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Journal of Pharmaceutical and Biomedical Analysis 47 (2008) 31–38
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Simultaneous estimation of phenolic acids in sea buckthorn (*Hippophaë rhamnoides*) using RP-HPLC with DAD

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Received 17 July 2007; received in revised form 21 September 2007; accepted 29 November 2007
Available online 5 December 2007

Abstract

A RP-HPLC-DAD method was developed and validated for the simultaneous analysis of nine phenolic acids including gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, salicylic acid, *p*-coumaric acid, cinnamic acid, caffiec acid and ferulic acid in sea buckthorn (SB) (*Hippophaë rhamnoides*) berries and leaves. The method was validated in terms of linearity, LOD, precision, accuracy and recovery and found to be satisfactory. Phenolic acid derivatives in anatomical parts of SB berries and leaves were separated into free phenolic acids, phenolic acids bound as esters and phenolic acids bound as glycosides and profiled in HPLC. Berry pulp contained a total of 1068 mg/kg phenolic acids, of which 58.8% was derived from phenolic glycosides. Free phenolic acids and phenolic acid esters constituted 20.0% and 21.2%, respectively, of total phenolic acids in SB berry pulp. The total phenolic acid content in seed kernel (5741 mg/kg) was higher than that in berry pulp and seed coat (Table 2). Phenolic acids liberated from soluble esters constituted the major fraction of phenolic acids (57.3% of total phenolic acids) in seed kernel. 8.4% and 34.3% of total phenolic acids in seed kernel were, respectively contributed by free and phenolic acids liberated from glycosidic bonds. The total soluble phenolic acids content in seed coat (448 mg/kg) was lower than that in seed kernel and pulp (Table 2). Proportion of free phenolic acids in total phenolic acids in seed coat was higher than that in seed kernel and pulp. Phenolic acids bound as esters and glycosides, respectively contributed 49.1% and 20.3% of total phenolic acids in seed coat. The major fraction (approximately 70%) of phenolic acids in SB berries was found to be concentrated in the seeds. Gallic acid was the predominant phenolic acid both in free and bound forms in SB berry parts and leaves.

Keywords: Sea buckthorn; *Hippophaë rhamnoides*; Phenolic acids; RP-HPLC-DAD; Gallic acid; Protocatechuic acid; Salicylic acid

1. Introduction

Sea buckthorn (SB) (*Hippophaë rhamnoides*), a thorny shrub of Elaeagnanceae family with very high nutraceautical and therapeutical values, is grown in Asia, Europe and North America. In India it is found in the cold deserts of Trans-Himalayan region between 3000 and 5000 m above sea level. Its berries and leaves have a long history of applications in Tibetan and Mongolian medicines. Ripe berries of SB are orange/red in color and have diameter of 10–15 mm with soft, fleshy edible outer tissue enclosing a hard seed. The berries are rich in vitamins, polyphenols, organic acids and bioactive lipids [1–5]. A wide spectrum of physiological effects of SB berries including inhibition of oxidation of low-density lipoprotein (LDL) [6], and antioxidant and immunomodulation effects [7] have been reported. Oral consumption of berry oil has been reported to inhibit platelet aggregation [8] and reduce atopic dermatitis [9] in human and protect rats from gastric ulcers [10]. Leaves of sea buckthorn are small and narrow with 2–6 cm in length. Leaves are very rich in polyphenolic compounds and are reported to have antitumor, antiviral and antioxidant properties [11]. SB leaves have been used for the preparation 'Hiporamin' a phytochemical drug with wide spectrum of antiviral activities [11]. There is a growing interest in the use of SB berries and leaves for medicinal and cosmetic applications. More than 200 products including nutraceuticals, cosmetics and pharmaceuticals, based on SB plant parts are currently available in market. Phenolics, including flavonols, flavones, phenolic acids, proanthocyanidins and hydrolysable tannins, are reported as the major contributors to the biological properties like antioxidant activities of SB berries and leaves [3]. Quercetin, kaempherol, and isorhamnetin are the major flavonoid compounds reported in SB berries

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doi:10.1016/j.jpba.2007.11.045

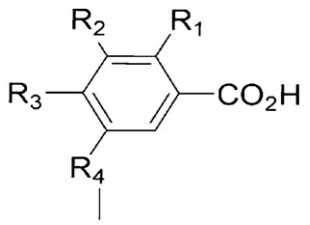

Fig-1a : Hydroxybenzoic acid derivatives

R1	R2	R3	R4	
OH	H	H	H	Salicylic acid
H	H	OH	H	p-hydroxy benzoic acid
H	OH	OH	H	3,4-dihydroxy benzoic acid
H	OH	OH	OH	Gallic acid
H	OH	OCH_3	H	Vanillic acid

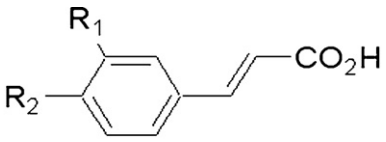

Fig-1b : Hydroxycinnamic acid derivatives

R1	R2	
H	H	Cinnamic acid
H	OH	p-coumaric acid
OH	OH	Caffiec acid
OH	OCH3	Ferulic acid

Fig. 1. Structures of phenolic acids.

[2]. HPLC-DAD analysis of flavonoids in SB leaves has been described by Zu et al. and reported the presence of catechin, quercetin, isorhamnetin and rutin [4].

Phenolic acids are simple compounds of non-flavonoid family, which constitute a large group of phenolic compounds in plants. Phenolic acids are reported to have a wide spectrum of pharmacological activities including antioxidant, antimutagenic, antitumor and anticarcinogenic properties [12]. Phenolic acids include two main groups namely, hydroxybenzoic acid and hydroxycinnamic acid derivatives with different number and position of hydroxylation and methoxylation in aromatic ring (Fig. 1). Phenolic acids are distributed as their free and bound forms in nature, more often bound forms occur as their esters and glycosides.

GC–MS profile of free and bound phenolic acids of six varieties of SB berries grown in Poland is reported by Zadernowski et al. [5]. The authors showed the presence of phenolic acids belonging to both hydroxybenzoic acid and hydroxycinnamic acid derivatives. Rosch et al. reported the amount of total GA and ProCA in SB berries from Finland by HPLC analysis [13]. Detailed reports on the nature, composition and distribution of phenolic acids in anatomical parts of SB berries and leaves are still lacking. Phenolic acids belonging to different classes show considerable variations in their UV absorption maxima. DAD (diode array HPLC detector) can be used to monitor the different classes of phenolic acids. In this study a HPLC-DAD analytical method was optimized for the quantification of nine phenolic acids in sea buckthorn and used for the profiling of free and bound phenolic acids in the anatomical parts of SB berries and leaves.

2. Materials and methods

2.1. Reagents and materials

2.1.1. Reference compounds and reagents

Reference compounds of gallic acid (GA), protocatachuic acid (ProCA), parahydroxybenzoic acid (*p*-HBA), vanillic acid (VA), salicylic acid (SA), cinnamic acid (CA), *p*-coumaric acid (*p*-CA), ferulic acid (FA) and caffiec acid (Caf A) were obtained from Sigma–Aldrich Inc. (St Luis MO, USA). Folin-Ciocalteau's reagent, methanol, water and acetic acid of HPLC grade were purchased from Merck Ltd. (Mumbai, India). Other chemicals and reagents used were of analytical grade.

2.1.2. Berries and leaves

Well-ripened berries, and leaves of SB (*H. rhamnoides* ssp. *turkestanica*) grown in Ladakh region of Himalayas (India) were harvested and authenticated with the help of Field Research Laboratory Leh (DRDO) India during October 2006. Berries and leaves were collected from three locations of Ladakh region and pooled. The fresh berries and leaves were ferried to Regional Research Laboratory, Trivandrum by air under cold condition (4–5 °C). The berries and leaves were freeze-dried and stored at 4 °C, till analyzed.

2.2. Optimization of extraction

Freeze-dried SB berries were manually separated into seeds and pulp. Seeds were coarse powdered and seed coat was separated from the seed kernel by winnowing. Pulp, seed kernel, seed coat and freeze-dried leaves were finally powdered to an average particle size of 0.5 mm. Powdered samples (2 g) were independently extracted with 20 ml absolute methanol, methanol with 10%, 20%, 30%, 40%, 50% and 75% water (v/v) and pure water. For extraction, the samples were homogenized at 200 rpm with solvent mixtures for 30 min in nitrogen atmosphere and subsequently centrifuged at $2000 \times g$ and supernatants were collected. The residues were resuspended in their respective solvent mixtures and the procedure was repeated. The supernatants were combined, dried under reduced pressure at ≤ 50 °C, and redissolved in 25 ml absolute methanol. The extracts, thus obtained were analyzed for their total phenolic content using Folin-Ciocalteau's reagent [3].

2.3. Preparation of crude phenolic extracts

Five grams of the powdered berry pulp, seed kernel, seed coat and leaves were homogenized at 200 rpm with methanol–water (60% for SB pulp, seed coat, and leaves, and 70% for seed kernel) mixtures (6×25 ml) at room temperature for 30 min in nitrogen atmosphere. The mixture was centrifuged at $2000 \times g$

for 15 min and supernatants were collected, combined and dried under vacuum at ≤50 °C and kept at −20 °C until analyzed.

2.4. Fractionation of phenolic acids

Free and bound phenolic acids in the extracts were fractionated according to the procedure described by Kozłowska et al. [14] and Zadernowski et al. [5]. Crude phenolic extracts were dissolved in 25 ml of water, acidified with 6N HCl to pH 2 and filtered to remove the precipitated phospholipids. It was then extracted with diethyl ether (5 × 25 ml) at room temperature. The diethyl ether extracts were combined and evaporated under vacuum at ≤40 °C and referred as free phenolic acid fraction. The water phase was neutralized with 2 M NaOH and evaporated under vacuum at ≤50 °C almost to dryness. The residue was treated with 20 ml 4N NaOH in nitrogen atmosphere for 4 h at room temperature. The reaction mixture was then acidified to pH 2 with 6N HCl and extracted with diethyl ether as before and analyzed as phenolic acids liberated from esters. The water phase was then neutralized and dried as before. The residue was hydrolyzed with 50 ml 2 M HCl for 30 min at 95 °C. The mixture was cooled, adjusted to pH 2 and extracted with diethyl ether as before. This extract was referred to as the phenolic acids liberated from their glycosides. The residual fatty material present in phenolic acid fractions thus obtained were removed by dissolving in 20 ml 5% $NaHCO_3$solution and extracting with diethyl ether (5 × 20 ml). Then the aqueous phase was acidified with 6N HCl to pH 2 and extracted with diethyl ether as before. The extracts were dried and redissolved in 5 ml 50% methanol and kept at −20 °C till analyzed.

2.5. Preparation of standard solutions

Standard solutions of nine phenolic acids at a concentration of 0.5 mg/ml in methanol were prepared and several dilutions in mobile phase were made. All standard solutions were filtered through 0.45 μm filters, and injected directly.

2.6. Chromatographic conditions

A Shimadzu LC-8A HPLC system (Shimadzu, Japan) with a binary solvent delivery system (LC-8A), a column temperature controller (CTO-10AV), a Rheodyne injector with 20 μl sample loop and a DAD detector (SPD-M 10 A) coupled with Class VP analytical software were used for analysis. A reverse phase (RP) column (Phenomenex C-18, ODS-2, 5 μm, 250 mm × 4.6 mm) with an extended guard column was used as stationary phase and column temperature was maintained at 35 °C. The mobile phase was a gradient elution of water containing 2% acetic acid (solvent A) and methanol (solvent B) at a flow rate of 1 ml/min. The gradient program of solvent A in B (v/v) was as follows: 0–5 min 100% A; 5–10 min 90% A; 10–15 min 90%; 15–20 min 80% A; 20–25 min 80%; 25–40 min 40% A; 40–60 min 0% A.

2.7. Statistical analysis

Standard deviation and anova calculations were performed using Microsoft Office Excel 2003 and Microcal Origin 6.0 Professional.

3. Results and discussion

3.1. Optimization of phenolic extraction

Phenolic acids in plant materials are found as their free, esterified and glycosidic forms. The extractability of phenolic acids from plant tissues largely depends on their chemical nature, solvent polarity and extraction conditions. The extraction conditions therefore were optimized as extractability of total phenolics. For this the effect of different compositions of methanol and water on extractability of polyphenols were evaluated as shown in Fig. 2. Polyphenol content in the extracts was estimated by using Folin-Ciocalteau's reagent. Methanol–water mixtures were found to be more effective for the extraction of polyphenols than absolute methanol. The increase in the extractability of polyphenols with the introduction of water to methanol can be attributed to the increase in permeability of plant tissues in the presence of water in extracting solvents, which enables better mass transfer by diffusion. 70% methanol in water was found to be optimum for the extraction of polyphenols from SB berry pulp ($P < 0.05$) and seed kernel ($P < 0.05$) and 60% methanol for seed coat ($P < 0.05$) and leaves ($P < 0.05$) (Fig. 2).

Total phenolic content in the crude phenolic extracts of berries and leaves prepared as described in Section 2.3 were estimated and expressed as gallic acid equivalents. Highest amount of polyphenols was found in seed kernel (11.4 ± 0.21 g/100 g of dry mass) and leaves (10.9 ± 0.45 g/100 g of dry mass) followed by pulp (2.6 ± 0.36 g/100 g of dry mass) and seed coat

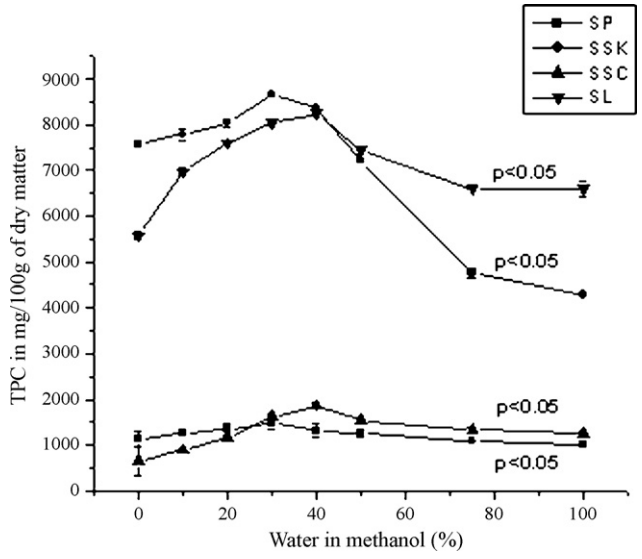

Fig. 2. Effect of methanol–water mixture on the extraction of polyphenols from SB berries and leaves. SP, berry pulp; SSK, seed kernel; SSC, seed coat; SL, leaves.

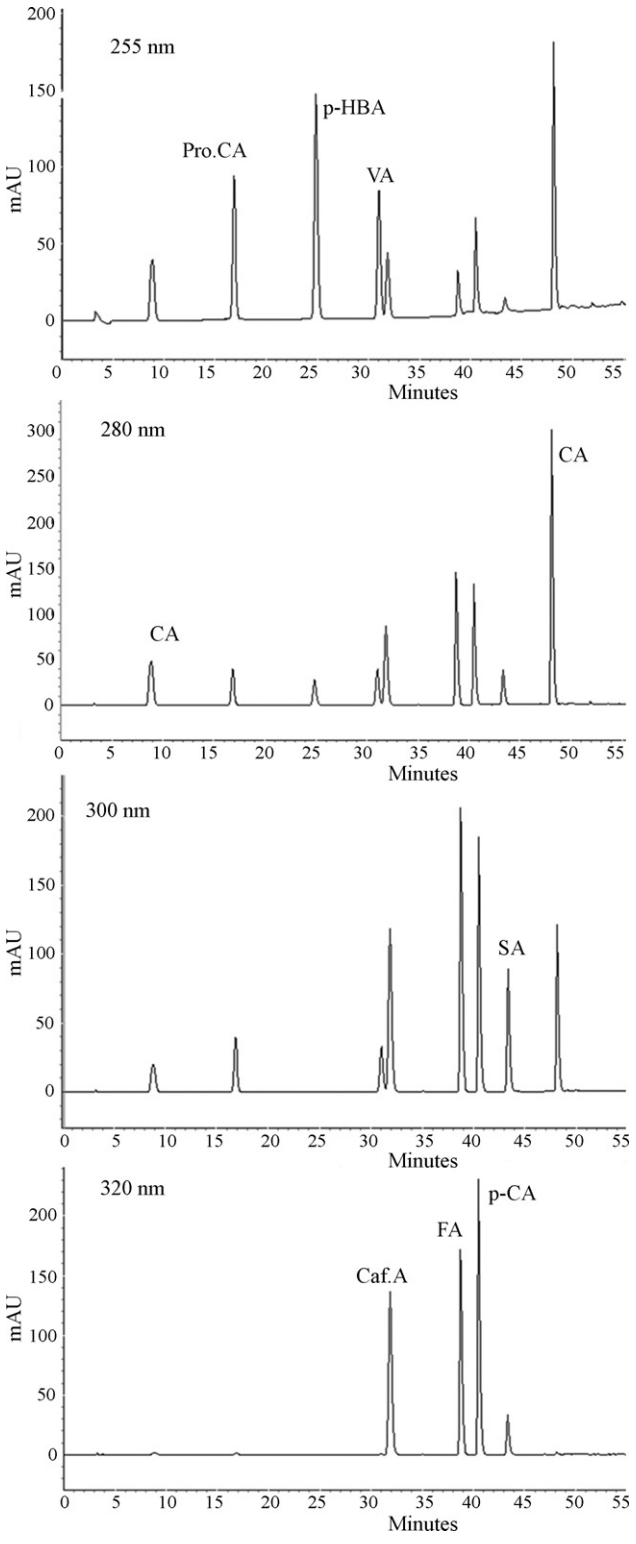

Fig. 3. HPLC chromatograms of phenolic acids at different wavelengths. ProCA, protocatechuic acid; *p*-HBA, *p*-hydroxybenzoic acid; VA, vanillic acid; GA, gallic acid; CA, cinnamic acid; SA, salicylic acid; *p*-CA, *p*-coumaric acid; FA, ferulic acid; Caf A, caffiec acid.

Table 1
Detection wavelength (λ), retention time (T_R), linear regression data, precision, accuracy and recovery of phenolic acids

Compounds	λ (nm)	T_R (min)	Regression equation[a]	Linear range (μg ml^{-1})	*r*	LOD[b] (μg ml^{-1})	Precision (R.S.D. %)[c]				Accuracy (%)[f]	Recovery (%)[g]
							Intra-day[d]		Inter-day[e]			
							T_R	Peak area	T_R	Peak area		
Gallic acid	280	8.4	$Y = 1.298 \times 10^5 x + 187$	10.8–150.2	0.999	1.20	0.92	1.62	1.12	2.05	99.6 ± 0.8	92.5 ± 1.2
Protocatechuic acid	260	16.8	$Y = 0.991 \times 10^5 x - 1101$	8.0–220.4	0.999	0.72	0.72	1.71	1.33	1.98	102.3 ± 1.0	94.3 ± 2.1
p-Hydroxybenzoic acid	255	25.6	$Y = 1.894 \times 10^5 x - 548$	12.5–175.0	0.992	2.78	0.85	1.25	1.28	1.79	98.1 ± 0.9	102.5 ± 1.9
Vanillic acid	260	31.8	$Y = 1.374 \times 10^5 x + 413$	13.2–150.0	0.989	1.97	1.01	1.38	1.78	2.23	101.7 ± 2.1	98.2 ± 1.8
Salicylic acid	300	42.5	$Y = 0.844 \times 10^5 x + 1332$	12.4–175.2	0.996	2.87	1.09	1.72	1.12	1.87	103.9 ± 0.4	95.9 ± 1.1
Cinnamic acid	275	49.8	$Y = 1.282 \times 10^5 x + 990$	10.1–166.8	0.997	1.77	1.13	1.41	1.36	1.08	98.9 ± 0.8	98.7 ± 1.6
Caffiec acid	325	32.4	$Y = 2.307 \times 10^5 x + 14489$	6.6–208.5	0.994	1.04	0.97	0.82	1.61	0.85	98.8 ± 1.9	98.1 ± 1.9
Ferulic acid	310	39.2	$Y = 2.357 \times 10^5 x - 15307$	5.0–220.0	0.998	1.65	0.78	1.66	1.19	0.97	100.6 ± 1.4	96.6 ± 2.2
p-Coumaric acid	325	40.8	$Y = 2.443 \times 10^5 x + 1495$	10.4–240.8	0.990	0.65	1.09	1.34	1.06	1.28	97.4 ± 0.6	97.2 ± 1.8

[a] *x*, peak area; *y*, concentration (mg/ml).
[b] Detection was expressed as LOD = 3.3*s*/*a*, where *a* is the slope and *s* is residual standard deviation of regression line.
[c] R.S.D.% = ((S.D./mean) × 100).
[d] *n* = 5.
[e] *n* = 6.
[f] Mean [(Found/nominal) × 100% (*n* = 6)] ± S.D., at medium analyte concentration of 50 μg ml^{-1}.
[g] Mean ± S.D. (*n* = 3).

(1.2 ± 0.19 g/100 g of dry mass) (data not shown in table). Major fraction of polyphenols (approximately 65%) in the berries was found to be concentrated in seeds. The amount of polyphenols contained in pulp was in accordance with previous reports [2,3] and higher than the values reported for berries of Polish origin (0.88–1.44 g/100 g of dry matter) [5]. Flavonols, flavones, phenolic acids and condensed and hydrolysable tannins are the major polyphenols reported in SB berries and leaves [2,4–6]. Polyphenolic compounds were reported as the major chemical entities responsible for the antioxidant activities of SB berries and leaves.

3.2. Standardization of chromatographic separation using reference phenolic acids

RP-HPLC-DAD conditions for the qualitative and quantitative profiling of nine major phenolic acids viz; GA, ProCA, *p*-HBA, VA, SA, CA, *p*-CA, FA and CA in SB berries and leaves were optimized using different proportions of methanol and water as the mobile phase with reverse stationary phase (Phenomenex, C-18, ODS-2, 5 μm, 250 mm × 4.6 mm). Variations in pH of mobile phase are reported to have significant effect on the resolution and tailing of polar compounds in RP-HPLC. Better separation for phenolic acids is achievable in the presence of acids in the mobile phase since these suppress the ionization of acidic groups [15–17]. A gradient of 2% acetic acid in water and methanol was optimized as mobile phase as described in Section 2.6. Retention times (T_R) of reference phenolic acids are presented in Fig. 3 and Table 1.

Fig. 3 shows the chromatograms monitored at different wavelengths, selected on the basis of maximum absorbance and maximum peak/noise ratio. These are summarized in Table 1. The UV spectra of the phenolic acids obtained by HPLC-DAD are shown in Fig. 4. VA and Caf A were found to be eluted closely, but the differences in their absorption maxima could be utilized for differentiating them by comparing their chromatograms recorded at 270 and 329 nm (Fig. 3). VA had highest absorbance at 270 nm and that was considerably smaller at 329 nm, the detection wavelength of Caf A.

3.3. Validation data

Regression equations were obtained by the external standard method. The linear range, regression equation and correlation coefficient of each analytes, LOD and LOQ values (representing good sensitivity) are summarized in Table 1. All the components showed good linearity ($R \geq 0.989$) in a relatively wide concentration range.

A solution mixture containing 50 μg/ml of each phenolic acids (GA, ProCA, PHBA, VA, SA, CA, PCA, FA and Caf A) was analyzed 5 times to determine the intra-day precision of the retention time and peak area. The inter-day precisions were determined by 6 analyses over 2 days for each phenolic acids (50 μg/ml). The R.S.D. values representing good precision and recovery data showing high accuracy are also summarized in Table 1. The sample solution kept at 4 °C was found to be stable for 48 h.

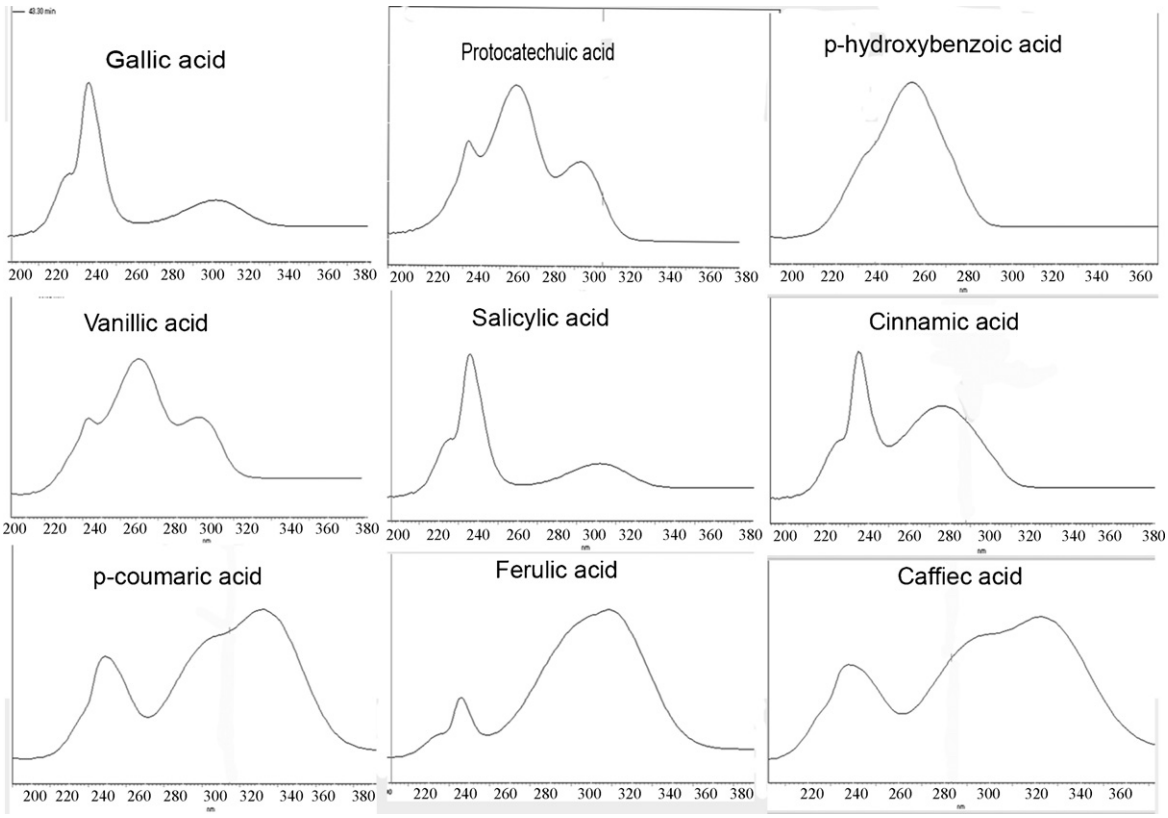

Fig. 4. UV spectra of phenolic acids recorded using DAD.

Table 2
Free and bound phenolic acids in SB berry pulp, seed kernel, seed coat and leaves (mg/kg of dry matter)[a]

Phenolic acids	Hydroxybenzoic acid derivatives					Hydroxycinnamic acid derivatives				Total
	Gallic acid	Proto catechuic acid	*p*-Hydroxy benzoic acid	Vanillic acid	Salicylic acid	Cinnamic acid	*P*-coumaric acid	Ferulic acid	Caffiec acid	
Berry pulp										
Free	88 ± 5.7	33 ± 2.5	8 ± 1.8	5 ± 0.4	46 ± 1.2	7 ± 0.2	11 ± 1.8	13 ± 2.9	3 ± 0.4	214 ± 9.6
Bound										
Esters	152 ± 1.0	23 ± 0.6	8 ± 3.2	1 ± 0.4	5 ± 0.8	5 ± 0.2	12 ± 0.3	18 ± 0.1	2 ± 0.3	226 ± 4.3
glycosides	465 ± 0.3	80 ± 0.1	24 ± 1.0	1 ± 0.1	3 ± 0.3	–	14 ± 4.8	38 ± 1.9	3 ± 0.1	628 ± 2.5
Total	705	136	40	7	54	12	37	69	8	1068
Seed kernel										
Free	329 ± 16.2	99 ± 12.1	28 ± 3.9	5 ± 0.2	ND	5 ± 0.3	5 ± 0.4	10 ± 0.8	3 ± 0.3	484 ± 21.6
Bound										
Esters	2198 ± 221.4	690 ± 77.7	144 ± 5.7	47 ± 7.5	ND	52 ± 3.2	65 ± 12.7	81 ± 4.6	12 ± 0.9	3289 ± 236.9
glycosides	914 ± 18.0	541 ± 12.1	93 ± 6.7	316 ± 34.3	ND	25 ± 1.0	4 ± 0.0	65 ± 2.2	10 ± 9.4	1968 ± 123.3
Total	3441	1330	265	368	ND	82	74	156	25	5741
Seed coat										
Free	30 ± 5.3	43 ± 10.8	17 ± 4.7	12 ± 4.5	ND	19 ± 1.2	ND	15 ± 2.2	1 ± 0.4	137 ± 5.6
Bound										
Esters	164 ± 8.3	27 ± 0.6	7 ± 0.8	5 ± 0.8	ND	11 ± 0.1	ND	2 ± 0.2	4 ± 0.1	220 ± 5.6.
glycosides	36 ± 2.4	12 ± 0.7	2 ± 0.3	22 ± 0.3	ND	14 ± 0.7	ND	–	5 ± 0.2	91 ± 2.5
Total	230	82	26	39	ND	44	ND	17	10	448
Leaves										
Free	689 ± 13.6	16 ± 1.8	144 ± 6.6	17 ± 1.7	ND	122 ± 0.5	1 ± 0.0	44 ± 2.9	18 ± 1.9	1051 ± 30.2
Bound										
Esters	2899 ± 23.2	19 ± 1.6	36 ± 4.3	6 ± 0.0	ND	54 ± 5.2	ND	119 ± 1.6	ND	3133 ± 17.7
glycosides	634 ± 0.2	15 ± 0.4	67 ± 0.7	14 ± 0.9	ND	62 ± 1.8	ND	12 ± 0.5	ND	804 ± 5.9
Total	4222	50	247	37	ND	238	1	175	18	4988

ND, not detected.
[a] Values are mean ± S.D. ($n = 3$).

3.4. Phenolic acids in SB berries and leaves

SB berries separated into pulp, seed kernel and seed coat as described before, and SB leaves were analyzed for their free and bound phenolic acids content. Phenolic acids in the SB berries and leaves were separated into three fractions, viz; phenolic acids present in free form, phenolic acid liberated from esters and phenolic acid liberated from glycosides and profiled by HPLC for their phenolic acid composition. Peaks in the chromatograms were identified by comparing the retention times and UV spectra with those of reference compounds and by spiking experiments (Fig. 5). The amount of each analyte was calculated from the corresponding calibration curve and represented as their mean ± standard deviation of three analyses.

3.4.1. SB pulp

Composition of free and bound phenolic acids in the dry matter of berry pulp is summarized in Table 2. Berry pulp contained 1068 mg/kg of total phenolic acids, of which 58.8% was derived from phenolic glycosides. Free and phenolic acids bound as esters, respectively, constituted 20.0% and 21.2% of total phenolic acids. Total phenolic acid content in the berry pulp was comparable with that of soybean flour [18]. Gallic acid was identified as the predominant phenolic acid both in free and bound forms, which accounted for 66.0% of total phenolic acids in pulp. Considerable amounts of ProCA (136 mg/kg), FA (69 mg/kg), SA (54 mg/kg), *p*-HBA (40 mg/kg) and *p*-CA (37 mg/kg) were found to be present in berry pulp. Presence of VA (7 mg/kg) CA (12 mg/kg) and Caf A (8 mg/kg) was also detected in SB berry pulp.

3.4.2. Seeds

Seeds were separated into seed kernel and seed coat and analyzed for their free and bound phenolic acids composition. The values on dry matter are summarized in Table 2. The total phenolic acid content in seed kernel (5741 mg/kg) was higher than that in berry pulp and seed coat (Table 2). Phenolic acids liberated from soluble esters (57.3% of total phenolic acids) were the most abundant phenolic acid fraction in seed kernel. Free and phenolic acids liberated from their glycosides, respectively, contributed 8.4% and 34.3% of total phenolic acids. The total phenolic acid content in the seed kernel was two times higher than those reported for sesame and cotton seed [19] and slightly lower than the values reported for rapeseed and canola meals [19] and flax seed [20]. GA (3441 mg/kg), ProCA (1330 mg/kg), *p*-HBA (265 mg/kg), VA (368 mg/kg) and FA (156 mg/kg) were the major phenolic acids present in seed kernel. Both free and bound phenolic acid profile contained GA as the predominant phenolic acid, which contributed 59.9% of total phenolic acids in seed kernel. In addition to this, presence of CA (82 mg/kg), *p*-CA (74 mg/kg) and Caf A (25 mg/kg) were also detected in seed kernel.

The total soluble phenolic acid content in seed coat (448 mg/kg) was lower than that in seed kernel and pulp (Table 2). Proportion of free phenolic acids in total phenolic acids in seed coat was higher than that in seed kernel and pulp. Phenolic acids bound as esters and glycosides, respectively contributed 49.1% and 20.3% of total phenolic acids in seed coat. GA (230 mg/kg), ProCA (82 mg/kg), CA (44 mg/kg), VA (39 mg/kg), *p*-HBA (26 mg/kg), FA (17 mg/kg) and Caf A (10 mg/kg) were identified in seed coat. ProCA (43 mg/kg) was

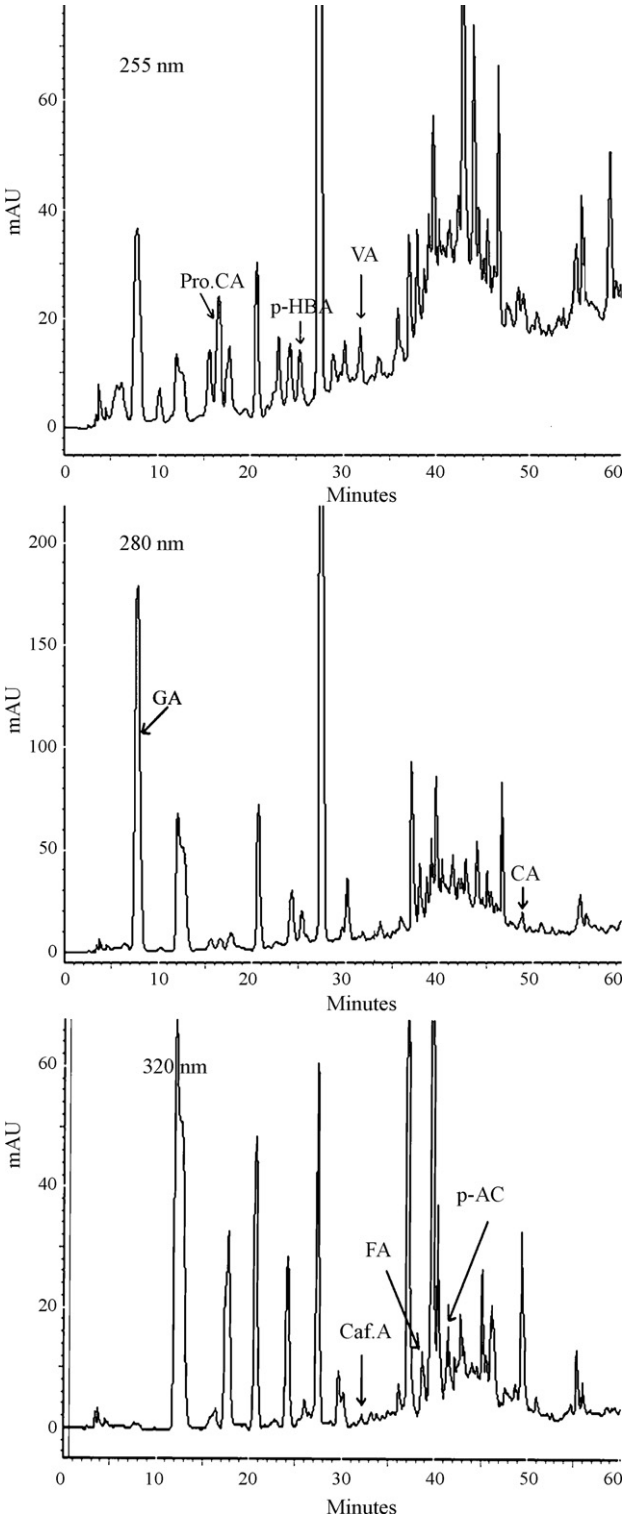

Fig. 5. HPLC chromatograms of phenolic acids liberated from esters of SB seed kernel at different wavelengths. ProCA, protocatechuic acid; *p*-HBA, *p*-hydroxybenzoic acid; VA, vanillic acid; GA, gallic acid; CA, Cinnamic acid; *p*-CA, *p*-coumaric acid; FA, ferulic acid; Caf A, caffiec acid.

present as the major phenolic acid of the free phenolic acid fraction, whereas GA (200 mg/kg) was the major constituent in bound phenolic acid fractions in seed coat. GA and ProCA, respectively, accounted for 51.3% and 18.3% of total phenolic acids in the seed coat.

3.4.3. SB leaves

SB leaves contained considerably high amount of phenolic acid esters (67.8% of total phenolic acids; Table 2). Free phenolic acid and phenolic acid glycosides, respectively, constituted 21.1% and 16.1% of total phenolic acids. GA was the dominant phenolic acid both in free and bound fractions, which contributed 84.6% of total phenolic acids in SB leaves. The present study showed that *p*-HBA (247 mg/kg), CA (238 mg/kg) and FA (175 mg/kg) were also present in SB leaves. As compared to the berries, the contribution of ProCA to the total phenolic acids in leaves was smaller. VA (37 mg/kg) and Caf (18 mg/kg) were also identified in SB leaves.

Detailed reports on the composition of phenolic acids in anatomical parts of SB berries and leaves are limited. Rosch et al. reported the amount of GA (1–2.6 mg/ml) and ProCA (2.1–2.9 mg/ml) in SB berry juice (*H. rhamnoides*, collected in Germany) and their contribution to its total antioxidant capacity [13]. Hakinen et al. reported the proportion of *p*-CA, FA, *p*-HBA and EA in total phenolic content of SB (*H. rhamnoides*) berries from Finland [21]. In another report on SB berries (*H. rhamnoides*) of Polish origin, salicylic acid was identified as the predominant phenolic acid both in free and bound forms by GC–MS analysis [5]. Presence of GA in SB leaves was previously reported [22]. In contrast to the present study, the distribution of phenolic acids among the anatomical parts of berries was not discussed in detail in the above mentioned papers. The major fraction (approximately 70%) of phenolic acids in SB berries was found to be concentrated in the seeds. The report of salicylic acid as the predominant phenolic acid in SB berries from Poland is contrary to the present study, where gallic acid was found as the major phenolic acid both in pulp and seeds of berries of Indian origin. The berry pulp in this study contained only 54 mg/kg of salicylic acid, which contributed 5.06% of total phenolic acids in pulp. These results warrant the detailed studies on the phenolic composition of SB berries from different varieties and geo-climatic regions.

4. Conclusion

RP-HPLC with DAD was found to be effective for the simultaneous detection and determination of phenolic acids in SB leaves and berries. The method standardized here was applied for the compositional analysis of free, ester-bound and glycoside-bound phenolic acids in SB berries and leaves of Indian origin. This is the first report on the free and bound phenolic acid profile of SB leaves and seeds. Hundreds of sea buckthorn based nutraceutical and health care products are available in market. The present method is suitable for the fingerprinting, qualitative and quantitative evaluation of health care products based on SB berries and leaves in terms of their phenolic acids profile.

Acknowledgements

The authors are grateful to the Defense Research and Development Organization (India) for financial support. We thank Dr. OP Chourasia and the Field Research Laboratory, Leh (DRDO) for supplying sea buckthorn berries and leaves.

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