

In vitro Antioxidant Activities of *Trianthema portulacastrum* L. Hydrolysates

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ABSTRACT: Hydrolysates of *Trianthema portulacastrum* in acidified methanol were evaluated for their total phenolic (TP) constituents and respective antioxidant activities using *in vitro* assays (i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, percent inhibition of linoleic acid peroxidation, and ferric reducing power). The observed results indicate that root, shoot, and leaf fractions of *T. portulacastrum* contain 50.75~98.09 mg gallic acid equivalents/g dry weight of TP. In addition, these fractions have substantial reducing potentials (0.10~0.59), abilities to inhibit peroxidation (43.26~89.98%), and DPPH radical scavenging capabilities (6.98~311.61 µg/mL IC₅₀). The experimental data not only reveal *T. portulacastrum* as potential source of valuable antioxidants, but also indicate that acidified methanol may be an ideal choice for the enhanced recovery of phenolic compounds with retained biological potential for the food and pharmaceutical industry.

Keywords: *Trianthema portulacastrum*, effective extraction, antioxidant phenolics, reducing power

INTRODUCTION

Trianthema portulacastrum L. (Family: *Azoiaceae*), commonly known as desert horse-purslane, carpet weed, pig, and it-sit (in Pakistan), is an annual or perennial weed plant. *T. portulacastrum* is recognized for its high potential for infestation of agricultural and vegetable crops and is primarily controlled by spraying herbicides (1,2). Epidemiological studies have revealed a therapeutic value of *T. portulacastrum* for bronchitis; heart disease; anemia; inflammation; piles; ascites; eye disorders; allergies; alcohol poisoning; and paracetamol-, thioacetamide-, and toxin-induced hepatocarcinogenesis (3-5). However, there is limited information available about the phytochemical attributes of *T. portulacastrum*. Awareness of the functional food and health benefits of phenolic antioxidants has enhanced the value of these biologically active compounds.

The advent of modern versatile chromatographic and spectroscopic techniques has revolutionized the era of analytical characterization. However, current methods used to extract phenolics for further characterization and other end uses lack fitness and are mostly selected because

of their yield, economic concerns, and environmental concerns. A number of attempts have been made to develop green extraction technologies (6,7). Enzyme-, ultrasound-, microwave- and heat pulse-assisted extraction strategies have shown promising yields for lipophilic compounds (8-10). In all approaches, the soaking or distribution potential of the extraction solvent can be modified to enhance the liberation of target compounds. Unfortunately, in most of these cases, the antioxidant potential of most of the phenolic compounds obtained is decreased.

The present work was undertaken to improve the soaking and distribution power of the most frequently used extraction solvent (i.e., methanol) to enhance the recovery of phenolics with maximized retained antioxidant capacity. For this purpose, methanol that had been acidified to variable pH levels was used to extract phenolic antioxidants from different parts of a potentially important medicinal weed (*T. portulacastrum*). The acidified methanol hydrolysates obtained from the different parts of *T. portulacastrum* were evaluated for their antioxidant activities using standard *in vitro* assays.

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MATERIALS AND METHODS

Materials and chemicals

T. portulacastrum weed samples were collected from the botanical garden of the University of Agriculture, Faisalabad (Pakistan). The specimens were identified by the Taxonomist of the Department of Botany, University of Agriculture, Faisalabad. Reagents, including Folin-Ciocalteu reagent (FCR), gallic acid (98.0%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (90.0%), and linoleic acid (99.0%) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals (anhydrous sodium carbonate, sodium hydroxide, sodium nitrite, ferrous chloride, ammonium thiocyanate, aluminum chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, isooctane, chloroform, acetic acid, potassium iodide, and sodium thiosulphate) were of analytical grade and procured from Merck (Darmstadt, Germany).

Sample preparation

The leaf, root, and shoot portions of healthy and mature *T. portulacastrum* weeds were separated with sharp scissors, dried under ambient conditions (25°C), pulverized to pass through an 80-mesh sieve and stored in airtight polythene bags until further analysis.

Extraction

Powdered material (5 g) from the leaf, root, and shoot portions of *T. portulacastrum* were soaked overnight with 50 mL of 0.01, 0.5, 0.1, 1, 2, and 5 N acidified methanol in an orbital shaker (Gallenkamp, Loughborough, Leicestershire, UK) at 120 rpm and 30°C. The hydrolysates were separated from the residue by filtering through Whatman No. 1 filter paper (Sigma). The residues were re-extracted twice with the same solvents, and the final hydrolysates obtained were concentrated to dryness under reduced pressure at 45°C using a rotary evaporator (EYELA, N-N Series, Rikakikai Co., Ltd., Tokyo, Japan), weighed to calculate the yield, and stored in a freezer (4°C) until further analysis.

Total phenolics (TP)

The concentrations of TP in the hydrolysates of different parts of *T. portulacastrum* were estimated using FCR as described by Aaby et al. (11), with slight modifications. Briefly, 50 mg of crude hydrolysate was diluted with 7.5 mL of deionized water and mixed with 0.5 mL of FCR. The mixture was incubated at 30°C for 10 min and then 1.5 mL of 20% w/v sodium carbonate was added followed by consistent heating at 40°C for 20 min. The resultant mixture was chilled in an ice bath and the absorbance was recorded at 755 nm (U-2001, Hitachi Instrument Inc., Tokyo, Japan) to calculate TP (milligram

gallic acid equivalents [GAE]/g dry weight [DW]) according to a gallic acid calibration curve (2~200 ppm; $R^2=0.9952$).

DPPH antiradical capacity

The antiradical potential of *T. portulacastrum* weed hydrolysates was determined spectrophotometrically as described by Ilahi et al. (12). Six different concentrations of hydrolysates (1, 10, 100, 1,000, 2,000, and 5,000 µg/mL) were mixed with 100 µL of DPPH radical solution in a 96-well microplate and incubated for 20 min at room temperature. The resultant mixture was read spectrophotometrically at 517 nm against a methanol blank and the following equation was used to calculate the % inhibition of each hydrolysate:

$$\% \text{ inhibition} = \frac{(A_{\text{Control}} - A_{\text{sample}})}{(A_{\text{Control}})} \times 100$$

where A_{Control} and A_{sample} indicate the absorbance of the DPPH solution and the reaction mixture, respectively. The effective dose of hydrolysate needed to neutralize 50% of the DPPH radical solution (IC_{50}) was obtained from a plot comparing percent inhibition to hydrolysate concentration.

Antioxidant activity against linoleic acid peroxidation

The antioxidant activities of acidified methanol extracts were determined by measuring the ability of the extracts to inhibit linoleic acid peroxidation (13). Briefly, 5 mg of each hydrolysate was diluted with 10 mL of 0.2 M sodium phosphate buffer (pH 7) and mixed with a solution of linoleic acid (0.13 mL) and ethanol (10 mL). The mixture was diluted with distilled water to 25 mL and incubated at 40°C for 96 h. The thiocyanate method was used to measure the degree of oxidation (14). Briefly, 10 mL of ethanol (75% v/v), 0.2 mL of an aqueous solution of ammonium thiocyanate (30% w/v), and 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl) were added sequentially to 0.2 mL of sample. A mixture containing all of the reagents except the sample was used as a blank, and synthetic antioxidants (i.e., butylated hydroxytoluene (BHT) and ascorbic acid [200 ppm]) were used as a positive control. The % inhibition of linoleic acid peroxidation was calculated as follows:

$$\% \text{ inhibition} = 100 - \frac{(\text{Abs. increase of sample at 96 h})}{(\text{Abs. increase of control at 96 h})} \times 100$$

Ferric reducing power

The antioxidant power of acidified methanol hydrolysates of *T. portulacastrum* was determined by measuring ferric reducing ability (15). Different amounts of each hydrolysate were suspended in 5 mL of phosphate buffer

to produce 2.5, 5, 7.5, and 10 mg/mL solutions. These solutions were then mixed with 5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v) and incubated at 50°C for 20 min. The resulting solution was mixed with 5 mL of trichloroacetic acid (10% w/v) and centrifuged at 3,000 rpm for 10 min. The upper layer (5 mL) was collected, diluted with an equal amount of distilled water and 1 mL $FeCl_3$ (0.1% w/v), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Statistical analysis

All *in vitro* assays measuring the antioxidant potential of hydrolysates were performed in triplicate. All results are reported as mean \pm standard deviation (SD) of triplicate experiments. Minitab 16 Statistical Software (Minitab Inc., State College, PA, USA) was used for all statistical analyses. Two-way analysis of variance was used to determine differences among plant parts and solvent acidification levels.

RESULTS AND DISCUSSION

Yield of *T. portulacastrum* hydrolysates

Plant materials are organized polymeric cellulosic structures of sugars (alcohols) and acids (organic and phenolic acids), which are further fortified by lignin and, in certain cases, shielded by pectin (16). The recovery of phenolic compounds from plant material depends upon the soaking and distribution potentials of the extraction media and the technique applied. Acid hydrolysis can depolymerize the constitutional monomers of cell walls, which in turn can be used as an energy source for the industrial production of valuable compounds and enzymes (17,18). Phenolic compounds are primarily bound in the form of their glycosidic esters. Therefore, acidic media will not only hydrolyze and swell plant cell walls, but also convert bound phenolic esters into phenolic acids, which will ultimately enhance the distribution power of

the extraction media. Therefore, in this study an organic solvent (i.e., methanol) that had been variably acidified with HCl was utilized to liberate phenolic antioxidants. Released phenolic compounds with a higher miscibility towards the polar solvent were simultaneously accumulated in the organic solvent and extracted (19).

The hydrolysate yield of *T. portulacastrum* root, shoot, and leaf portions was 10.43~54.23 g/100 g DW, 10.30~26.29 g/100 g DW, and 5.53~38.40 g/100 g DW, respectively. The extent of methanol acidification (0.01~5 N) produced significantly variable amounts of hydrolysate ($P<0.05$), as indicated by different subscripted letters (Table 1). The highest hydrolysate yield was produced with 0.1 N acidified methanol from root of *T. portulacastrum*, while the lowest yields were achieved with 80% aqueous methanol (i.e., control). Overall, the acidification of methanol enhanced the recovery of phytochemicals from *T. portulacastrum* up to four fold. The higher hydrolysate yield at a relatively lower acidification level (i.e., 0.1 N acidified methanol) might be attributed to the optimum maceration of the *T. portulacastrum* cell wall. Significant variations in the percent yield for root, shoot, and leaf portions were due to difference in the chemical composition of the plant parts.

A thorough review of previously published research indicates that this is the first report of the extraction of antioxidant phenolics from *T. portulacastrum*. The methanol extract used in the current study results in a similar yield to that of fruits, as reported by our group (20,21), and that of *Lantana camara* (12.68%), as reported by Ali-Emmanuel et al. (22). Together, these results indicate that acidified methanol is a suitable choice for the recovery of phenolic antioxidants.

Total phenolics

The concentrations of TP in the crude hydrolysates of root, shoot, and leaf portions of *T. portulacastrum* obtained using variably acidified methanol were 63.60~98.09 mg GAE/100 g DW, 50.75~68.63 mg GAE/100 g DW, and 54.21~91.86 mg GAE/100 g DW, respectively. Overall, the yield of TP, as determined using FCR

Table 1. Hydrolysate yields and total phenolics (TP) concentrations of different parts of *T. portulacastrum*

Acidified methanol (N)	Extraction yield (g/100 g)			TP (mg GAE/100 g DW)		
	Root	Shoot	Leaf	Root	Shoot	Leaf
5	10.43 \pm 0.40 ^{CA}	10.30 \pm 0.28 ^{BA}	5.53 \pm 0.47 ^{AA}	63.60 \pm 2.73 ^{EA}	50.75 \pm 0.11 ^{AA}	54.21 \pm 2.86 ^{DA}
2	27.36 \pm 1.56 ^{CB}	12.63 \pm 0.15 ^{AB}	15.56 \pm 0.15 ^{BB}	66.46 \pm 0.34 ^{EA}	56.47 \pm 1.67 ^{AA}	61.18 \pm 0.08 ^{DA}
1	28.43 \pm 0.15 ^{CB}	15.43 \pm 0.45 ^{AB}	16.60 \pm 0.21 ^{BB}	75.23 \pm 5.06 ^{EB}	58.51 \pm 3.32 ^{AB}	67.18 \pm 0.61 ^{DB}
0.5	33.60 \pm 0.39 ^{CB}	16.30 \pm 0.36 ^{AB}	22.26 \pm 0.25 ^{BB}	76.20 \pm 2.12 ^{EC}	60.87 \pm 0.08 ^{AC}	83.37 \pm 0.33 ^{DC}
0.1	54.23 \pm 0.25 ^{CD}	26.29 \pm 0.89 ^{AD}	38.40 \pm 0.29 ^{BD}	98.09 \pm 0.50 ^{ED}	68.63 \pm 5.99 ^{AD}	91.86 \pm 3.86 ^{DD}
0.01	44.30 \pm 0.38 ^{CC}	17.53 \pm 0.25 ^{AC}	23.33 \pm 0.41 ^{BC}	86.08 \pm 2.88 ^{EC}	62.16 \pm 4.57 ^{AC}	87.77 \pm 0.11 ^{DC}
Aqueous methanol	16.55 \pm 0.45 ^{CA}	9.99 \pm 0.72 ^{AA}	10.42 \pm 0.37 ^{BA}	61.56 \pm 0.67 ^{EA}	52.27 \pm 1.25 ^{AA}	58.13 \pm 0.63 ^{DA}

The results are mean \pm SD of experiments conducted in triplicate for each *T. Portulacastrum* part and solvent acidification level. Superscripted capital and small letters denote variation ($P<0.05$) among solvent acidification levels and plant parts, respectively.

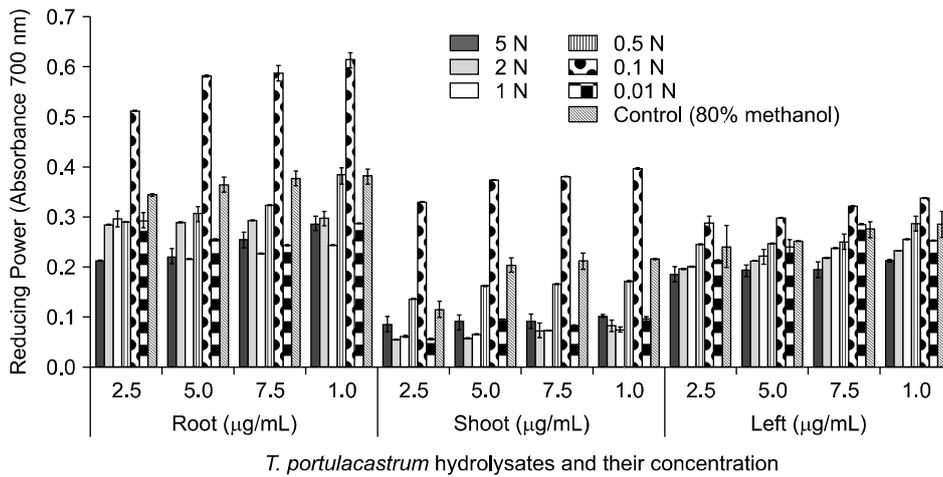


Fig. 1. Comparison of the reducing power of acidified methanol hydrolysates of *T. portulacastrum*.

and gallic acid as positive controls, varied significantly ($P < 0.05$) with the level of acidification applied and the plant part investigated (Table 1). The results indicate that acidification of the extraction solvent (i.e., methanol) increases phenolic recovery 1~1.5 fold. The root, shoot, and leaf hydrolysate fractions obtained using 0.1 N acidified methanol contained the highest concentrations of TP, whereas further acidification lowered the amount of phenolic liberation. The reason for this may be a change in the structural features of the hydrolysate substituents or the deterioration of phenolic compounds under severe acidic conditions. No previous technical data were found regarding phenolic and antioxidant attributes for comparison. The concentration of TP found in *T. portulacastrum* was higher than that of methanol extracts from *Vitex doniana* (0.20~7.60 mg GAE/100 g) reported by Muanda et al. (23) and methanol extracts from ambient dried cauliflower (24); however, the concentrations of TP found in this study were comparable to those found in certain oriental herb medicines (25).

The markedly high concentrations of TP that were observed in different parts of *T. portulacastrum* during the present investigation indicate that the reported folk medicinal applications of *T. portulacastrum* may be due to the presence of biologically active phenolic metabolites. The observed levels of TP also suggest that acidified methanol is a good choice for the recovery of phenolic compounds. However, there is some concern in the literature that acid or alkaline hydrolysis causes a degradation of phenolic compounds that decreases the biological activity of the hydrolysate (26). Therefore, to confirm that appropriate antioxidant activities were retained, all extracts were subjected to *in vitro* experiments to determine radical scavenging potential, peroxidation inhibition capacity, and reducing power.

Ferric reducing power (FRP)

The antioxidant activities of *T. portulacastrum* hydrolysates were evaluated by determining FRP. FRP is thought to

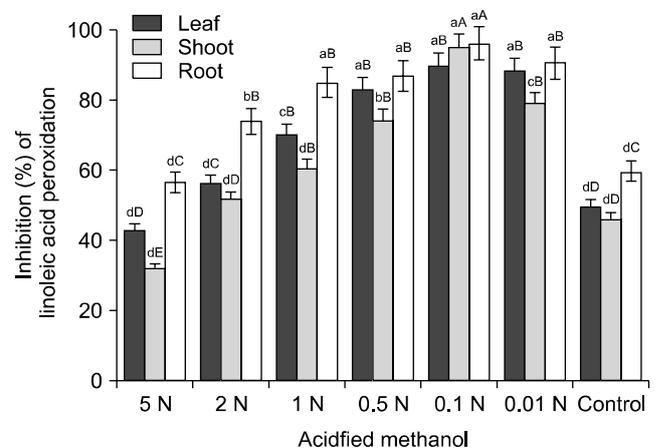


Fig. 2. Antioxidant potential offered by *T. portulacastrum* hydrolysate in linoleic acid peroxidation system. Means with capital and small letters indicate variation ($P < 0.05$) among solvent acidification levels and plant parts, respectively.

imitate some aspects of the antioxidant activity of extracts. According to a few reports, FRP is controlled by the hydrogen donating ability of the compounds under investigation (27). In this assay, ferric ions are reduced to ferrous ions, resulting in a change of color from yellow to bluish-green. The intensity of the bluish-green color reflects the reducing potential of the compounds: a more intense color is associated with a higher absorption and a greater antioxidant activity (28).

The reducing potentials of *T. portulacastrum* root, shoot, and leaf hydrolysates were assessed over a concentration range of 2.5 to 10 mg/mL. The graphical presentation of assimilated data (Fig. 1) reveals that the root of *T. portulacastrum* contains the highest amount of reducing species, followed by the leaf and the shoot portions of *T. portulacastrum*. In addition, the reducing potentials of the 0.1 N acidified methanol hydrolysates were higher for all *T. portulacastrum* concentrations tested (Fig. 2). Therefore, the ferric reducing power of *T. portulacastrum* phenolic metabolites is maintained when phenolic metabolites are liberated using acidified methanol. FRP varied sig-

Table 2. Pearson's correlation between yield, TP, and antioxidant activities

	Root				Shoot				Leaf			
	Yield	TP	DPPH	Inh	Yield	TP	DPPH	Inh	Yield	TP	DPPH	Inh
TP	0.821*				0.695*				0.849*			
DPPH	0.000	0.491*			0.000	0.459			0.000	0.000		
Inh	-0.016 ^{ns}	0.02	0.118 ^{ns}		-0.201 ^{ns}	0.360	-0.672*		-0.386 ^{ns}	-0.725*	0.474	0.000
RP	0.945	0.904*	0.612	0.389 ^{ns}	0.456*	0.800*	0.001	0.939*	0.734*	0.962*	-0.306 ^{ns}	0.177
	0.000	0.000	-0.330 ^{ns}	0.082	0.038	0.682*	-0.794*	0.000	0.000	0.000	0.000	0.604*
	0.297 ^{ns}	0.904*	0.145	0.098	0.371 ^{ns}	0.001	0.000	0.000	0.397 ^{ns}	0.572*	0.000	0.004
	0.191	0.000			0.098	0.001	0.000	0.000	0.075	0.007	0.000	0.004

TP, total phenolics; DPPH, DPPH radical scavenging activity of hydrolysates; Inh, inhibition of linoleic acid peroxidation; RP, reducing power. ns: Not significant.

*Significant at $P < 0.05$.

nificantly ($P < 0.05$) in response to solvent system, plant part, and hydrolysate concentration. At the maximum hydrolysate concentration investigated (10 mg/mL), FRP was greatest in the root portion (0.268~0.595), followed by the shoot portion (0.103~0.398) and the leaf portion (0.213~0.339). Among methanol acidification levels (0.01~5 N), the 0.1 N acidified methanol condition produced hydrolysates with the highest concentrations of valuable reductants (phenolics). Further acidification lowered the reducing potential of extracts, possibly because of a change in structural features or the availability of interchangeable hydrogen atoms. Furthermore, a strong correlation was found between the observed reducing power and concentration of TP (Table 2). This finding supports the hypothesis that the antioxidant character of plant biodiversity may be due to the presence of phenolic compounds (28).

No previous reports describing the reducing power of *T. portulacastrum* plant parts were available for comparison. However, the FRP values of *T. portulacastrum* reported in this study are comparable with previously published values from certain Brazilian vegetables (29) and fruits (21), but lower than values from medicinal plants investigated by Sultana et al. (30).

Antioxidant activity against linoleic acid peroxidation

The potential of *T. portulacastrum* hydrolysates to inhibit the peroxidation of linoleic acid *in vitro* was assessed using the thiocyanate method (14). Linoleic acid, a polyunsaturated fatty acid, is highly susceptible to autoxidation, which leads to the formation of peroxides. These peroxides convert oxidized Fe^{2+} to Fe^{3+} . The latter of the two then forms a complex with SCN^- , which can be measured spectrophotometrically. The final concentration of the formed complex is inversely related to the antioxidant activity of hydrolysate. The extent of linoleic acid peroxidation was assessed after a 96-h incubation period. Ascorbic acid was used as positive control for this assay.

All hydrolysate fractions (root, shoot, and leaf) obtained with acidified methanol and aqueous methanol extracts (i.e., control) exhibited appreciable inhibition of linoleic acid peroxidation (43.26~89.98%). As shown in Fig. 2, the percent inhibition varied by plant part and solvent acidification level. Overall, hydrolysates obtained using 0.1 N acidified methanol were more effective at preventing the peroxidation of linoleic acid than other acid concentrations and aqueous methanol. Among the plant parts investigated, the root of *T. portulacastrum* produced hydrolysates offering a substantially greater linoleic acid peroxidation inhibition potential for all solvent acidification levels, followed by leaf fractions and shoot fractions.

The considerably higher linoleic acid peroxidation inhibition potential of the hydrolysates obtained with the 0.1 N acidified methanol solvent is thought to be due to the presence of an optimal concentration of liberated phenolics. This was confirmed by a number of previous studies (31,32) and a statistical analysis of the data, which revealed a significant correlation (Table 2) between TP and the inhibition potential of extracts.

DPPH radical scavenging activity

DPPH, a stable organic free radical with a deep violet color, is frequently used for the assessment of antioxidant activity (12,33). Upon accepting an electron, a yellow coloration that can be quantified spectrophotometrically is produced. As the concentration of phenolic compounds or the degree of hydroxylation of the phenolic compounds increases, the yellow color produced becomes more intense (34). *T. portulacastrum* weed hydrolysates were evaluated at six different concentrations (1, 10, 100, 1,000, 2,000, and 5,000 $\mu\text{g/mL}$) to assess their DPPH radical scavenging potential. The data revealed that the DPPH radical scavenging capacity of *T. portulacastrum* hydrolysates increases linearly with concentration ($R^2 > 50\%$). Therefore, the minimum inhibitory concentration (IC_{50}) was calculated. *T. portulacas-*

Table 3. DPPH radical scavenging activity of *T. portulacastrum* hydrolysates obtained with acidified methanol

Acidified methanol (N)	Plant parts		
	Root	Shoot	Leaf
5	163.95±4.16 ^{dB}	116.98±1.13 ^{dB}	190.74±4.27 ^{dB}
2	380.43±3.42 ^{dE}	201.47±0.84 ^{aE}	192.91±4.15 ^{dE}
1	134.61±4.34 ^{dD}	230.70±3.05 ^{aD}	257.95±3.43 ^{dD}
0.5	417.23±3.35 ^{dD}	48.17±4.16 ^{aD}	179.32±5.56 ^{dD}
0.1	40.02±0.55 ^{dA}	28.87±1.33 ^{aA}	54.50±4.03 ^{dA}
0.01	116.97±0.76 ^{dC}	311.61±1.02 ^{aC}	198.56±4.26 ^{dC}
Control (80% aqueous methanol)	120.78±3.11 ^{dB}	112.76±1.57 ^{aB}	190.89±3.53 ^{dB}

Values are mean±SD of the minimum inhibitory concentration (IC₅₀).

IC₅₀ was determined by *in vitro* experiments that were conducted in triplicate.

Superscripted capital and small letters indicate significant variation between solvent fractions used and plant parts investigated, respectively.

trum stem hydrolysates produced with 0.1 N acidified methanol were the most effective at quenching DPPH radicals (28.87±1.33 mg/mL), followed by root hydrolysates (40.02±0.55 mg/mL) and leaf hydrolysates (54.50±4.03 mg/mL) (Table 3). The results of present investigation revealed that the recovery of phenolic compounds with retained antioxidant potential is greater with an optimally acidified extraction than it is with an aqueous methanol extraction, which is a more frequently used for phenolic extraction. The substantial level of antioxidant activity observed in this study may be related to the altered hydrolytic potential of acidified methanol, which allowed for the liberation of glycosidically entrapped plant phenolics. It should be noted that 0.1 N acidification was sufficient to increase antioxidant potential. Further increases in acidity level enhanced extraction yield but lowered the antioxidant potential of the hydrolysates, indicating that severe acidic conditions may deteriorate plant phenolics.

CONCLUSION

The results of the present analysis revealed that optimally acidified methanol is ideal for enhanced liberation of antioxidant phenolics for food and pharmaceutical industries. Furthermore, the data revealed that *T. portulacastrum* is a rich source of potentially valuable antioxidants. Further work is necessary to determine nutraceutical and chemopreventive applications of *T. portulacastrum*. Among the plant parts tested, the root fraction of *T. portulacastrum* exhibited the highest concentration of phenolic compounds and the greatest antioxidant activity.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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