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Microwave-Assisted Efficient Extraction of Different Parts of *Hippophae rhamnoides* for the Comparative Evaluation of Antioxidant Activity and Quantification of Its Phenolic Constituents by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)†

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The outcome of different extraction procedures (microwave, ultrasound, Soxhlet, and maceration) on the antioxidant activity of seeds, leaves, pulp, and fruits of *Hippophae rhamnoides* (sea buckthorn or SBT) was investigated by two different bioassays: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. The SBT extracts were found to possess strong antioxidant activity measured in terms of TEAC (2.03–182.13 and 6.97–282.75 mg/g) with ABTS and DPPH assays, respectively. In general, the antioxidant capacity of microwave-assisted extracts was found to be significantly higher than those obtained by ultrasound-assisted extraction (UAE) and maceration while being slightly higher than Soxhlet extracts. Further, microwave extracts of seeds were found to possess maximum antioxidant capacity followed by leaves, fruits, and pulp. Also, the chemical composition of extracts, studied in terms of the total phenolic content, was found to be in the range of 1.9–23.5 mg/g Gallic acid equivalent (GAE), which indicates a strong correlation between antioxidant activity and phenolic content present in the SBT. In addition, some of its bioactive phenolic constituents, such as rutin (**1**), quercetin-3-*O*-galactoside (**2**), quercetin (**3**), myricetin (**4**), kaempferol (**5**), and isorhamnetin (**6**), were also quantified in different extracts by reverse-phase high-performance liquid chromatography (RP-HPLC).

KEYWORDS: ***Hippophae rhamnoides*; sea buckthorn; antioxidant activity; DPPH; ABTS; HPLC**

INTRODUCTION

In recent years, intensive research on natural antioxidants has received considerable importance because synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been shown to have one or the other side effects (*1, 2*). Several studies have revealed that plants have potent antioxidants in the form of vitamins, flavonoids, and other phenolic compounds that act as scavengers of free radicals and inhibitors of lipid peroxidation (*3–5*). Among the various plants reported for antioxidant activity, sea buckthorn (*Hippophae rhamnoides* L., Elaeagnaceae) stands out. This plant is a native of Eurasia and has been domesticated in several countries (India, China, Nepal, Pakistan, Myanmar, Russia, Britain, Germany, Finland, Romania, France, etc.) at an altitude of 2500–4300 m (*6*). It has been recognized as a versatile nutraceutical crop with diverse uses, from controlling soil erosion to being a source of horse fodder, nutritious foods, drugs, and skin-care products (*7*). Different parts of this plant are used in traditional medicine for the treatment of diseases, such as flu, cardiovascular diseases, mucosal injuries, and skin disorders (*8, 9*). Various studies of alcoholic and hydroalcoholic extracts of fruits, seeds, and leaves of sea buckthorn have confirmed its medicinal and nutritional value (*6, 10–14*). All parts of this wonder plant are considered to be a good source of a large number of bioactive compounds, including carotenoids, tocopherols, sterols, flavonoids, lipids, vitamins, tannins, minerals, etc. (*15–20*), which contribute to its wide usage as a natural antioxidant.

Nowadays, there has been a huge upsurge for developing rapid, reliable, and reproducible methods for the efficient extraction of bioactive compounds from plants to increase their therapeutic functionality. In the literature, different extraction techniques, such as maceration, Soxhlet, ultrasound-assisted

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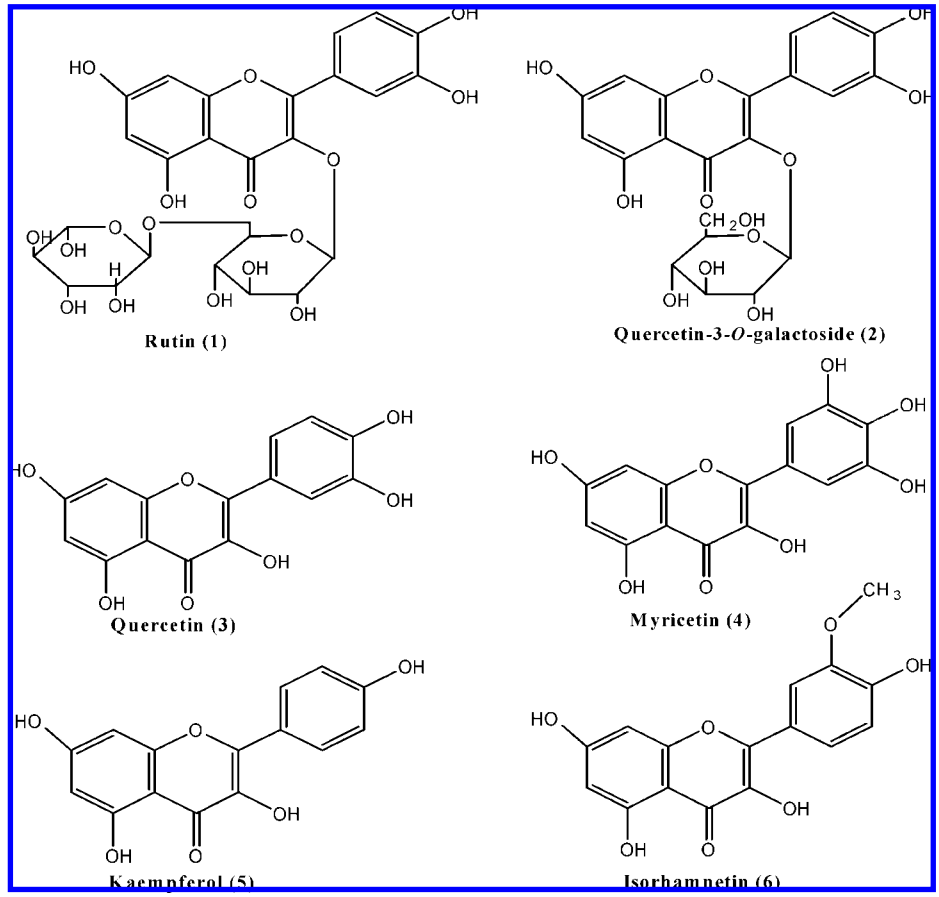

Figure 1. Structures of six phenolic compounds quantified in the different extracts of SBT: rutin (**1**), quercetin-3-*O*-galactoside (**2**), quercetin (**3**), myricetin (**4**), kaempferol (**5**), and isorhamnetin (**6**).

extraction (UAE), and microwave-assisted extraction (MAE), are reported (*21, 22*). Among these, MAE is the simplest and most economical technique in terms of lesser solvent consumption and considerable reduction in extraction time (*23–25*). In recent years, many papers have been published on the applicability of MAE for the extraction of bioactive compounds from plants. Nevertheless, to the best of our knowledge, there is no report available that could illustrate the feasibility of MAE as a rapid and efficient extraction tool for the determination of antioxidant activity of different SBT parts.

Because the various bioactive properties of SBT, including antioxidant, are attributed to the presence of phenolic compounds in it; hence, evidently, the other objective of this work was to investigate the feasibility of MAE for the rapid and efficient extraction of bioactive phenolics from the plant and comparing it to other extraction techniques (maceration, Soxhlet, and UAE). Keeping this in mind, the antioxidant activity of extracts of different parts of SBT (seeds, leaves, pulp, and fruits) was analyzed using the above-mentioned extraction methods. Simultaneously, the content of some of its phenolic constituents [rutin (**1**), quercetin-3-*O*-galactoside (**2**), quercetin (**3**), myricetin (**4**), kaempferol (**5**), and isorhamnetin (**6**); **Figure 1**] was determined with the help of reverse-phase high-performance liquid chromatography (RP-HPLC) to demonstrate the enhanced extraction efficiency and, hence, antioxidant activity.

MATERIALS AND METHODS

Plant Material and Chemicals. Leaves and fruits of SBT were collected from hilly regions (Spiti valley, HP) of Western Himalayas, India, and dried under shade (temperature, 25 ± 2 °C; and relative humidity, 50 ± 5%). About 100 g of fully dried fruits were deseeded for obtaining seeds and pulp. All of the materials (seeds, leaves, fruits, and pulp) were powdered separately in an electric grinder. Absolute ethanol used was from Bengal Chemicals, India. HPLC-grade acetonitrile (MeCN), acetic acid, and Folin−Ciocalteu reagent were purchased from E. Merck. HPLC-grade water was purchased from J.T. Baker. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, trolox, quercetin, rutin, and myricetin standards were purchased from Sigma. Quercetin-3-*O*-galactoside, isorhamnetin, and kaempferol were from Chromadex. All of the samples and solvents were filtered through a 0.45 μm membrane filter (Millipore, Germany) and degassed prior to use.

Extraction Procedure. *Microwave-Assisted Extraction (MAE).* About 5 g of each powdered plant material (seeds, leaves, fruits, and pulp) was extracted with 50 mL of absolute ethanol in a focused microwave (CEM Discover) for 10–40 min. On mass yield basis, an extraction time of 20 min at 150 W microwave power and 60 °C temperature was taken as optimum. The extracts were filtered and concentrated to dryness under vacuum (temperature, 40–45 °C) and then subjected to lyophilization until a constant weight was obtained.

Ultrasound-Assisted Extraction (UAE). About 5 g each of powdered plant material (seeds, leaves, fruits, and pulp) was sonicated with 50 mL of absolute ethanol in an ultrasonicator bath (Elma Ultrasonic, Germany) at a controlled temperature (30 ± 5 °C) for 40–80 min. An extraction time of 60 min was taken as optimum on mass yield basis. The extracts were filtered and concentrated to dryness under vacuum (temperature, 40–45 °C) and then subjected to lyophilization until a constant weight was obtained.

Soxhlet Extraction. About 5 g each of powdered plant material (seeds, leaves, fruits, and pulp) was extracted with 150 mL of absolute ethanol for 6−10 h in a Soxhlet apparatus. An extraction time of 8 h was taken as optimum on the basis of mass yield. The extracts were filtered and concentrated to dryness under vacuum (temperature, 40–45 °C) and then subjected to lyophilization until a constant weight was obtained.

Maceration. About 5 g each of powdered plant material (seeds, leaves, fruits, and pulp) was macerated overnight in 50 mL of absolute ethanol at room temperature. The extract obtained was filtered and concentrated fully under vacuum (temperature, 40–45 °C) and lyophilized until a constant weight was obtained.

All of the extractions were performed in triplicate. All samples 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 for fruits and pulp, 1 mg/2 mL for leaves, and 1 mg/4 mL for seeds. For the quantitative determination of compounds by HPLC, concentrated extracts were dissolved in methanol (analytical grade) to obtain a sample solution of 10 mg/mL. The extracts were filtered through a 0.45 μm membrane filters prior to use.

Determination of Total Phenolic Content. The total phenolic content was measured using Folin−Ciocalteu's method (*26*). 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 ambient temperature. The total phenolic content of the sample is expressed as mg of gallic acid equivalent (GAE)/g of plant material. A total of 50 μL of ethanolic extract of SBT samples were mixed with the same reagent as described above, and after 35 min of incubation, the absorption was measured at 730 nm for determination of total phenolics. All determinations were performed in triplicate.

Determination of Antioxidant Capacity. *ABTS Radical Cation Scavenging Activity.* The ABTS radical cation decolorization test is a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The experiment was carried out using an improved ABTS decolorization assay (*27*). ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation was produced by reacting an 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. For the study of SBT extracts, $ABTS^{\bullet+}$ was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. After the addition of 2.0 mL of diluted $ABTS^{\bullet+}$ solution ($A = 0.700 \pm 0.020$) to 50 μL of extracts, an absorbance reading was taken exactly after 4 min, with an appropriate solvent blank run in each assay. All determinations were carried out in triplicates on each occasion. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of the concentration of antioxidants.

DPPH Radical Scavenging Activity. Radical scavenging activity of extracts against stable $DPPH^{\bullet}$ was also determined spectrophotometrically at 517 nm. The radical scavenging activity of extracts was measured by a slightly modified method of Brand-Williams et al. (*28*). $DPPH^{\bullet}$ (100 μM) was prepared in 80% aqueous methanol. The extracts of SBT measuring 0.1 mL were added to 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 after 30 min. A control consisted of 0.1 mL of ethanol and 2.9 mL of $DPPH^{\bullet}$ solution. All readings were taken in triplicate.

TEAC of SBT Samples. Trolox standard curves that relate the concentration of trolox and the amount of absorbance reduction caused by trolox were obtained using the ABTS and DPPH assays (*29*). For preparation of a calibration curve, a 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 a 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 (*30, 31*). For samples, 50 μL of extracts was assayed as described above. The radical scavenging activity was calculated using the formula

$$\text{percent inhibition} = [(A_B - A_A)/A_B] \times 100$$

where A_B is the absorption of the blank sample and A_A is the absorption of the tested extract solution.

The calculation of TEAC of each compound at the various concentration levels was made using trolox standard curves (*32, 33*). For SBT samples, properly diluted extracts were assayed by both ABTS and DPPH procedures.

HPLC Analysis. The HPLC system consisted of a Shimadzu HPLC (Model LC-20AT pump, DGU-20A5 degasser) equipped with photodiode array detector (CBM-20A; Shimadzu, Kyoto, Japan) interfaced with an IBM Pentium 4 personal computer. The separation was performed on a Phenomenex Luna C-18 column (250 × 4.6 mm i.d., 5 μm). The temperature of the column was set at 25 °C. Elution of standards and samples (20 μL) was performed with gradient solvent program, at a flow rate of 1.0 mL/min for 30 min. The mobile phase consisted of 0.05% trifluoroacetic acid (TFA) in water (A) and acetonitrile (B) with the following gradient: 15−60% B in 0–30 min and 15% B in 35 min. The detection wavelength was set at 355 nm. Identification of compounds was performed on the basis of the retention time, coinjections, and spectral matching with standards. For the preparation of the calibration curve, standard stock solutions of compounds **1**–**6** (1 mg/2 mL) were prepared in methanol, filtered through 0.45 μm filters (Millipore), and appropriately diluted (0.01–100 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.

RESULTS AND DISCUSSION

Solvent Extraction. In this study, the effect of various extraction methods viz. microwave, Soxhlet, ultrasound, and maceration for the efficient extraction of antioxidative compounds from SBT parts 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 procedures on the antioxidant power of SBT seeds, leaves, fruit, and pulp extracts. For the extraction purpose, ethanol was chosen as the solvent because alcohols are most widely used in antioxidant extraction work (*34*). 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 best feasible approach for the rapid and efficient extraction of bioactive phenolic constituents.

Antioxidant Activity of SBT Fruit, Seeds, Leaves, and Pulp. Concerning the study of antioxidant effectiveness, it has recently been recommended to employ at least two different *in vitro* models because of the differences between various free-radical scavenging assay systems (*35–37*). Thus, the extracts were subjected to two different antioxidant bioassays employing $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ (expressed as mg/g). The results of both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assay are listed in **Table 1**. The TEAC values obtained for the extracts submitted to the ABTS assay were in the range of 2.03–182.13 mg/g, while the values for the $DPPH^{\bullet}$ assay were in the range 6.97–282.75 mg/g. In addition, the higher antioxidant activity exhibited by the microwave-assisted extracts (**Table 1**) over other extraction techniques clearly demonstrates the relative advantage of MAE for obtaining formulations with high nutraceutical value.

On the basis of the results of the ABTS and DPPH radical scavenging assays, the ethanolic extracts of SBT seeds and leaves were shown to exhibit significant inhibitory activity against radicals, while it was comparatively less in pulp and

Table 1. Total Phenolic Content and Radical Scavenging Activities of Different Extracts (for $n = 3$)

	maceration		ultrasound		microwave		Soxhlet	
	TP[a,b]	TEAC[a,c]	TP[a,b]	TEAC[a,c]	TP[a,b]	TEAC[a,c]	TP[a,b]	TEAC[a,c]
seed	9.4 ± 0.05	44.05 ± 0.09[d] 128.03 ± 0.63[e]	15.6 ± 0.39	93.74 ± 0.80[d] 131.29 ± 0.12[e]	23.5 ± 0.19	182.13 ± 0.10[d] 282.75 ± 0.12[e]	21.9 ± 0.11	166.16 ± 0.48[d] 235.12 ± 1.13[e]
leaves	2.7 ± 0.39	9.80 ± 0.40[d] 17.52 ± 0.12[e]	5.9 ± 0.39	13.72 ± 0.41[d] 18.91 ± 0.25[e]	10.8 ± 0.38	41.14 ± 0.40[d] 56.82 ± 0.19[e]	9.7 ± 0.35	37.16 ± 0.46[d] 39.55 ± 0.43[e]
pulp	1.9 ± 0.38	2.03 ± 0.60[d] 6.97 ± 0.16[e]	3.8 ± 0.19	4.86 ± 0.60[d] 7.07 ± 0.12[e]	4.8 ± 0.19	16.82 ± 0.70[d] 10.11 ± 0.63[e]	4.4 ± 0.18	6.24 ± 0.64[d] 8.73 ± 0.44[e]
fruit	2.3 ± 0.39	2.13 ± 0.50[d] 14.28 ± 0.31[e]	4.4 ± 0.39	6.13 ± 0.30[d] 16.72 ± 0.70[e]	9.3 ± 0.39	18.81 ± 0.19[d] 28.40 ± 0.19[e]	4.9 ± 0.35	8.33 ± 0.28[d] 21.37 ± 0.24[e]

[a] Data expressed as mean ± standard deviation (SD) of three replicates. [b] Data expressed as mg of GAE/g of plant material (DM basis). [c] Data expressed as mg of trolox equivalent/g of plant material (DM basis). [d] TEAC assayed by the ABTS method. [e] TEAC assayed by the DPPH method.

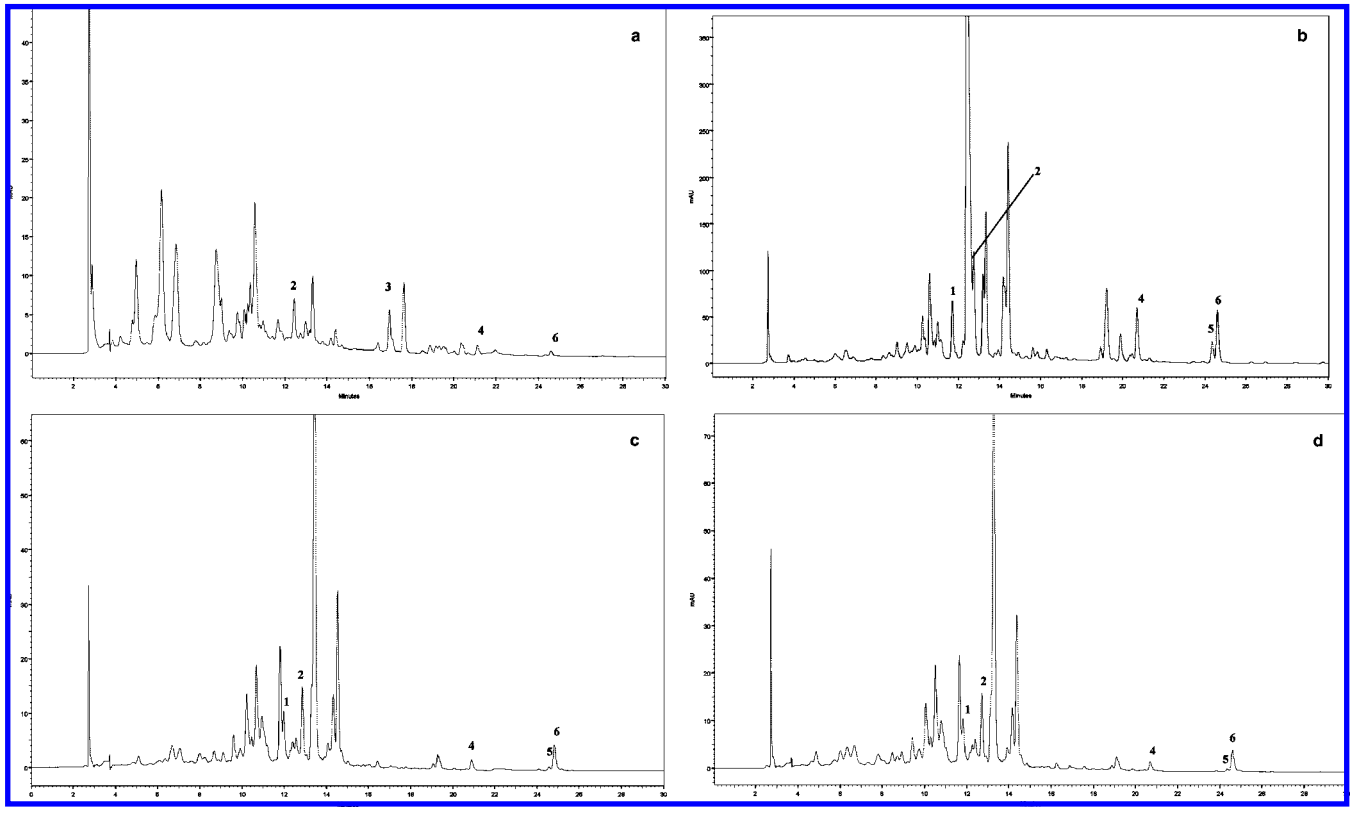

Figure 2. HPLC chromatogram of microwave-assisted (a) seeds extract of SBT, (b) leaves extract of SBT, (c) pulp extract of SBT, and (d) fruit extract of SBT.

fruit, which, in turn, indicates the higher concentration of antioxidant constituents in seeds and leaves.

It will be relevant to mention here that earlier papers (*37, 38*) have demonstrated the correlation between the phenolic content of plants to their antioxidant power. In this study also, a good correlation was indicated between the phenolic content and the antioxidant power of extracts. For the measurement of total phenolic content, the absorbance of ethanolic extracts of SBT was determined spectrometrically according to the Folin−Ciocalteu method and calculated as GAE. It is clear from **Table 1** that the total phenolic content of SBT seeds was highest among all, which is evidently in accordance with the observed antioxidant activity.

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 six phenolics (**1**–**6**) in various extracts. For the development of an effective mobile phase, various solvent systems, including different combinations of acetonitrile and methanol with TFA were tried. Finally, a solvent system consisting of 0.05% TFA in water and acetonitrile proved successful because it allows for the separation of maximum compounds with good resolution. Six phenolic compounds (**1**–**6**) that might contribute to the antioxidant behavior of the plant were identified in extracts in varying concentrations as evident from parts **a**−**d** of **Figure 2**. Quantitation was carried out by integration of the peak using an external standard method, and results are presented in **Table 2**. The calculated amount is given in μg/g of the dry plant material for three replicate injections. It is also clear from **Table 2** that the microwave-assisted extracts were found to display the highest content of six identified phenolics, which further corroborates the observed trends in total phenolic content and antioxidant activity.

In addition to compounds **1**–**6**, some of the unidentified peaks in the HPLC chromatogram of leaves, whole fruit, and pulp of SBT, especially in the region of 10–15 min, showed UV maxima around 254 and 355 nm, which is characteristic of flavonols. It may also be mentioned that a couple of earlier papers (*39, 40*) have indicated SBT to possess flavonol glycosides, especially of isorhamnetin and quercetin. Thus, the identity of these peaks

Table 2. Phenolic Composition of Extracts of Different SBT Parts[a]

	RT	QC-3-*O*-G	QC	MY	KMP	ISR
seeds						
maceration		2.94	2.66			
ultrasound		16.9	6.41			
microwave		34.98	29.7	2.71		3.74
Soxhlet		23.9	22.9	1.26		2.22
leaves						
maceration	43.09	43.9		18.84	7.98	19.0
ultrasound	45.7	45		27.5	11.3	29.2
microwave	365	334		161.7	54.6	147.0
Soxhlet	313.8	280.8		136.8	45.8	120.0
pulp						
maceration	102.2	77.6		11.8	3.63	32.5
ultrasound	194.1	148.9		25.96	5.3	46.4
microwave	281.8	170.2		37.9	8.98	53.2
Soxhlet	300.2	194.7		50.1	12.61	61.5
whole fruit						
maceration	52.2	43.6		8.03	2.22	20.9
ultrasound	94.9	76.3		10.75	2.87	25.7
microwave	155	123.2		22.4	4.29	42.8
Soxhlet	136	108.2		17.3	3.71	41.2

[a] Amount represented in μg/g of the dry plant material $\pm$ SD of three replicates (SD = 0.54–1.3%). RT, rutin; QC-3-*O*-G, quercetin-3-*O*-galactoside; QC, quercetin; MY, myricetin; KMP, kaempferol; ISR, isorhamnetin.

could be attributed to these flavonol glycosides, which was further confirmed by the disappearance of the above peaks when the berries and leaves were subjected to acid hydrolysis. However, in the case of seeds (which showed maximum phenolic content), the amount of above-mentioned compounds (**1**–**6**) was found to be quite low, leading to the presumption that some other phenolics instead of these flavonols might be responsible for the higher antioxidant behavior of seeds. This was found true while examining the UV spectra of major unidentified peaks. The peaks show a UV maxima in the region of 270 nm, characteristic of catechins, which indicates the presence of gallocatechins or high-molecular-weight proanthocyanidins that have been reported to be present in SBT seeds (*7*). However, further studies in this direction need to be targeted for their identification and determination of their role toward higher antioxidant behavior of SBT seeds.

To conclude, MAE of different parts of SBT (seeds, leaves, pulp, and fruits) was found to be a better approach than Soxhlet, ultrasound, and maceration because the use of microwave imparted higher antioxidant activity to the extracts besides ensuring low solvent consumption, ease, and rapidity of the overall method than Soxhlet and other extraction methods. Simultaneously, a simple RP-HPLC method was developed for the identification and quantification of six phenolic compounds (**1**–**6**) present in the extracts of SBT, to demonstrate the increased antioxidant power. The results are promising and demonstrate the practical feasibility of MAE to substitute the traditional time-consuming techniques for efficient extraction of antioxidative compounds to provide nutraceutical-rich formulations.

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