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PHYTOCHEMICAL AND ANTIOXYDANT ACTIVITIES OF *SCHINUS MOLLE* L. EXTRACT

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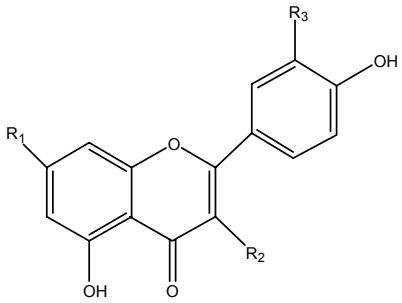
For the first time, the current study was designed to evaluate the chemical composition as well as the antioxydant capacity of *Schinus molle* L. aqueous extracts. It was found that root *Schinus molle* L. extract had the highest total phenolic, flavonoids and condensed tannin contents. Therefore, this extract was subjected to LC-MS analysis in order to identify and quantify the phenolic composition of this extract. This analysis allowed us to identify fifteen phenolic compounds in which six phenolic acids, one coumarin and eight flavonoid compounds. Rutin, Hyperoside, Kaempferol, Quercetin, quinic acid and gallic acid were tentatively identified in the root of this plant. The highest antioxidant activities using two methods, DPPH and FRAP assays were obtained with aqueous root extract from *Schinus molle* L. (54.41 µg.mL^{-1} and 207.42 µg.mL^{-1} of extracts, respectively). This study could provide useful information for industry to produce potentially bioactive plant extract.



INTRODUCTION

Food processors and consumers have expressed a desire to reduce the use of synthetic chemicals in food preservation. Recently, there has been a considerable interest in extracts and essential oils (EOs) from common culinary herbs, spices and aromatic plants characterized by a notable antimicrobial activity. *Schinus molle* L. (Anacardicaceae); commonly known as Peruvian pepper tree; is a native species at South America but is also found as an introduced and invasive plant worldwide.[1] The successful introduction of this exotic species in a non-native range is attributed to its high drought and heat tolerance, great potential to compete for nutritive resources and light, as well as high growth rate and prolific seed production.[2] It was introduced and naturalised in Southern Europe, including Portugal, as a nornamental plant. In traditional cuisine, *Schinus molle* fruits (berries) have been used as a replacement for black pepper and also to prepare alcoholic drinks and beverages.[3] Furthermore, the research interest on *S. molle* have increased significantly and several biological activities were identified to its essential oils which were reported as allelopathic, antibacterial, antifungal, anticancer, antimicrobial, antitumoral, and insecticidal properties.[1] In Tunisia, *S. molle* L. is rather used as urban greening tree throughout the country and no real exploitation as medicinal plant was being noted especially to its root extracts. Therefore, the main task of the present study was to assess the chemical composition via colorimetrical methods and using LC-MS apparatus in order to identify the phenolic compounds as well as their antioxidant activities.

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MATERIALS AND METHODS

Plant material

The *Schinus molle* were harvested in January 2017 from Gafsa region in the South-West of Tunisia. Fruits, leaves and roots of *Schinus molle* L. was previously cleaned with tap water, cuted into slices and dried in the open air. After drying, each *Schinus molle* L. part were ground to a fine powder, used for the extraction of phenol compounds. The identification of the plant material was done by Dr Maher Boukhris and a voucher specimen (SMR2017) of the plant was deposited at the Sciences Faculty of Sfax (Tunisia).

Sample preparations

Extraction of phenolic compounds. Fifty grams of each *Schinus molle* L. part were extracted by maceration with distilled water (500 mL) for 24 hours at room temperature and under continuous agitation. The resulting extract was then filtered through Whatman no. 4 paper and centrifuged for 10 min at 4500 g. The supernatant was lyophilized and stored at -21°C, until use.[4]

Extraction of condensed Tannin (CT). Briefly, 40 g dry weight each *Schinus molle* L. part were soxhlet-extracted with hexane for six hours to remove lipids and lipophilic substances. Subsequently, the CT was isolated from the solid residue by an acetone/water solution (70:30, v/v) after addition of ascorbic acid (0.1%, w/v). Subsequently, acetone were evaporated using a rotary evaporator, and the aqueous phase was washed successively with chloroform and ethyl acetate in order to remove chlorophyll, carotenoids, low molecular weight phenolics, and tannin monomers.[4] The extract was filtered through Whatman no. 4 paper then stored at -21 °C until further use.

Total Phenols, Flavonoids, tannins contents

Total phenols were determined with Folin-Ciocalteu reagent according to the procedure described by Singleton and Rossi[5] with slight modifications.[4] Gallic acid was used as the standard for the calibration curve and results are expressed as mg of gallic acid equivalent.g^{-1} DW.

Total flavonoids content was based on the method described by Yahyaoui *et al.*[4] with some modifications, the results were expressed as milligram of quercetin equivalents per 1 g of dry weight (mg of quercetin equivalent.g^{-1} DW).

In addition, the condensed tannins were tested colorimetrically as described previously by Yayaoui *et al.*[4] To 400 µL of 3 mL of 4% vanillin reagent, a 1.5 mL volume of 4% concentrated H_2SO_4 were added. After 15 min, the absorbance was measured at 500 nm. The content of condensed tannins in the aqueous extract was expressed as mg catechin equivalents per g of dry weight.

LC-ESI-MS analysis

Extracts (20 mg/mL) were filtered through a 0.45 µm pore size membrane (Merck, Darmstadt, Germany) before be inginjected into the ultra-fast liquid chromatography system. The LC-Electrospray Ionization-Tandem Mass Spectrometry (LC-ESI-MS) analysis wasd one using a LCMS-2020 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source (ESI) and operated in negative ionization mode. The mass spectrometer was coupled in-line with an ultra-fast liquid chromatography system consisting of a LC-20 CE XR binary pump system, SIL-20AC XR auto-sampler, CTO-20AC column oven and DGU-20A 3 R degasser (Shimadzu). An Aquasil C18 column (Thermo Electron, Dreieich, Germany) (150mm×3 mm, 3 µm) preceded by an Aquasil C18 guard column (10 mm×3 mm, 3 µm, Thermo Electron) were used for the analysis. The mobile phase was A (0.1% formic acid in H_2O, v/v) and B (0.1% formic acid in methanol, v/v) with a linear gradient elution: 0–45 min, 10–100% B; 45–55 min, 100% B. Re-equilibration duration was 5 min between individual runs. The column temperature was maintained at 40°C, the mobile phase flow rate was 0.4 mL/min and the injection volume was 5 µL. High-purity (>99%) nitrogen was used as a nebulizer and an auxiliary gas. The spectra were monitored in selected-ion monitoring (SIM) mode and processed using Shimadzu Lab Solutions LC-MS software. The mass spectrometer was operated in negative ion mode with a capillary voltage of −3.5 V, a dry gas flow rate of 12 L/min, a nebulizing gas flow rate of 1.5 L/min, a block source temperature of 400°C, a dissolving line temperature of 250°C, a voltage detector of 1.2 V and the full scan spectra from 50 to 2000 m/z.

Detection and quantification were performed at 280 and 335 nm. Each phenolic compound was quantified in comparison to its standard when it was available. Quantitative evaluation of individual phenolic was performed by means of a four-point regression curve (r^2=0.989) using authentic external standards.

Table 1

Total Phenols, Flavonoids and condensed tannins in *Schinus molle* L. extract

	Total phenols (mg GAE/g DW)	Flavonoids (mg QE/g DW)	Condenced Tannins (mg CE/g DW)
Leaves	25.42± 0.22[a]	7.09± 0.03[a]	7.8± 3.22[a]
Fruits	73.75±1.25[b]	35± 4.22[b]	22.95± 3.22[b]
Roots	427.76 ± 3.22[c]	218.44 ± 9.14[c]	114.83 ± 6.44[c]

DW: dry weight. Means with different letters in the same column were significantly different at $p<0.05$. Results are expressed as mean± standard deviation of 3 determinations.

Antioxidant activities

DPPH radical scavenging activity: The free radical-scavenging capacity was measured using the DPPH method described in a previous study.[6] The concentration of the test extract providing 50% inhibition (IC_{50}, expressed in $\mu g.mL^{-1}$) was calculated from the graph plotted with inhibition percentage against the extract concentration. All tests were carried out in triplicate.

Ferric reducing antioxidant power (FRAP) assay: FRAP assay was estimated following the procedure originally described by Pulido *et al*.[7] with some modification.[6] Results were calculated as IC_{50} of reducing power expressed in $\mu g.mL^{-1}$.

Statistical Analysis

All data were expressed as the average ± standard deviation of the measurements. Quantitative differences was assessed by Tukey's test (at $p < 0.05$) followed by Dunn's post–hoc multiple comparison test (SPSS. v15).

RESULTS AND DISCUSSION

Phenolic, flavonoid and Condenced tannin content in *Schinus molle* L. extract

Considering the growing interest in the use of medicinal plants in both food and pharmaceutical industries, the total phenolic contents of Tunisian *Schinus molle* L. from Gafsa were presented in Table 1. The amount of total polyphenols was higher in roots (427.76 mg GAE/g dry weight extracts) than in fruits (73.75 mg GAE/g dry weight extracts). The same tendency was observed for flavonoid and condensed tannins content (Table 1). Results showed also that the highest condensed tannin content was found in the *Schinus molle* L. roots (114.83 mg CE/g DW) while the lowest one was in the *Schinus molle* L. leaves (7.8 mg CE/g DW). From these results, and for the first time, our research team concluded that the change in the polyphenolic content varies with *Schinus molle* L. parts.

Identification and quantification of phenolic compounds in *Schinus molle* L. roots extract

Taking into account the results from the last section, we aimed to identify the major phenolic compounds present in the *Schinus molle* L. roots. Therefore, using LC-MS analysis, it was possible to reveal the presence of several phenolic acids, coumarin and flavonoid compounds. Table 2 lists each of the identified phenolic in elution order. The structure assignment of all phenolic compounds for which no standards were available was based on a systematic search for molecular ions using extracted ion mass chromatograms and comparing those with literature. [8-10] For example, the ESI-mass spectrum in negative mode of compound 8 (retention time 24.00 min) exhibited a base peak at m/z 609 in negative ion mode and strong peaks at m/z 301. This observation can be a diagnosis of quercetin derivatives, and a fragment at m/z 463, due to the loss of 146 Da corresponding to rhamnose. These results confirm the presence of rutin, having a molecular mass of 610 Da (Fig. 1). In addition, Compound 9 exhibited a base peak $[M-H]^-$ at m/z 463 and fragment ions at m/z 301, 273, 179 and 151. The fragmentation of the pseudomolecular ion $[M-H]^-$ of compound 9 at m/z 463 yielded a fragment at m/z 301 by neutral loss of 162 mass units. The ion m/z 273 was formed by the loss of CO from the fragment at m/z 301. On the other hand, the ion at m/z 179 was a glucoside moiety while m/z 151 was a result of a retro- Diels-Alder (RDA) fragmentation of the heterocyclic ring system. Those signals suggested that compound 9 was probable Hyperoside (quercetin-3-*O*-galactoside). Compound 10 had a deprotenated molecule $[M-H]^-$

at m/z 447, and a strong fragment at m/z 285 suggested that this compound was the luteolin-7-*O*-glucoside. On the other hand, the mass spectrum of compound 12 showed a strong peak at m/z 301 whose MS² fragmentation spectrum indicated various ionic species (Data not shown). The principal fragment was originated by the loss of 28 Da, giving rise to the intense signal at m/z 273. The ionic species at m/z 257 was also obtained by the loss of 44 Da, which can be justified by the loss of a CO_2 molecule. While the ions at m/z 193 and 121 were obtained by the elimination of $C_6H_4O_2$ and $C_7H_4O_2$ molecules from the ion at 301, respectively. These results were consistent with the presence of quercetin as previously described by Rigane *et al.*, (2012). While, the mass spectrum of compound 15 showed a high-intensity ion at m/z 269. The ESI-MS² spectrum of that ion showed m/z at 225 $[M-H-44]^-$ and 201 $[M-H-68]^-$. The m/z 225 can be explained by the loss of CO_2 molecule from A ring, while the m/z 201 was obtained by the elimination of C_3O_2 from β-dihydroxy in A ring. The obtained mass spectra suggested that compound 15 was Apigenin. These compounds were detected in the *Schinus molle* L. roots growing in Tunisia, for the first time.

Table 2

Phenolic compounds detected in *Schinus molle* L. root extracts
with their HPLC retention times, Molecular formula and molecular mass

No[a]	Compounds[b]	Molecular formula	Molecular mass	$[M-H]^-$ m/z	Retention time (min)	Content (µg/g extract)*
1	Quinic acid	$C_7H_{12}O_6$	192	191	2	1288
2	Gallic acid	$C_7H_6O_5$	170	169	4	550
3	Catechin (+)	$C_{15}H_{14}O_6$	290	289	11.4	1028
4	Caffeic acid	$C_9H_8O_4$	180	179	14.8	3
5	Syringic acid	$C_9H_{10}O_5$	198	197	16.4	0.6
6	*p*-coumaric acid	$C_9H_8O_3$	164	163	21.2	2
7	*trans*-Ferulic acid	$C_{10}H_{10}O_4$	194	193	23.5	2
8	Rutin (quercetin-3-*O*-rutinoside)	$C_{27}H_{30}O_{16}$	610	609	24	26
9	Hyperoside (quercetin-3-*O*-galactoside)	$C_{21}H_{20}O_{12}$	464	463	24.8	14
10	Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	448	447	24.9	1
11	Quercetrin (quercetin-3-*O*-rhamonoside)	$C_{21}H_{20}O_{11}$	448	447	27.1	1
12	Quercetin	$C_{15}H_{10}O_7$	302	301	32.2	2.5
13	Kaempferol	$C_{15}H_{10}O_6$	286	285	32.3	19
14	Naringenin	$C_{15}H_{12}O_5$	272	271	34.2	1
15	Apigenin	$C_{15}H_{10}O_5$	270	269	34.9	1

[a]The numbering refers to elution order of compounds from an Aquasil C18 column. [b]Phenolic compound quantified in comparison to its standard. * Concentration expressed as $\mu g.g^{-1}$ of extract.

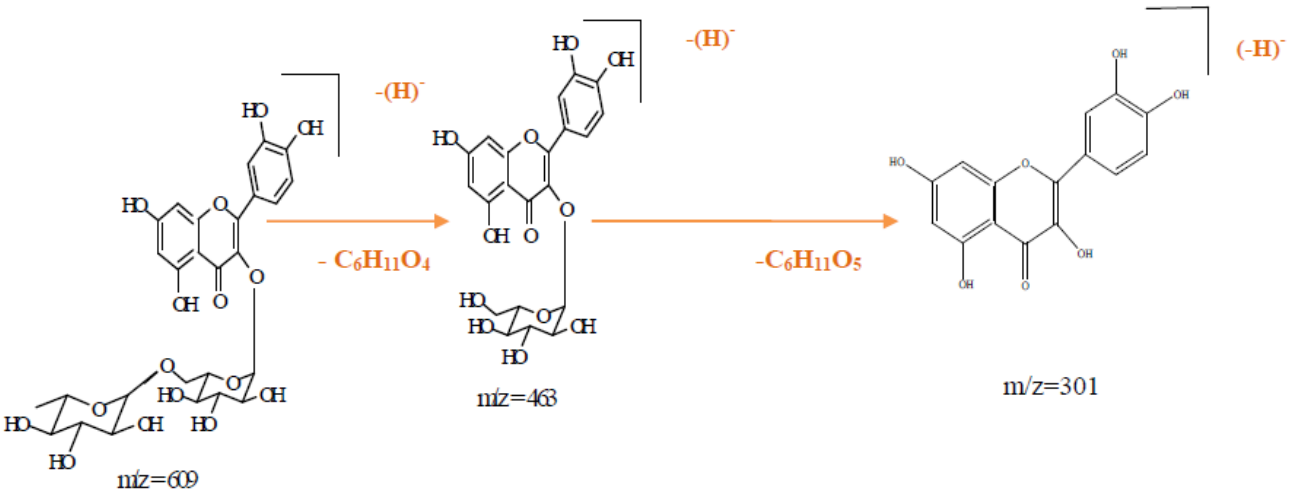

Fig. 1 – Proposed fragmentation from the pseudomolecular anion of compound 8.

Table 3

Antioxidant activities of aqueous *Schinus molle* L. extract

	IC_{50} of DPPH radical scavening activity (µg/mL)	IC_{50} of reducing power (µg/mL)
Fruits	217.63± 2.87[a]	260± 6.25[a]
Leaves	274.85± 1.59[b]	475.9± 1.92[b]
Roots	54.41 ± 4.57[c]	207.42 ± 18.76[c]

Means with different letters in the same column were significantly different at $p<0.05$. Results are expressed as mean± standard deviation of 3 determinations.

The analysis of phenolic substances using reversed phase-HPLC from *Schinus molle* L. roots extracts, as described in the experimental section, allowed to the quantification of the phenolic compounds previously identified by LC-MS apparatus. The results obtained revealed the presence of six phenolic acid, one Catechin and eight flavonoids. The main phenolic acid found in *Schinus molle* L. roots was quinic acid which was present with1288 µg/g of extract folowed by gallic acid. Table 2 reveals a low concentration of syringic acid (0.6 µg/g of extract). From Table 2, we could conclude that Rutin was the major flavoinoids compounds quantified in the *Schinus molle* L. roots extract (26 µg/g of extract), followed by Kaempferol (19 µg/g of extract), Hyperoside (14 µg/g of extract) and Quercetin (2.5 µg/g of extract). On the other hand, Luteolin-7-*O*-glucoside, Quercetrin, Naringenin Apigenin were present in very low amounts (1 µg/g of extract). From these results, our research team concluded that this study could provide useful information for industry to produce the potentially bioactive compound extracted from *Schinus molle* L. roots using distillated water as solvent.

Antioxidant activity

The fruits, leaves and roots extracts of *Schinus molle* L. were subjected to *in vitro* tests to evaluate their antioxidant activities. In particular, we carried out two tests: the DPPH radical-scavenging and FRAP assays. DPPH is a free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. [9] The reduction capability of DPPH radical was determined by the decrease in absorbance induced by plant antioxidants. The scavenging effect of aqueous-methanol extracts and standard on the DPPH radical expressed as IC_{50} values was in the following order: fruits (217.63 µg/mL), leaves (274.85 µg/mL) and roots (54.41 µg/mL) (Table 3). The radical scavenging properties of natural products are frequently linked with their aptitude to shape stable radicals. For instance, the aqueous root extract from *Schinus molle* L. was the most effective in this test as well as FRAP assays (Table 3). Taking into account the obtained reseults in the last section, these results confirm the importance of the flavonoid B-ring catechol structure and the presence of a 3-hydroxyl free or glycosylated group. For example, luteolin (3', 4'-dihydroxylation of the B ring) was a more powerful radical scavenger than apigenin (4'-monohydroxylation of the B ring), suggesting that further hydroxylation at the C-3' position contributes to a greater inhibition and increase of the antioxidant activity. Also, the hydroxylation of the C-3 of the non-phenolic C ring seems to be important, as the flavonol quercetin, which differs from the flavone luteolin in being 3-hydroxylated, has a stronger antioxidant activity than the latter compound. The glucosylation of the 7-hydroxyl group of flavones (luteolin-7-*O*-glucoside) reduced the antioxidant activity respect to their aglycones (quercetin, rutin and luteolin). The 7-*O*-glucosylation produces conformational changes in the flavonoid molecule that might make electronic delocation difficult as well as decreasing the electron donor capacity of the 7-hydroxyl group.[11] It seems that there is a correlation between total phenol and flavonoid contents and antioxidant activity. [9]

CONCLUSION

Schinus molle L. is perfectly growing to a very saharien climate. This shrub is not yet appropriately exploited especially its roots part, except as wild dry pastures or in some folk medicine remedies. It showed a wealth of powerful antioxidant activities. This study clearly indicated that it is important to consider both the associated antioxidant activity and phenolic content. Indeed, aqueous extract of root containing higher level of phenolics possessed more powerful antioxidant potential. This plant, mainly its root, constitutes an excellent source of flavonoids. Indeed, its elevated

phenolic compound content makes it a potential source of dietary regime and a protection against numerous diseases and infections.

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