of interest

The authors declare no conflict of interest.

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