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Effects of Extraction Methods on Phenolic Contents and Antioxidant Activity in Aerial Parts of *Potentilla atrosanguinea* Lodd. and Quantification of Its Phenolic Constituents by RP-HPLC†

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The effects of different solvent systems (methanol, ethanol, acetone, and their 50% aqueous concentrations) and extraction procedures (microwave, ultrasound, Soxhlet and maceration) on the antioxidant activity of aerial parts of *Potentilla atrosanguinea* were investigated by three different bioassays: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays and ferric reducing antioxidant potential (FRAP). The 50% aqueous ethanol extracts exhibited strong antioxidant activity measured in terms of Trolox equivalent antioxidant capacity (TEAC) [(54.34 to 122.96, 29.82 to 101.22 and 13.64 to 41.43) mg of Trolox/g] with $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP assays, respectively. In general, TEAC of Soxhlet extracts was found to be 1.8 and 3 times higher than ultrasound and maceration but slightly (1.2 times) higher than microwave. A positive correlation ($r^2 = 0.931$ to 0.982) was observed between total polyphenol (TPC) and total flavonoid (TFC) contents which ranged between 26.7 to 30.7 mg/g gallic acid equivalent and 16.8 to 20.8 mg/g quercetin equivalent respectively, with antioxidant activity. In addition, some of its bioactive phenolic constituents which contribute largely toward antioxidant potential such as chlorogenic acid, catechin, caffeic acid, *p*-coumaric acid and quercetin were also quantified in different extracts by RP-HPLC.

KEYWORDS: ***Potentilla atrosanguinea*** **(Lodd.); polyphenols; MAE; Soxhlet; antioxidant activity; HPLC**

INTRODUCTION

Antioxidants and their chemical properties are currently a subject of intensive research. This is stimulated by the fact that oxidative stress in vivo may cause various problems, associated with numerous degenerative aging diseases, such as cancer and arteriosclerosis (*1*). Oxidative stress caused by reactive oxygen species (ROS) is a specific feature in the pathogenesis of various diseases, including cancer, cardiovascular diseases, diabetes, tumors, rheumatoid arthritis and epilepsy (*2, 3*). In recent years, considerable attention has been paid to antioxidant properties of plants that may be used for human consumption. Phenolic compounds of plants are attracting considerable interest in the field of food, chemistry and medicine due to their promising antioxidant potential (*4*). Keeping in view the relevance of free radical theory of diseases as one of the objective parameters, the amenability of extracts of *Potentilla atrosanguinea* was examined in the present study as no scientific report on antioxidant activity of this plant is available to date.

Potentilla is a genus of about 500 species of annual, biennial and perennial herbs of the Rosaceae family, native to most of the Northern Hemisphere (*5*). Recent investigations have shown that some extracts of different parts of plant from *Potentill*a species exhibit antioxidant, hypoglycemic, anti-inflammatory, antitumor and antiulcerogenic potential properties (*6*−*12*). Phytochemical studies have demonstrated the presence of proanthocyanidins, catechin polyphenols, flavonoids (quercetin and myricetin glycosides) and secondary metabolites like tannins of the ellagic-acid type, with monomeric and dimeric ellagitannins, similar to those found in green tea in various species of *Potentilla* (*13, 14*).

Potentilla atrosanguinea Lodd. var. argyrophylla (Wall. ex. Lehm) is a perennial herb native to western Himalayas and commonly known as Himalayan cinquefoil (*15*). The starchy roots of *P. atrosanguinea* which are said to taste like parsnips, sweet potatoes, or chestnuts have served as a human food, while its leaves are valued as a healthful tea (*16, 17*) and deserve attention for their investigation into chemical composition and antioxidant properties as various bioactive properties of the

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plants including antioxidant are attributed to the presence of phenolic compounds in them. In recent years, many papers have been published on the extraction of bioactive compounds from plants for antioxidant activities (*18*−*21*) but studies on the antioxidant activity of *P. atrosanguinea* are scarce. Hence, the objective of this work was to investigate the effects of extraction solvents and methods on phenolic contents and antioxidant activities in aerial parts of *P. atrosanguinea* vis-à-vis quantification of some of its phenolic constituents [chlorogenic acid (CA,1); catechin (CN, 2); caffeic acid (CFA, 3); *p*-coumaric acid (PCA, 4); and quercetin (QC, 5)] with the help of RP-HPLC to demonstrate the further correlation of phenolics with antioxidant activity.

MATERIALS AND METHODS

Chemicals. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), ferric chloride, Folin-Ciocalteu's phenol reagent, gallic acid, Trolox, quercetin, chlorogenic acid, (+)-catechin, caffeic acid and *p*-coumaric acid were purchased from Sigma−Aldrich Chemie (Steinheim, Germany). All of the extracts and solvents were filtered through a 0.45 μm membrane filter (Millipore, Germany) and degassed prior to use. HPLC-grade acetonitrile (MeCN) and water were purchased from J.T. Baker. All other solvents and chemicals were of analytical grade and obtained from Merck (Mumbai, India). Green and black tea were obtained from Institute's experimental tea factory.

Plant Material. The aerial parts of *P. atrosanguinea* weighing 1.0 kg were collected from Kunzum Pass (light intensity, 2500 μEinstein/m^2/s; daytime air temperature, 3−10 °C; altitude, 4517 m; 32° 24′ 20″ N; 077° 38′ 40″ E) in Lahaul and Spiti district of Himachal Pradesh in Western Himalayas of India in the month of September 2006 and voucher specimens (PLP 4353) collected were identified, processed and deposited in the Herbarium of Institute of Himalayan Bioresource Technology (CSIR), Palampur, India. After harvest, the plant material was air-dried under shade (temperature, 27 ± 2 °C; and relative humidity, 34 ± 3%) on the laboratory bench for five days and then pulverized and stored at an ambient temperature (27 °C) in a desiccator before extraction and analysis.

Extraction of Plant Material. *Standardization of Extraction Solvents for Phenolics.* Different solvent systems (methanol, 50% aqueous methanol, ethanol, 50% aqueous ethanol, acetone, 50% aqueous acetone) were used to determine the effectiveness of solvent type on the extraction of phenolics from *P. atrosanguinea*. The phenolic compounds were extracted from 1 g of dry sample using 50 mL of solvent. The mixtures were sonicated with 50 mL solvents in an ultrasonicator bath (Elma Ultrasonic, Germany) at a controlled temperature (30 ± 5 °C) for 60 min. The mixtures were centrifuged at 10000*g* for 10 min at 4 °C. Supernatants were filtered through a funnel with glass wool, which was washed with 3−4 mL of solvent and concentrated to dryness under vacuum (temperature, 40−45 °C) and subjected to lyophilization using Savant (Savant, USA) vacuum concentrator until a constant weight was obtained.

Standardization of Extraction Methods for Phenolics. Different extraction methods like Soxhlet, microwave, ultrasonic and cold extraction were used to determine the effectiveness of extraction type on the extraction of phenolics from *P. atrosanguinea*.

Microwave-Assisted Extraction (MAE). Five grams of powdered plant material was extracted with 50 mL of solvent in a domestic microwave (Solo-M1630N, Samsung: South Korea) for 1−6 min at 450 W microwave power. The samples were heated according to the preset programs of microwave irradiation. The extract was filtered, processed and dried as explained in the section 'Extraction of Plant Material'.

Ultrasound-Assisted Extraction (UAE). Five grams of powdered plant material was sonicated with 50 mL of solvent in an ultrasonicator bath (Elma Ultrasonic, Germany) at a controlled temperature (30 ± 5 °C) for given certain time. After extraction, the extract was processed as above for analysis.

Soxhlet Extraction. Five grams of powdered plant material was put into a 150 mL Soxhlet thimble. The apparatus was fitted with a 500 mL round-bottom flask containing 150 mL of extraction solvent. The extraction temperature was controlled at 80 °C with a regulator. The flask was heated for a certain time, and the solvent was refluxed. After extraction, the extract was similarly processed as above.

Maceration. Five grams of powdered plant material was macerated overnight in 50 mL of solvent at room temperature (27 ± 3 °C). The extract obtained was filtered and processed as done previously with other methods for further analysis.

All of the extractions were performed in triplicate. All extracts were kept in a nitrogen atmosphere and −20 °C until further use. For evaluation of antioxidant activity, concentrated extracts were dissolved in ethanol (analytical grade) to get final sample solutions of 1 mg/mL.

Determination of Total Polyphenol Contents. Total polyphenol contents (TPC) in extracts were measured using Folin-Ciocalteu's method (*22, 23*). For preparation of a calibration curve, 20, 40, 60, 80, and 100 μL aliquots of aqueous gallic acid (0.2%) were mixed with 0.5 mL of 1 N Folin-Ciocalteu's phenol reagent and 1.0 mL of 35% Na_2CO_3 in a 25 mL volumetric flask and the solution was made to 25 mL in distilled water. The absorbance relative to that of the blank was measured using a Hitachi 150-20 UV spectrophotometer (Hitachi Corp.; Tokyo, Japan) at 730 nm after 35 min of incubation at an ambient temperature. The total polyphenol contents of the extracts were expressed as mg of gallic acid equivalent (GAE)/g of dry plant material. A total of 50 μL of aqueous ethanol extracts was mixed with the same reagent as described above, and after 35 min of incubation, the absorbance was measured at 730 nm for determination of total polyphenol contents. All determinations were performed in triplicate.

Determination of Total Flavonoid Contents. Total flavonoid contents (TFC) in the extracts were measured using a modified colorimetric method (*24*). For preparation of calibration curve, 0.5 mL of 12.5, 25.0, 50.0, 80.0 and 100 μg/mL quercetin in 80% ethanol (v/v) was mixed with 1.5 mL of 95% ethanol (v/v), 0.1 mL of 10% aluminum chloride (w/v), 0.1 mL of 1 M potassium acetate and 2.8 mL of water in a 5.0 mL volumetric flask. After incubation at room temperature for 30 min, the absorbance of the reaction mixture relative to blank was measured using spectrophotometer at 415 nm. The total flavonoid contents of extracts were expressed as mg of quercetin equivalent (QE)/g of dry plant material. Aliquots (0.5 mL) of aqueous ethanol extracts were mixed with the same reagent as described above, and after 30 min, the absorption at 415 nm was measured for determination of total flavonoids. All measurements were done in triplicate.

Evaluation of Antioxidant Activity. *ABTS Radical Cation Scavenging Activity.* The ABTS radical cation decolorization test is a spectrophotometric method widely used for assessment of antioxidant activity of various substances. The experiment was carried out using an improved ABTS decolorization assay (*25*). In brief, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulphate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study of *P. atrosanguinea* extracts, the $ABTS^{\bullet+}$ was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm and equilibrated at 30 °C. After addition of 2.0 mL of diluted $ABTS^{\bullet+}$ solution ($A = 0.700 \pm 0.020$) to 50 μL of antioxidant compounds the absorbance reading was taken exactly after 4 min, appropriate solvent blank was run in each assay. All determinations were carried out in triplicate on each occasion and at each separate concentration of the standard and extracts. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants.

DPPH Radical Scavenging Activity. Radical scavenging activity of extracts against stable $DPPH^{\bullet}$ was also determined spectrophotometrically. When $DPPH^{\bullet}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The change in color (from deep-violet to yellow) was measured at 517 nm. The radical scavenging activity

of extracts was measured by slightly modified method of Brand-Williams (*26*). The extracts of *P. atrosanguinea* measuring 0.1 mL were added to 2.9 mL of the 100 μM DPPH$^{\bullet}$ solution prepared in 80% aqueous ethanol. The mixture was shaken vigorously and allowed to stand at 23 °C in the dark for 30 min, and decrease in absorbance of the resulting solution was monitored at 517 nm against a blank consisted of 0.1 mL of 50% aqueous ethanol and 2.9 mL of DPPH$^{\bullet}$ solution. All measurements were done in triplicate.

Ferric-Reducing Antioxidant Power Assay (FRAP). The FRAP assay was carried out according to the procedure of Benzie and Strain (*27*) with slight modification. The principle of this method is based on the reduction of a ferric 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (Fe^{3+}-TPTZ) to its ferrous, colored form (Fe^{2+}-TPTZ) in the presence of antioxidants. Briefly, the FRAP reagent was prepared from 0.3 M acetate buffer (pH 3.6), 10 mmol of TPTZ solution in 40 mmol of HCl and 20 mmol of iron(III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared daily and warmed to 37 °C prior to use. 50 μL of extract was added to 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min against blank. All measurements were done in triplicate.

Trolox Equivalent Antioxidant Capacity (TEAC). Trolox standard curves that relate the concentration of Trolox and the amount of absorbance reduction caused by it were obtained using the ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ assays (*28, 29*). The absorbance reduction at 734 and 517 nm of Trolox was measured at various concentrations by ABTS and DPPH assay respectively. For preparation of a calibration curve, 0.025% ethanolic solution of Trolox in various concentrations (2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μL) was mixed with 2.0 mL of diluted ABTS$^{\bullet+}$ solution ($A = 0.700 \pm 0.020$) and the absorbance reading was taken exactly 4 min after initial mixing at 734 nm. A similar experiment was performed with the DPPH radical, wherein 0.05% ethanolic solution of Trolox in various concentrations (10, 20, 30, 40, 50, 60, 70, and 80 μL) was mixed with 2.9 mL of 100 μM DPPH$^{\bullet}$ solution. The mixture was shaken vigorously and allowed to stand at 23 °C in the dark for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm at 30 min. The radical scavenging activity was calculated by the formula % inhibition $= [(A_B - A_A)/A_B] \times 100$, where A_B = absorption of blank sample and A_A = absorption of tested extract solution.

The calculation of TEAC of each extracts at the various concentrations levels was made using Trolox standard curves (*30, 31*). For extracts, properly diluted extracts were assayed by both ABTS and DPPH models.

For calculation of TEAC in case of FRAP assay, a calibration curve was prepared by mixing 0.01% ethanolic solution of Trolox in various concentrations (15, 30, 45, 60, 75, 90 and 105 μL) with 1.5 mL of FRAP reagent and absorbance of the reaction mixture was then recorded at 593 nm after 4 min. The results were expressed as mg of Trolox/g weight of plant material/extracts.

HPLC Analysis. HPLC-DAD analysis was performed with a Waters HPLC system equipped with 600 quaternary gradient pump, 717 plus autosampler, 996 PDA detector, and Millennium (*32*) software (version 3.05.01). The chromatographic separation was performed on Phenomenex (Torrance, CA) analytical column (LUNA C_{18} (2) 250 mm × 4.6 mm i.d.) packed with 5 μm silica. The temperature of the column was set at 27 °C. Elution of standards and extracts (20 μL) was performed with gradient solvent program, at a flow rate of 1.0 mL/min for 25 min. The mobile phase consisted of 0.01% (v/v) trifluoroacetic acid (TFA) in water (A) and acetonitrile (B) with the following gradient: A−B (80:20), B was gradually increased to 30% at 8 min, to 40% at 12 min, to 50% at 17 min and back to 20% from 17 to 25 min. The column was than re-equilibrated with the initial conditions for 5 min before the next injection. The detection wavelength was set at 280 nm (spectral acquisition in the range 200−400) nm. Identification of compounds was performed on the basis of the retention time, co-injections, and diode array spectral matching with standards. For the preparation of the calibration curve, standard stock solutions of compounds (1 mg/2 mL) were prepared in methanol, filtered through 0.45 μm filters (Millipore), and appropriately diluted (0.1−500 μg/mL) to obtain the desired concentrations in the quantification range. The calibration graphs were plotted after linear regression of the peak areas versus concentrations.

Table 1. Total Polyphenol Contents in Different Solvent Extracts

sr. no.	solvent system	TPC[a]
1	methanol	16.86 ± 0.02
2	50% methanol	24.54 ± 0.16
3	ethanol	25.65 ± 0.03
4	50% ethanol	26.72 ± 0.11
5	acetone	1.75 ± 0.09
6	50% acetone	20.44 ± 0.16

[a] Data expressed as mg of gallic acid equivalent (GAE)/g of plant material (DM basis).

Statistical Analysis. Results were expressed as mean ± standard error. Correlation between polyphenol contents, flavonoid contents and antioxidant activity was established by regression analysis.

RESULTS AND DISCUSSION

Solvent Extraction. In this study, the effect of various solvents on the efficient extraction of phenolics from aerial parts of the plant was investigated. Until now, to the best of our knowledge, there is no such report available that could highlight the comparative role of various extraction solvents on the phenolic contents of aerial part of *P. atrosanguinea*. It was seen that 50% aqueous concentration of each solvent was found to be most efficient for extraction of polyphenols in comparison to other concentrations and 50% aqueous ethanol was more efficient than all other solvent systems (**Table 1**). From these results, it is clear that the addition of some amount of water enhances the extraction efficiency. A similar result was also reported previously in microwave-assisted solvent extraction of effective constituents from *Herba epimedii* (*32*). The reason for the extraction efficiency with aqueous solvents is primarily due to water soluble nature of plant phenolics enhanced by the presence of solvent which facilitate solubilization through penetration in plant cell structure.

Extraction Yields. In this study, the effect of various extraction methods for the efficient extraction of antioxidative compounds from aerial parts of *P. atrosanguinea* was investigated. All the extractions were carried out in 50% aqueous ethanol solvent system as it results into efficient extraction of phenolics as optimized (**Table 1**). On mass yield basis, extraction time for MAE, UAE, Soxhlet and maceration was optimized as 4 min, 60 min, 8 and 20 h respectively. During extraction, it was seen that maximum extraction yield was achieved with Soxhlet extraction followed by MAE, UAE, and maceration. However, taking into consideration the solvent consumption and time needed for extraction, MAE was found to be the most practical approach for the rapid and efficient extraction of bioactive phenolic constituents (**Table 2**). The slight increase in extraction efficiency seen in case of Soxhlet extraction is mainly due to extraction for extended period of time as compared to others.

Total Polyphenol and Flavonoid Content. The total polyphenol contents in different extracts were determined from regression equation of calibration curve and expressed in gallic acid equivalent between 26.7 ± 0.11 to 30.7 ± 0.05 mg of GAE/g of dry plant material (**Table 2**). Similarly, total flavonoids in aqueous ethanol extracts were determined from regression equation of calibration curve and expressed in quercetin equivalent between 16.8 ± 0.07 to 20.8 ± 0.02 mg of QE/g of dry plant material (**Table 2**). It is clear from **Table 2** that the phenolic contents in Soxhlet extracts were highest among all. Total phenolic levels of these plants used as herbal tea in public

Table 2. Extraction Yield, Total Phenolics and Antioxidant Activity of *P. atrosanguinea*

sr. no.	extraction method	yield (wt %)	TPC[a]	TFC[b]	TEAC[c]
1	Soxhlet	24.2 ± 0.03	30.7 ± 0.05	20.8 ± 0.02	122.96 ± 0.38 * 101.22 ± 0.41 ** 41.43 ± 0.22 ***
2	microwave	21.8 ± 0.28	29.1 ± 0.27	19.6 ± 0.04	103.40 ± 0.69 * 88.70 ± 0.53 ** 26.88 ± 0.05 ***
3	ultrasonic	18.8 ± 0.14	27.8 ± 0.15	17.4 ± 0.04	78.51 ± 0.48 * 55.04 ± 0.46 ** 19.41 ± 0.03 ***
4	maceration	17.2 ± 0.21	26.7 ± 0.11	16.8 ± 0.07	54.34 ± 0.77 * 29.82 ± 0.42 ** 13.64 ± 0.01 ***

[a] Data expressed as mg of gallic acid equivalent (GAE)/g of plant material (DM basis). [b] Data expressed as mg of quercetin equivalent (QE) /g of plant material (DM basis). [c] Data expressed as mg of Trolox equivalent/g of plant material (DM basis). (*) TEAC assayed by ABTS method. (**) TEAC assayed by DPPH method. (***) TEAC assayed by FRAP method.

are important by reducing the risk of atherosclerosis and coronary heart disease, which can be caused by oxidation of low-density lipoproteins (*33*).

Antioxidant Activity of *P. atrosanguinea.* In the light of the differences among the wide number of test systems available, the results of a single-assay can give only a reductive suggestion of the antioxidant properties of extracts toward food matrices and must be interpreted with some caution. Moreover, the chemical complexity of extracts could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays in screening work is highly advisable (*34*−*36*). Thus, the extracts were subjected to three different antioxidant bioassays employing $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP methods. In this study, the antioxidant activity was expressed as mg of Trolox equivalents per gram of plant material on a dry basis as it is a more meaningful and descriptive expression than assays that express antioxidant activity as the percentage decrease in absorbance. As such the results provide a direct comparison of the antioxidant activity with Trolox. The results of $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP by standard antioxidant Trolox are summarized in **Table 2**. As displayed in **Table 2**, the TEAC values for the extracts had wide variability and ranged between 54.34 ± 0.77 to 122.96 mg of Trolox/g by ABTS assay, 29.82 ± 0.42 to 101.22 ± 0.41 mg of Trolox/g by DPPH and 13.64 ± 0.01 to 41.43 ± 0.22 mg of Trolox/g by FRAP assay. The higher antioxidant activity exhibited by the Soxhlet extracts (**Table 2**) over other extraction techniques clearly demonstrates the relative advantage of SAE for obtaining formulations with high nutraceutical value. On the basis of the results of the $ABTS^{\bullet+}$, $DPPH^{\bullet}$and FRAP assays, the Soxhlet extracts of aerial parts of *P. atrosanguinea* obtained using 50% aqueous ethanol were shown to exhibit significant inhibitory activity against free radicals, while it was comparatively less in microwave, sonication and maceration, which, in turn, indicates the higher concentration of antioxidant constituents in Soxhlet extracts. The antioxidant activity of *P. atrosanguinea* was also compared with green and black tea. The TEAC of different samples as determined by $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and FRAP assays, decreased in the order green tea > black tea > *P. atrosanguinea* as shown in **Table 3**. The TEAC of *P. atrosanguinea* was found to be 2.5- to 3-fold lower than green tea but almost comparable to that of black tea. A linear relationship was also established between total polyphenol and flavonoids contents with TEAC values and it was observed that antioxidant activity increased proportionally to the polyphenol and flavonoid contents. It will

Table 3. Comparison of Antioxidant Activity among Soxhlet Extract of *P. atrosanguinea*, Green and Black Tea

sr. no.	samples	ABTS[a]	DPPH[a]	FRAP[a]
1	green tea	352.56 ± 0.24	272.13 ± 0.56	172.90 ± 0.40
2	black tea	139.84 ± 0.64	108.09 ± 0.46	47.60 ± 0.47
3	*P. atrosanguinea*	122.96 ± 0.38	101.22 ± 0.41	41.43 ± 0.22

[a] Data expressed as mg of Trolox equivalent/g of plant material (DM basis).

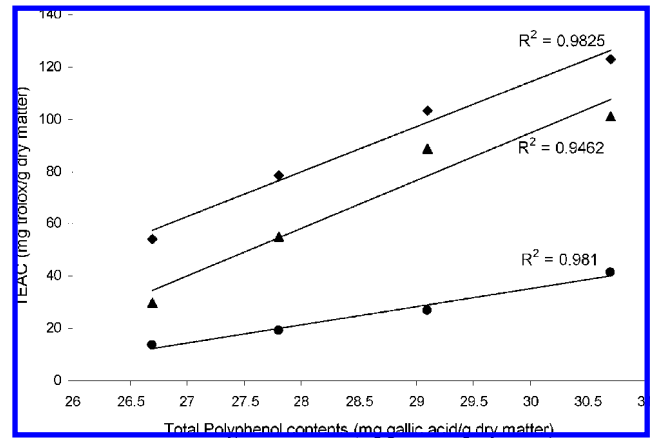

Figure 1. Correlation between total polyphenol contents and TEAC measured by ◆, ABTS; ▲, DPPH; ●, FRAP assays in different extracts of *P. atrosanguinea*.

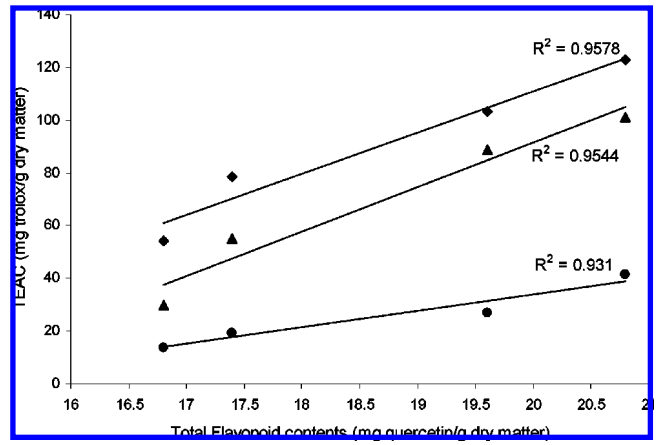

Figure 2. Correlation between total flavonoid contents and TEAC measured by ◆, ABTS; ▲, DPPH; ●, FRAP assays in different extracts of *P. atrosanguinea*.

be relevant to mention here that earlier reports (*37, 38*) have demonstrated the correlation between phenolic content of plant to their antioxidant power. In this study also, the contents of polyphenols and flavonoids in the extracts positively correlate with their antiradical activity confirming participation to the radical scavenging activity of the *P. atrosanguinea* extracts (**Figures 1** and **2**) which in general was observed in other plants as well (*39, 40*).

Identification and Quantification of Phenolics by RP-HPLC. A simple and gradient elution-based RP-HPLC method was developed for the analysis and quantification of five major phenolics (**1**−**5**) in various extracts. For the development of an effective mobile phase, various solvent systems, including different combinations of acetonitrile and water with TFA were tried. Finally, a solvent system consisting of 0.01% TFA in water and acetonitrile proved successful because it allows for the separation of maximum compounds with good resolution. Five phenolic compounds (**1**−**5**) that might contribute to the anti-

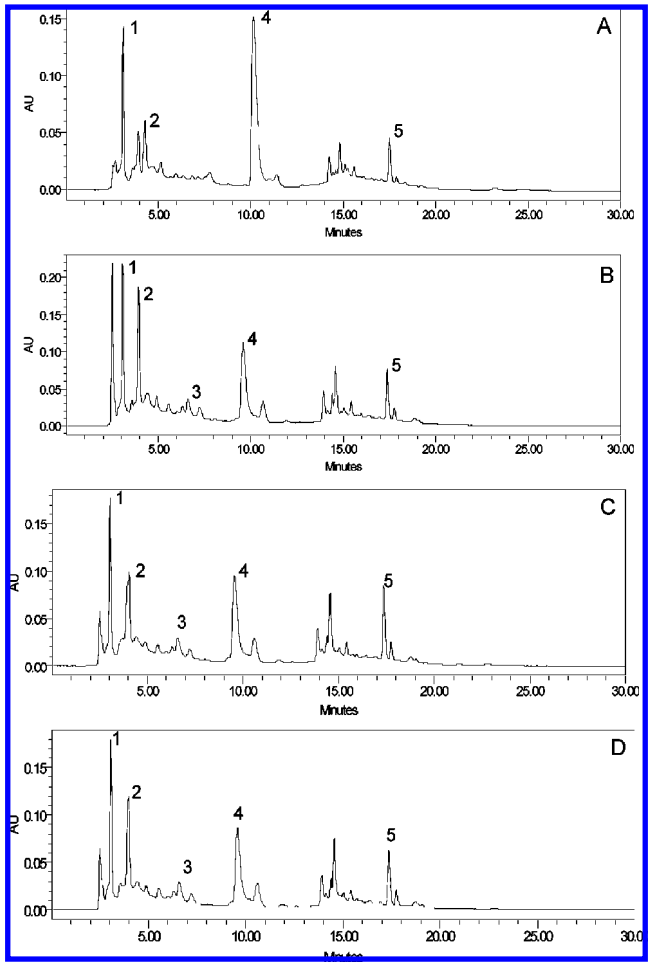

Figure 3. HPLC chromatogram of extracts of *P. atrosanguinea* (**A**) Soxhlet extract, (**B**) microwave extract, (**C**) ultrasound extract, and (**D**) maceration extract.

Table 4. Phenolic Composition of Extracts of *P. atrosanguinea*[a]

extracts	CA	CN	CFA	PCA	QC
Soxhlet	2.89 ± 0.07	6.72 ± 0.11		0.95 ± 0.06	0.53 ± 0.05
microwave	2.48 ± 0.08	3.02 ± 0.14	0.88 ± 0.07	0.20 ± 0.04	0.21 ± 0.04
ultrasonic	2.15 ± 0.08	3.36 ± 0.06	0.44 ± 0.04	0.11 ± 0.03	0.12 ± 0.03
maceration	1.43 ± 0.05	3.79 ± 0.08	0.48 ± 0.05	0.23 ± 0.03	0.20 ± 0.03

[a] Amount represented in mg/g of the dry plant material [SD of three replicates]. CA, chlorogenic acid; CN, (+)-catechin; CFA, caffeic acid; PCA, *p*-coumaric acid; QC, quercetin.

oxidant behavior of the plant were identified in extracts in varying concentrations as evident from parts **A**−**D** of **Figure 3**. Quantification was carried out by integration of the peak using an external standard method, and results are presented in **Table 4**. The calculated amount is given in mg/g of the dry plant material for three replicate injections. It is also clear from **Table 4** that the Soxhlet extracts were found to display highest content of five identified phenolics, which further corroborates the observed trends in total phenolic contents and antioxidant activity. It may also be mentioned here that an earlier paper (*13, 14*) has also reported certain polyphenolic compounds such as chlorogenic acid, caffeic acid, quercetin, catechin and its dimers in other *Potentilla* species. In addition to compounds **1**−**5** some of the unidentified peaks in the HPLC chromatogram of aerial parts of *P. atrosanguinea* extracts, especially in the region of 12−18 min, showed UV maxima around 254 and 360 nm, which is characteristic of flavonols. Thus, the identity of these peaks could be attributed to these flavonol glycosides, which was further confirmed by the disappearance of the above peaks when the extracts were subjected to acid hydrolysis. However, further studies in this direction need to be targeted for their identification and determination of their role toward higher antioxidant behavior of *P. atrosanguinea*.

To conclude, Soxhlet assisted extraction of aerial parts of *P. atrosanguinea* was found to be a better approach than ultrasound and maceration but almost comparable to that of microwave. The use of microwave imparted almost similar antioxidant activity besides ensuring low solvent consumption, ease, and rapidity of the overall method compared to Soxhlet and other extraction methods; therefore, for large scale extraction and evaluation of antioxidants from *P. atrosanguinea*, MAE is a better approach over Soxhlet. Owing to excellent protective features exhibited in antioxidant activity tests comparable to that of black tea, the aerial parts of *P. atrosanguinea* could be concluded as a natural source that can be freely used in the food industry as a culinary herb, but, first, immediate and necessary measurements should be taken for the protection of these plant species; otherwise their bioactive properties and secrets could be lost forever without being tapped.

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