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Polyphenolic burden and *in vitro* antioxidant properties of *Moringa oleifera* root extracts

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ABSTRACT

Introduction: *Moringa oleifera* is a small tree with very significant nutritional but also pharmacological properties and various preparations from almost all parts of the plant (leaves, fruit, stems, bark, roots) are used in folk medicine for treating several ailments. This study aimed to investigating the polyphenolic burden and *in vitro* antioxidant properties of *M. oleifera* root extracts, obtained with solvents covering a range of polarities.
Methods: Lyophilised *Moringa oleifera* roots were extracted with solvents of increasing polarity, including ethyl acetate, butanol, methanol and water. The generated extracts were screened for polyphenolic load and antioxidant activities, by determining the total polyphenol, total flavonoid, total flavanol, antiradical activity and reducing power. Liquid chromatography-mass spectrometry was also employed to obtain some evidence regarding the nature of the main constituents.
Results: The results indicated that both aqueous and methanolic extracts were the richest in total polyphenols, but the aqueous extract also exhibited high reducing power. The liquid chromatography-diode array-mass spectrometry analysis also revealed that the major substances occurring in the aqueous extract were relatively polar molecules, but the mass spectral data were ambiguous to assign tentative structures.
Conclusion: Polar solvents, such as water, may be effectively used to recover high amounts of *M. oleifera* root phenolics, which possess important antioxidant properties.

Implication for health policy/practice/research/medical education:
In spite the concrete evidence regarding the pharmacological potential of *Moringa oleifera* root extracts, the data available to-date on the polyphenolic composition of this tissue are particularly limited. It is anticipated that this study will further expand the knowledge pertaining to the major polyphenol classes occurring in fractions of *M. oleifera* root extracts, with respect to composition, polarity and *in vitro* antioxidant properties.
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Introduction

Moringa oleifera Lam (syn. *M. ptreygosperma* Gaertn.), referred to as the 'drumstick tree' or the 'horse radish tree', is one of the most widely distributed species of a monogeneric family Moringaceae (1). *M. oleifera* is an important food commodity and the leaves, fruits, flowers and immature pods of this tree are used as a nutritive vegetable in many countries, being rich in β-carotene, phenolics, protein, vitamin C, calcium and potassium etc.
In addition to its high nutritional value, *M. oleifera* is also important for its medicinal properties. Various parts of this plant such as the leaves, roots, seeds, bark, fruits, flowers and immature pods act as cardiac and circulatory stimulants, possess anitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and are routinely used in folk medicine for the treatment of different ailments in several countries (2,3). Regarding the roots, they have been found to possess several distinct pharmacological properties and root preparations are used for the cure of bronchitis, stomatitis,

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urinary discharges and asthma (4). However, despite the aforementioned activities, *M. oleifera* roots extracts have been scarcely investigated with regard to their polyphenolic content and composition, hence the data available in the literature are particularly limited. Therefore, this study had a scope to illustrate some aspects of the polyphenolic composition and antioxidant potency of *M. oleifera* root extracts. To the best of our knowledge, the present study is the first investigation reporting on the polyphenolic burden and *in vitro* antioxidant properties of *M. oleifera* root extracts, obtained with solvents covering a range of polarities.

Materials and methods

Chemicals

Ferric chloride hexahydrate ($FeCl_3$ $6H_2O$) was from Acros Organics (New Jersey, USA). Gallic acid, Folin-Ciocalteu reagent, rutin (quercetin 3-*O*-rutinoside), ascorbic acid, 2,2-diphenyl-*β*-picrylhydrazyl (DPPH•) radical, *p*-(dimethylamino)-cinnamaldehyde (DMAC), (+)-catechin and 2,4,6-tripyridyl-s-triazine (TPTZ) were from Sigma Chemical Co. (St. Louis, MO). Aluminium chloride ($AlCl_3$) was from Fisher Scientific (New Jersey, USA).

Plant material

M. oleifera roots were obtained from an experimental plantation (Karditsa prefecture, Central Greece). Roots were washed well to remove soil debris, cut into small pieces with a sharp cutter and lyophilised (mean moisture content 81.4%). The lyophilised material was pulverised in a laboratory blender and kept in sealed plastic tubes, at – 40°C, until used.

Batch extraction procedure

An amount of 1 g of lyophilised material was mixed with 20 mL of solvent (water, methanol, butanol, ethyl acetate) in a stoppered plastic tube. The material was subjected to extraction under stirring at 600 rpm, with a teflon-coated magnetic stirrer, for 180 minutes, at room temperature (23 ± 1°C). Following extraction, samples were centrifuged in a table centrifugator (Hermle, Wehingen, Germany) at 10 000 rpm for 10 minutes. The clear supernatant was used for further analysis.

Determination of total polyphenol yield (Y_{TP})

A previously reported protocol was used (5). Briefly, 0.78 mL of distilled water, 0.02 mL of sample and 0.05 mL of Folin-Ciocalteu reagent were mixed. After exactly 1 minute, 0.15 mL of aqueous sodium carbonate 20% was added, and the mixture was vortexed and allowed to stand at room temperature in the dark, for 60 minutes. The absorbance was read at 750 nm (A_{750}) and the total polyphenol concentration (C_{TP}) was calculated from a calibration curve, using gallic acid as a standard (25-500 mg L^{-1}). Yield in total polyphenols (Y_{TP}) was determined as mg gallic acid equivalents (GAE) per g of dry weight (dw), using the following equation:

$$Y_{TP}(mg\ GAE\ g^{-1}\ dw) = \frac{C_{TP} \times V}{m} \quad (1)$$

Where V is the volume of the extraction medium (L) and m the dry weight of the lyophilized material (g).

Determination of total flavonoid yield (Y_{TFn})

A previously published methodology was employed (6). An aliquot of 0.25 mL sample was mixed with 0.75 mL $AlCl_3$ reagent [0.16% (w/v) $AlCl_3$ and 5% (v/v) acetic acid in methanol] and allowed to stand for 30 minutes, at room temperature. The absorbance was obtained at 415 nm (A_{415}) and the total flavonoid concentration (C_{TFn}) was calculated from a calibration curve, constructed with rutin (quercetin 3-*O*-rutinoside) as the calibration standard (10-160 mg L^{-1}). Yield in total flavonoids (Y_{TFn}), expressed as mg rutin equivalents (RtE) per g of dry weight was determined using Eq. (1).

Determination of total flavanol yield (Y_{TF})

Total flavanols were determined following derivatization with DMAC, based on a previously established protocol (7), with modifications. Briefly, an aliquot of 0.02 mL sample was mixed with 0.88 mL HCl (2M in MeOH) and 0.1 mL DMAC (0.1% in MeOH). The mixture was left to react for 15 minutes and then the absorbance was read at 640 nm. The total flavanol concentration (C_{TF}) was calculated from a calibration curve, constructed with (+)-catechin as the calibration standard (1-80 mg L^{-1}). Yield in total flavanols (Y_{TF}), expressed as mg catechin equivalents (CtE) per g of dry weight was determined using Eq. (1).

Determination of the reducing power (P_R)

A methodology reported previously was used (5). Sample (0.05 mL) appropriately diluted with methanol was mixed thoroughly with 0.05 mL $FeCl_3$ solution (4 mM in 0.05M HCl), and incubated for 30 minutes in a water bath at 37°C. Following this, 0.9 mL TPTZ solution (1 mM in 0.05M HCl) were added, and the absorbance was recorded at 620 nm after exactly 10 minutes. P_R was determined as μmol ascorbic acid equivalents (μmol AAE) per g of dw.

Determination of the antiradical activity (A_{AR})

The assay was performed on the basis of a well-established protocol (8). A volume of 0.025 mL sample was mixed with 0.975 mL DPPH solution (100 μM in methanol) and the absorbance at 515 nm was read immediately after mixing ($A_{515(i)}$) and after exactly 30 minutes ($A_{515(f)}$). The A_{AR} was determined using the following equation:

$$A_{AR}(\mu mol\ DPPH\ g^{-1}\ dw) = \frac{C_{DPPH}}{C_{TP}} \times \left(1 - \frac{A_{515(f)}}{A_{515(i)}}\right) \times Y_{TP} \quad (2)$$

Where C_{DPPH} is the initial molar concentration of DPPH (μmol L^{-1}) and C_{TP} is the total polyphenol concentration of the extract, expressed as mg GAE L^{-1}. Results were ex-

pressed as μmol DPPH per g of dry weight.

Qualitative liquid chromatography-diode array-mass spectrometry (LC-DAD-MS)

A previously described method was used (8). A Finnigan MAT Spectra System P4000 pump was used coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on an end-capped Superspher RP-18, 125×2 mm, 4 μm, column (Merck, Germany), protected by a guard column packed with the same material, and maintained at 40°C. Analyses were carried out employing electrospray ionization (ESI) at the positive ion mode, with acquisition set at 5 and 50 eV, capillary voltage 4 kV, source voltage 25 V, detector voltage 650 V and probe temperature 400°C. Eluent (A) and eluent (B) were 2% acetic acid and methanol, respectively. The flow rate was 0.33 mL min^{-1}, and the elution programme used was as follows: 0-2 minutes, 0% B; 2-52 minutes, 100% B; 60 minutes, 100% B.

Statistics

All extractions were carried out in duplicate. All determinations were carried out at least in triplicate and values were averaged. Statistical differences among values were ascertained by performing Student's *t* test, at least at a 95% significance level. Statistics was performed with Microsoft™ Excel 2010 and SigmaPlot™ 12.0.

Results

In this study, the assessment of the polyphenolic content of the extracts obtained was based on the determination of three representative indices, the Y_{TP}, Y_{TFn} and Y_{TF}. As can be seen in Table 1, the highest Y_{TP} was achieved with methanol, but water extraction provided Y_{TP} of comparable magnitude. Extracts of butanol and ethyl acetate were much poorer and the corresponding Y_{TP} values were by 88.6 and 96.2% lower compared with that of methanolic extract. Unlike Y_{TP}, the Y_{TFn} determined for the water extract was 92.7% higher compared with that of methanol, suggesting that the flavonoid fraction occurring in *M. oleifera* roots might consist of compounds with relatively high polarity. This claim was corroborated by the fact that extracts obtained with both butanol and ethyl acetate, which are even less polar solvents, were also poor in flavonoids. Regarding Y_{TF}, a level of 3.24 mg CtE g^{-1} dw was determined in the methanolic extract (Table 1) and it was statistically higher that the levels found in all other extracts ($P < 0.05$). Nevertheless, it is to be noted that the water extract also had Y_{TF} of 2.87 mg CtE g^{-1} dw, as opposed to butanol and ethyl acetate extracts, which had Y_{TF} lower than 0.28 mg CtE g^{-1} dw. This outcome suggested that the flavanol fraction is rather represented by relatively polar constituents.

P_R determination showed that the water extract was by far the most effective, yielding levels that were by 80.9%, 81.44% and 85% higher than the corresponding of methanol, butanol and ethyl acetate extracts (Figure 1), which did not exhibit large differences and their P_R varied from 1.06 to 1.35 μmol AAE g^{-1} dw. The order of efficacy was also very similar with regard to A_{AR}, and the water extract was by 78.9% and 88.2% more effective than the butanol and ethyl acetate extracts. However, a major difference was observed with the methanol extract, which displayed 11.5% higher A_{AR} compared with the water extract (Figure 2).

The LC-MS analysis confirmed that the major phenolics occurring in the extracts obtained were quite polar, since the principal peaks detected were eluted very early. However, three minor peaks were also detected at longer retention times (Table 2), indicating the presence of less polar substances too. The peak eluted at 1.75 minutes yielded a molecular ion with $m/z = 649$, but no derivative ions could be identified. The peak with retention time 2.13 minutes gave a distinct mass spectrum with a molecular ion at $m/z = 543$ and several fragment ions (Table 2). Finally, the peak at 3.50 minutes showed a molecular ion at $m/z = 553$ with no derivative ions.

Discussion

The highest Y_{TP} level found in this study (7.19 mg GAE g^{-1} dw) was close to 10.10 mg GAE g^{-1} dw (9), but significantly higher than 2.20 mg GAE g^{-1} dw (10), reported for methanolic extracts of *M. oleifera* roots. Furthermore, based on Y_{TFn} obtained with the solvents used, it was concluded that this class of polyphenols might consist of polar compounds. This assumption is also supported by other investigations, which demonstrated that *M. oleifera* root extracts obtained with 80% aqueous methanol contained higher amounts of total flavonoids, compared with extracts obtained with either pure methanol or ethanol (10).

Table 1. Y_{TP}, Y_{TFn}, and Y_{TF} achieved by extracting lyophilised *M. oleifera* roots with solvents of variable polarity[a]

Extract	Y_{TP} (mg GAE g^{-1} dw)	Y_{TFn} (mg RtE g^{-1} dw)	Y_{TF} (mg CtE g^{-1} dw)
Water	7.02 ± 0.10	2.73 ± 0.05[b]	2.87 ± 0.06
Methanol	7.19 ± 0.15[b]	0.20 ± 0.01	3.24 ± 0.06[b]
Butanol	0.82 ± 0.02	0.09 ± 0.00	0.28 ± 0.02
Ethyl acetate	0.27 ± 0.01	0.17 ± 0.01	0.03 ± 0.00

[a] All extractions were carried out under stirring (600 rpm) for 180 min, at ambient temperature ($T = 23 \pm 2$°C) and solvent-to-solid ratio of 20 mL g^{-1}. Results are given as average values of triplicate determination (±SD).
[b] Indicates statistically different values ($P < 0.05$).

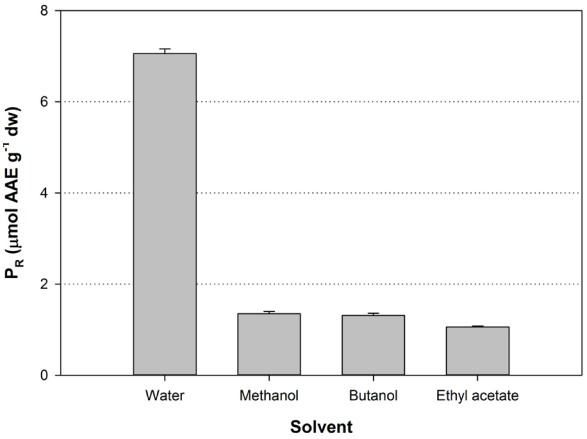

Figure 1. Reducing power (P_R) of *M. oleifera* root extracts obtained with solvents of variable polarity. All extractions were carried out under stirring (600 rpm) for 180 minutes, at ambient temperature (*T* = 23 ± 2°C) and solvent-to-solid ratio of 20 mL g^{-1}. Results are given as average values of triplicate determination (±SD).

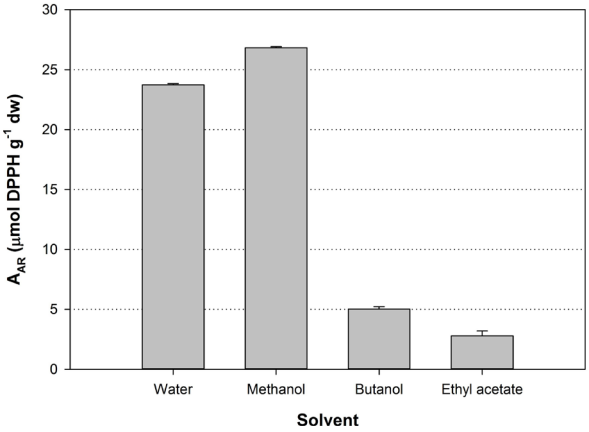

Figure 2. Antiradical activity (A_{AR}) of *M. oleifera* root extracts obtained with solvents of variable polarity. All extractions were carried out under stirring (600 rpm) for 180 minutes, at ambient temperature (*T* = 23 ± 2°C) and solvent-to-solid ratio of 20 mL g^{-1}. Results are given as average values of triplicate determination (±SD).

Table 2. UV-vis and mass spectral characteristics of the main polyphenolic phytochemicals tentatively identified in *M. oleifera* root aqueous extracts

Peak	Rt (min)	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	Other ions (*m/z*)
1	1.75	292	649	-
2	2.13	258	543	485, 381, 365, 323
3	3.50	292	553	-
4	17.00	256, 362	479	303
5	41.00	240, 278	579	301
6	41.50	240, 278	579	301

The Y_{TFn} determined in the extract obtained with 80% methanol was 2.94 mg catechin equivalents g^{-1} dw, which is very close to 2.73 mg RtE g^{-1} dw determined in this study (Table 1). However, other studies failed to detect flavonoids in water extracts of *M. oleifera* roots, but reported levels of 0.18 mg g^{-1} dw in extracts obtained with methanol (9). This value is similar to Y_{TFn} found in this study for the methanolic extract (0.20 mg RtE g^{-1} dw), but also the extract with ethyl acetate (0.17 mg RtE g^{-1} dw). Contradictory results were also reported by earlier examinations, where methanolic extracts (11) but also ethanolic extracts (12) of *M. oleifera* roots were shown to be free from flavonoids. In addition, all the extracts obtained were found to give positive response to reaction with DMAC, indicating the presence of flavanols, since this reagent is very specific and does not react with any other class of flavonoids (13,14). Although there are no similar reports in the literature, there have been claims for the presence of proanthocyanidins in methanolic extracts of *M. oleifera* roots (15).

The evaluation of the antioxidant potency of the extracts was carried out on the basis of two complementary tests, namely the reducing power (P_R) and the antiradical activity (A_{AR}). It has been pointed out that extracts deriving from different plant tissues, thus possessing significantly different polyphenolic composition, may have almost identical response to these two tests and yield highly correlated values (16). However, the first tests examines the ability of the compounds in question (polyphenols) to exchange electrons in a purely redox reaction, but hydrogen transfer mechanisms cannot be detected. To the contrary, the use of DPPH in the measurement of A_{AR} may also assess the ability of antioxidants to donate hydrogen atoms, depending on the conditions (17).

Apparently, the differences in the antioxidant potency of the extracts could reflect to some extent the differences found in the polyphenolic composition. In particular, the high A_{AR} of the methanolic extract could be justified by the higher flavanol concentration, because flavanols have been proven very powerful antioxidants in several systems (18) and their content has been significantly correlated with A_{AR} in grape seed extracts, but correlation with P_R was not consistent (19). Other studies demonstrated that the various forms of flavanols (monomers, type of dimers) affected in an important manner the scavenging ability of $DPPH^{\bullet}$, indicating flavanol dimers to be more efficient than monomers (20). Further to that, mixture effects resulting in synergism or antagonism amongst the various polyphenols occurring in the extracts could not be ruled out. Evidence emerged from studies on the A_{AR} of mixtures with pure compounds (21) but also with plant extracts (22) demonstrated the manifestation of antagonistic effects, as a result of regeneration of a compound with higher oxidation potential, at the expense of another with lower oxidation potential, by donating H atoms. Mixture effects, based on the estimation P_R, of pure antioxidants (23) or antioxidants in combination with extracts (24) were in the same line.

Regarding the polyphenolic composition, it could be speculated that substances eluted early are rather simple phenolics, as judged by the UV characteristics given in Table 2. On the other hand, the compound eluted at 17 minutes showed a typical flavonol spectrum and on the basis of

the mass spectral data it could be tentatively assigned to quercetin 3-*O*-glucuronide. Likewise, the peaks eluted at 41-41.5 minutes were tentatively identified as flavanol derivatives (25).

Conclusion

The polyphenolic composition of *M. oleifera* roots remains largely unexamined, despite the considerable volume of literature highlighting its important pharmacological properties. The present study represents a first approach to this issue, by screening some widely used solvents of increasing polarity with regard to their potential in extracting polyphenols from lyophilised *M. oleifera* roots. The results indicated that water extracts were the richest in total polyphenols, displaying strong antioxidant activity. The liquid chromatography-mass spectrometry study confirmed that the major in *M. oleifera* roots are quite polar, but minor substances corresponding to flavonoids were also detected.

Authors' contributions

IK: Laboratory experimentation and antioxidant tests; SG: Sample handling, LC-MS analysis, and data handling; SL: Raw material handling and laboratory experimentation; DPM: Paper writing, data handling, data interpretation, and statistics.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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