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Research Article

Impact of Methanol-Water Ratio of Leaf and Root Extracts of *Ampelodesma mauritanica* on the Antioxidant Activity, and Effect of Different Solvent Extraction on Phenolic Compounds Quantification

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Graphical Abstract

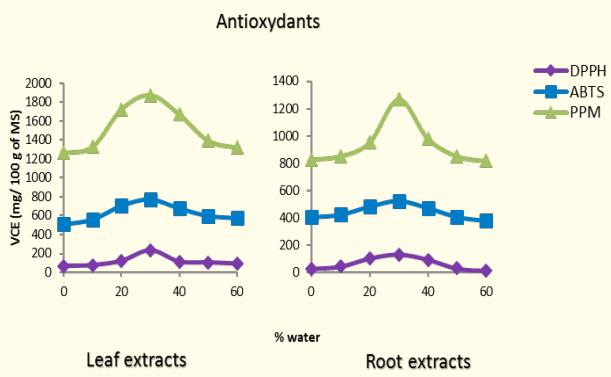

Figure 1

Abstract

This work evaluates the antioxidant capacity of roots and leaves extracts of *Ampelodesma mauritanica* using ABTS, DPPH and PPM *in vitro* tests and, investigates the influence of the variations of the solvent extraction (methanol-water concentrations) on the anti-oxidant activity. The results exhibit that the highest antioxidant activity both for the leaves extracts and for the roots extracts were obtained for the methanol-water at a ratio of 70:30.

Moreover, some polyphenolic compounds contained in extracts of *Ampelodesma mauritanica* was identified using HPLC. The results showed that gallic acid, protocatechuic acid, catechin hydrate acid, and caffeic acid are the most representative compounds. Furthermore, owing to its high polarity, gallic acid is more extracted with alcoholic solvents like methanol or butanol than the other acids.

Keywords: Extraction; HPLC; Polyphenolic Compounds; Antioxidant Activity; ABTS; DPPH; PPM

Introduction

The modern pharmaceutical industry is always looking for new active compounds inspiration which still comes mainly from plant secondary metabolites [1]. These later are chemically varied in nature from polar to non-polar; Polyphenols are one of their largest classes which are distributed in all parts of plants. They are synthesized by the plant to defend itself against environmental stresses [2].

Polyphenols are known for their physiological properties (antioxidant activity, antibacterial activity, anti-inflammatory activity, antidiabetic activity… etc.), these activities depend on their chemical structure; for example, antioxidant activity comes from the hydroxyl functional group. Polyphenolic compounds can be classified into several groups: simple phenolics, phenolic acids, coumarins, flavonoids, stilbenes, lignans, lignins, and tannins [3].

Algeria is an important geographical source of more than 3000 plant species belonging to several botanical families which are little investigated [4]. This work aims to explore natural substances coming from the Algerian flora which is rich in medicinal and aromatic plants. We choose to expand the studies concerning *Ampelodesma mauritanica "Diss"* known for its remarkable antioxidant and antibacterial properties as well as for its antidiabetic activity, as reported in our previous studies [5-9].

Owing to the biological interest of *Ampelodesma mauritanica* and the lack of knowledge concerning their secondary metabolites, we planned to identify some polyphenolic compounds responsible for the free radical scavenging properties and to evaluate the antioxidant capacity of methanol-water extracts of roots and leaves.

The *Ampelodesma mauritanica*, which belongs to the family *Poaceae,* is a great grass widespread in North Africa and the Southern Europe. It is a herbaceous plant that blooms between April and June, it forms large rigid clumps.

Materials and Methods

Plant Material

Ampelodesma mauritanica was collected from El Kala National Park in the north east of Algeria in October 2011. The plant was identified by Dr. G. Debelair (Department of Biology, University of Badji Mokhtar-Annaba, Algeria). Roots and leaves were dried in shade at room temperature, ground on the mill to obtain coarsely powder and stored in a dark glass flask to protect it from humidity and light.

Chemicals

Vitamin C, gallic acid, protocatechuic acid, catechin hydrate acid, caffeic acid, catechin, rutine, and quercetine were purchased from Acros Organics. Potassium persulfate, sulfuric acid, sodium hydrogen phosphate, Ammonium phosphomolybdate, methanol, 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) diammonium salt (ABTS) were obtained from Sigma and Roth (France). The used chemicals were all of the analytical grades.

Extractions procedures

For the antioxidant activity evaluations

5g of plant powder (roots or leaves) was extracted by percolation (48h) in hydro-methanolic solutions of 50 ml. The organic phase was filtered through Whatman paper N°1, concentrated under reduced pressure and freeze at 4°C before use.

For the HPLC analysis

The extracts used for the chromatographic analysis are carried out either using a gradient polarity of the solvent or under methanol.

Extraction using a polarity gradient

100g of plant part powder was extracted with 1000 ml of petroleum ether and set stirring for 24 hours. After filtration, the marc is then macerated in 1000 ml of a methanol/water solution (7/3) for 48 hours with stirring. The obtained hydro-alcoholic extract was filtered and concentrated at 35°C under vacuum, and then the residue was diluted with distilled water to 100 ml. firstly, the aqueous solution was defatted with petroleum ether and then was submitted to successive liquid-liquid extractions using: chloroform, ethyl acetate, and butanol respectively. For each solvent, the organic phases were concentrated under vacuum at 35°C and the obtained residues were stored in the dark at 4°C before use.

Extraction using methanol

In this case, the plant material was defatted by petroleum ether. Then after filtration, 2 grams of the marc is extracted with 10 ml of methanol under agitation for 24 hours. This alcoholic extraction was conducted two times. The collected solution was centrifuged and filtered through a 0.45 µm filter then concentrated under vacuum at 35°C. The obtained residue was stored in the dark at 4°C before use.

Antioxidant Activities

Three different tests were used to determine the antioxidant capacity. The ABTS test, the DPPH test and the phosphomolybdenum (PPM) test [10-12].

All tests were replicated at least three times.

ABTS radical scavenging test

Total antioxidant activity was determined by scavenging blue-green ABTS radicals and was expressed as mg vitamin C equivalent (VCE) per g dry weight. ABTS radical cation ($ABTS^{\cdot+}$) was generated according to the experiment using an improved method [13]. It is produced by reacting of ABTS solution (7 mM in water) with 2.5 mM potassium persulfate (final concentration) for 16h at ambient temperature in the dark (stock solution). Then the $ABTS^{\cdot+}$ stock solution was diluted with methanol to an absorbance of 0.7 ± 0.2 at 734 nm. A quantity of 1 mg of the extract was dissolved in 5 ml of 70% aqueous methanol. A quantity of 50 µl of extract solution, was added to 2.0 ml of diluted $ABTS^{\cdot+}$ solution (A = 0.7 ± 0.2). The decrease of absorbance was measured after 5 minutes of incubation at room temperature in the dark and plotted as a function of the concentration of antioxidants. All determinations were carried out in triplicate on each occasion and at each separate concentration of the standard. Methanol and L-ascorbic acid were used as a negative and positive control, respectively. All radical stock solutions were prepared fresh daily.

DPPH radical scavenging activity

The antioxidant activity of plant extract was estimated using a slight modification of the DPPH radical scavenging protocol reported by [14]. Two and nine-tenths mL of 100 mM DPPH solution (2,2-Diphenyl-1-picrylhydrazyl radical) in methanol was mixed with 0.1 ml of plant extract. The reaction mixture was incubated in the dark for 30 minutes and thereafter the optical density was monitored at 517 nm against the blank. Vitamin C equivalent antioxidant capacity (VCEAC) was calculated by using ascorbic acid as a reference compound to prepare the standard curve and was expressed as mg/100 g of dry matter of VCEAC. For the control, 2.9 ml of DPPH solution in methanol (100 mM) was mixed with 0.1 ml of methanol. The radical solution was prepared daily.

PPM test

The total antioxidant capacity of the plant extracts was measured by the method described by Prieto., *et al.* [15]; 100 µl of the sample solution was mixed with 900 µl of the reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium phosphomolybdate) against a blank containing 100µl of methanol mixed with 900 µl of reagent solution. The absorbance of the test sample was measured at 695 nm. The antioxidant activity was expressed as vitamin C equivalent (mg 100 dry matter).

Statistical analysis

All experimental data were expressed as a mean ± standard deviation. Statistical analysis was done using one-way analysis of variance (ANOVA). For all statistical comparisons, the level of significance was set at $P < 0.05$. All statistical analyses were carried out using the Statview® 4.5 statistical package (Abacus Concepts, Inc).

Identification and quantification of some polyphenolic compounds

Standard solutions

The concentration of standard solutions of catechin, rutin, quercetin and gallic, protocatechuic, catechin hydrate and caffeic acids were 1 mg in 1 ml of methanol-water (1:1) solution.

Analysis using HPLC

The apparatus used is a Waters Pump 600, equipped with an injection loop of 20 µl. Analyses were performed on a column Alltech Intertsil ODS-5; 4.6 mm, the particle size is 5 µm. The detector is a Waters 486 Tunable.

Measurements were carried out at two wavelengths, 280 and 320 nm. The solvents were degassed (placed in an ultrasonic bath for 25 minutes); extracts and standards were filtered through a Millipore membrane (0.45 µm).

The eluent system proposed by Nakatani., *et al.* [16] is slightly modified. To perform this study a gradient of three mobile phases was used. Solvent A: 50 mM ammonium phosphate ($NH_4H_2PO_4$) pH 2.6 (adjusted with phosphoric acid), solvent B: (80: 20 (v/v)) acetonitrile/ solvent A, and solvent C: 200 mM of phosphoric acid pH 1.5 (pH adjusted with ammonium hydroxide).

20 µl of the eluent solution was injected according to the table 1 with a flow rate of 1 ml/min.

Instants (min)	%A	%B	%C
0 - 5	100	0	0
5 - 11	92	8	0
11 - 16	0	12	88
16 - 22 ,5	0	14	86
22,5 - 27,5	0	16	84
27,5 - 37,5	0	20	80
37,5 - 50	0	25	75
50 - 55	0	80	20
55 - 60	100	0	0

Table 1: Gradient elution.

Results and Discussion

Antioxidant Activity

The results of the three tests for antioxidant activity of the methanol-water extracts are summarized in table 2. The variation of the antioxidant activity according to the proportion of methanol-water is illustrated.

	VCE (mg/100 g d.w) - leaves-			VCE (mg/100 g d.w) - Roots -		
(MeOH/H_2O) extract	DPPH	ABTS	PPM	DPPH	ABTS	PPM
100 / 0	68 ± 1,04	509 ± 2,44	1263 ± 1,48	27 ± 0,83	405 ± 1,22	827 ± 4,07
90 / 10	76 ± 2,50	561 ± 0,73	1330 ± 3,33	43 ± 2,08	423 ± 1,46	851 ± 3,70
80 / 20	123± 1,67	706 ± 2,68	1724 ± 10,37	101 ± 1,25	481 ± 1,95	950 ± 1,48
70 / 30	236 ± 2,29	772 ± 2,93	1871 ± 2,22	129 ± 1,46	522 ± 1,46	1265 ± 4,81
60 / 40	114 ± 2,08	678 ± 0,76	1670 ± 16,67	92 ± 0,83	471 ± 0,49	979 ± 6,66
50 / 50	106 ± 1,87	599 ± 3,41	1393 ± 3,70	29 ± 1,25	407 ± 0,24	850 ± 2,96
40 / 60	93 ± 2,29	576 ± 0,73	1320 ± 1,85	13 ± 2,92	379 ± 1,22	816 ± 3,33

Table 2: Antioxidant activity of different extracts in methanol-water mixtures. Values are expressed as mean ± SEM, $P < 0.05$ when compared with control

Table 2 reveals that whatever the selected plant part and regardless of the test used, the antioxidant capacity of the extract increased in concentration from 40% to 70% of methanol, in contrast to a concentration from 70% to 100% of methanol. Showing that the highest antioxidant capacity was always obtained from the solution of methanol/water in proportion 70/30 (v/v). In contrast to what reported by Nagendra., *et al.* [17] who worked on the seeds of Litchi, using different extraction of solvents, they found that the 50% ethanol extract had better antioxidant capabilities compared to the other extracts.

Furthermore, under this condition, the leaves extracts exhibited the best antioxidant capacity.

On the other hand, table 2 shows that compared to the different used tests; the DPPH test gave the lowest values. This behavior was previously reported by Arnao., *et al.* [18] and by Kim., *et al.* [19], they explained this observation owing to the interferences occurring at 417 nm, and the insolubility of DPPH in an aqueous medium.

HPLC Analysis

The chromatographic profiles of standards are represented by figures 2 and 3.

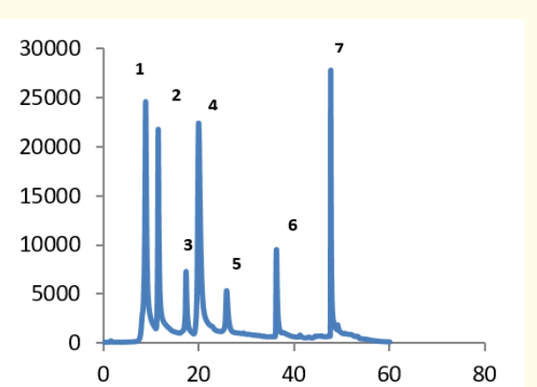

Figure 2: Chromatographic profiles of standards at 280 nm at 1mg/ml: (1) Gallic acid; (2) Protocatechuic acid; (3) Catechin hydrate acid; (4) Caffeic acid; (5) Catechin; (6) Rutin; (7) Quercetin.

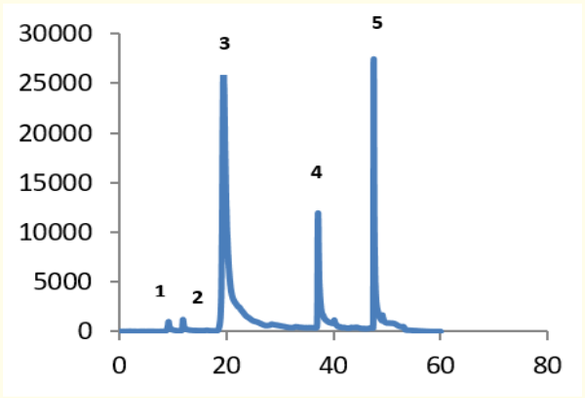

Figure 3: Chromatographic profiles of standards at 320 nm at 1mg/ml: (1) Gallic acid; (2) Protocatechuic acid; (3) Caffeic acid; (4) Rutin; (5) Quercetin.

The compounds contained in the various parts of the plants are identified by matching the retention time of standards with the chromatograms of different extracts.

The results of chromatographic analysis of the different leaves extracts are collected in table 3.

The identified and quantified compounds were gallic acid, protocatechuic acid, catechin hydrate acid, and caffeic acid.

Although epicatechic acid, rutin, and quercetin were identified, they're too small amount in the samples does not permit their quantification.

Gallic acid, which has the highest polarity in this series of polyphenolic compounds [20,21] is better extracted by the hydro-alcoholic solvent (70 / 30). In contrast, for the other compounds, they are mainly extracted by chloroform and ethyl acetate, explaining why only traces appear in the n-butanol extract.

	Retention time	Successive Extractions			Extraction under MeOH/ H_2O (70/30)
		Chloroform	Ethyl Acetate	n-butanol	
Gallic acid	8,93	-	-	0.021 ± 0.003	0.022 ± 0.002
Protocatechuic acid	11,55	0.021 ± 0.002	0.037 ± 0.004	-	0.039 ± 0.004
Catechin hydrate acid	17,38	0.016 ± 0.002	0.043 ± 0.004	-	0.047 ± 0.005
Caffeic acid	20,02	0.013 ± 0.001	0.049 ± 0.008	-	0.056 ± 0.006

Table 3: Chromatographic analysis (HPLC).
(-) not quantified compound.

Conclusions

The study of the antioxidant capacity which assessed by three different methods: DPPH test, ABTS test and PPM test revealed that all extracts (methanol-water) exhibited good antioxidant activity.

Indeed, the extract methanol-water in a proportion 70:30 had the best antioxidant activity of both leaves and roots. These results can be explained by the fact that this proportion, extracted as the best as possible of the polyphenol compounds and the flavonoids which are mainly responsible for the antioxidant activity of plants.

This study clearly shows that *Ampelodesma mauritanica* had a high level of antioxidant capacity and that this plant could be used as a good source of natural antioxidants.

For identification of the polyphenolics compounds present in the aerial part (leaves) of *Ampelodesma mauritanica*, we used the high-performance liquid chromatography. The results show that the methanol/water (70/30) was the better extraction solvent. The amounts found in the hydro-methanol extract for gallic acid, protocatechuic acid, catechin hydrate acid, and caffeic acid were 0.022, 0.039, 0.047 and 0.056 mg/ml respectively.

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