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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Metabolic fingerprinting of different populations of *Phyllanthus niruri* L. from Punjab using electrospray ionization mass spectrometry (ESI–MS)

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Abstract There is growing interest of healthcare and food industry in ingredients of plant origin as they are potent sources of antioxidant, anti-inflammatory, antimicrobial and anticancer agents. In the present work different extracts of *Phyllanthus niruri* L. from various regions of Punjab were screened for their phenolic profiles and antioxidant properties. Crude extracts obtained by solid–liquid extraction with different solvents were tested for total anthocyanins, flavonoids, phenolic content, and free radical scavenging activity. Out of all the solvents used, methanol was regarded as best to be used for soxhlet extraction of plant metabolites, as it provided highest phenolic, flavonoid, antioxidant, and anthocyanin contents. Similarly, ESI–MS was employed to obtain mass profiles of phenolic and other metabolites present in various *P. niruri* populations. Out of 72 compounds detected, 51 are reported for the first time in *P. niruri* L. Similarly, different populations of *P. niruri* were discriminated through metabolic fingerprinting using ESI–MS.

Keywords *Phyllanthus niruri* · ESI–MS · Metabolic fingerprinting · Antioxidant activity · Phenolic profile · Phytochemicals · Biochemical characterization

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Introduction

Naturally occurring, plant-based antioxidants such as flavonoids, polyphenols, carotenoids, and vitamins A, B, C, and E (tocopherols), have beneficial effects of preventing or delaying aging and of inhibiting common cancers. Due to increased interest in antioxidants, the need for high resolution assays for their rapid screening in multi-component extracts has also been increased (Kim and Um, 2011).

Electrospray ionization mass spectrometry (ESI)–MS is a soft-ionization technique in which molecular ions are generated having relatively unexplored potential for metabolic fingerprinting in plants. The 'molecular' ions in a complex sample are sufficiently distinguished by their *m/z* values alone, omitting the need for conventional LC column (Liquid chromatography) and the unfractionated sample is directly introduced by flow injection or direct infusion into the ESI–MS. The technique has found recent applications for the rapid characterization of micro-organisms, for rapid estimation of secondary metabolite expression in actinomycetes, and for semi-quantitative determination of specific plant metabolites (Goodacre et al., 2003).

ESI–MS has been exploited previously for *Pharbitis nil* to discriminate metabolic fingerprints of leaves under different physiological states (Goodacre et al., 2003). Similarly, it has also been used in combination with other methods such as HPLC-DAD, HPLC-DAD-ESI-QTOF-MS/MS and HPLC–ESI/MS for the analysis of phenolic composition and characterization of metabolites in *Phyllanthus simplex*, *Phyllanthus urinaria* and fruits of *Phyllanthus emblica* (Huang et al., 2009; Niu et al., 2012; Yang et al., 2012).

Phyllanthus niruri L. (Euphorbiaceae) is a medicinal plant widely distributed in tropical and subtropical regions of both hemispheres, and largely used in folk medicine to treat various

disorders such as asthma, arthritis, poor appetite, constipation, cuts and bruises, corneal opacity, conjunctivitis, flu and colds, blennorrhagia, colic, diabetes, dropsy, dysentery, dyspepsia, fever, flu, gout, gonorrhea, itch, jaundice, kidney aliments, leucorrhea, malaria, menorrhagia, menstrual troubles/complaints, obesity, proctitis, stomachache, tenesmus, tumor, typhoid fever, and vaginitis (Paithankar et al., 2011). Pharmacological experiments and phytochemical examinations on *P. niruri* confirm its therapeutic efficacy and safety (Asare et al., 2011; Asare et al., 2012; Narendra et al., 2012). Bioactive constituents such as alkaloids, flavonoids, lignans, tannins, phenols, and terpenes have been identified (Rajeshkumar et al., 2002). However, the composition of the different extracts, used for medicinal purposes, has not been adequately studied. Although the specific compounds have not been precisely defined, some research results credit the therapeutic action of *P. niruri* to the phenols. Thus, to use this plant in pharmaceutical industry or as a herbal drug, valid quality control methods need to be developed in order to comply with regulatory requirements (De Souza et al., 2002).

The present study evaluates different populations of *P. niruri* L. from Punjab for various phytochemicals and assay of their antioxidant activity. ESI–MS technique, the most popular tool for rapid metabolic fingerprinting, was employed for semi-quantitative characterization of the complex phytochemicals/metabolites.

Materials and methods

Plant material

The *P. niruri* samples were collected from various regions of Punjab, India viz. Bathinda (Krishi Vigyan Kendra, Kheti Bhawan, Rose Garden, and Chetak Park), Amritsar (Guru Nanak Dev University, Khalsa College), Roopnagar (Chamkaur Sahib, Bhakra nangal dam, and Gurudwara Sadabarat) and Patiala (Punjabi University, Baradari Garden, and Urban Estate). The plant samples were identified by Botanist Dr. Geetika Sirhindi, Department of Botany, Punjabi University, Patiala. The collected populations were washed thorougly under running water to remove soil and other extraneous matter. The samples were then shade dried properly for atleast a month and dried samples were crushed using electric grinder to get fine powder.

Extract preparation

Aqueous extract (Dineshkumar et al., 2010)

P. niruri plant powders (500 g) were macerated using 1 L of millipore water in a sterile glass container, stirred intermittently and then left overnight under hygienic conditions. After maceration, it was filtered through Whatmann filter paper (110 mm) and the filtrate was separated and stored aseptically in an air-tight container at −20 °C until further analysis.

Ethanolic and methanolic extracts (Aarthi and Murugan, 2011)

For ethanolic extract soxhalation of the powder was achieved using ethanol as solvent. 500 g of powder was soxhalated with 1,000 ml of ethanol for 24 h. Positive pressure (2–3 bar) was provided to evaporate solvent and to obtain ethanolic extract. Methanolic extract was prepared in the same way by using methanol as solvent.

Hydroalcoholic extracts (de Souza et al., 1998)

Air dried plant powder of *P. niruri* was minced and extracted with 50 % ethanol and water in 1:3 ratio. The fraction was then macerated at room temperature (25 ± 3 °C) for 15 days. The solvent was evaporated and the extract was concentrated to desired level using vaccum evaporator and stored at−20 °C until furthur analysis.

Biochemical characterization of extracts

Total phenolic content (TPC)

The total phenolic content (TPC) content in extracts was assessed using Folin-Ciocalteu reagent according to Siddique et al. (2010). Briefly, the solvent extract (0.1 ml) was mixed with 1.5 ml of Folin-Ciocalteu reagent, 4 ml of sodium carbonate, and final volume was made to 10 ml using deionized water. The mixture was kept at room temperature for 30 min and the absorbance of the samples was read at 738 nm using a spectrophotometer (Genesys 10S UV-Vis Spectrophotometer). TPC content was calculated using Gallic acid caliberation curve within range of 20–100 mg/ml ($R^2 = 0.9994$). The results were expressed as Gallic Acid Equivalents or GAE (mg/ml). All the samples were analyzed thrice and results averaged.

Total flavonoid content (TFC)

Aluminium chloride colorimetric technique was used for total flavonoids estimation (Hasanlooet al., 2011). Each extract (0.5 ml) was mixed with 0.5 ml of methanol, 0.1 ml of 10 % aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The mixture was kept at room temperature for 30 min after which absorbance of the reaction mixture was read at 415 nm using spectrophotometer (Genesys 10S UV-Vis spectrophotometer). Calibration curve was plotted using quercetin within range

of 20–100 g/mL ($R^2 = 0.999$) and used for quantification of TFC's. All samples were analyzed in triplicate and results averaged.

Total antioxidant content

The total antioxidant content of extracts was assessed using the 1,1′–diphenyl-2-picrylhydrazyl (DPPH) method according to standard methodology of Siddique et al. (2010) with some modifications. To the solvent extract (1 ml), 3 ml of freshly prepared solution of DPPH was added. The mixture was incubated for 30 min in dark and the absorbance of the samples was measured at 517 nm using spectrophotometer (Genesys 10S UV-Vis spectrophotometer). Ascorbic acid was used as the standard for preparing the calibration curve ($R^2 = 0.998$). All samples were analyzed in triplicate and results averaged. Percent inhibition was calculated using free radical scavenger, i.e., DPPH.

Calculations:

$$\text{DPPH radical scavenging activity}(\%) : \frac{100 \times (A_o - A_t)}{A_0}$$

A_o = initial absorbance

A_t = Absorbance of antioxidant measured at t = 30 min.

Total anthocyanin content (TAC)

Total anthocyanin content (TAC) was estimated by using a pH differential method (Humadi and Istudor, 2009). 1 ml of extract was diluted with 10 ml of buffer of pH 1 in an Erlenmeyer flask 1. Again 1 ml of extract was diluted with 10 ml of buffer of pH 4.5 in a Erlenmeyer flask 2. The flasks were kept at room temperature for 15 min and then absorbance was measured at 520 nm and 700 nm. Millipore water was taken as blank. The anthocyanins are calculated as cyanidin-3-glucoside equivalents, mg/L.

Calculations:

$$\text{Total anthocyanin content}(\text{mg/ml}) = \frac{\text{A} \times \text{Mol.wt. of anthocyanin} \times \text{DF} \times 10^3}{\epsilon \times \text{L}},$$

where, A = (A 520 nm−A 700 nm) pH 1.0 − (A 520 nm−A 700 nm) pH 4.5

Mol.wt. = 449.2 g/mol for cyanidin-3-glycoside

DF = 1:10

L = path length in cm

ϵ = 26900 molar extinction coefficient, in L mol^{-1} cm^{-1}, for cyanidin-3-glucoside

10^3 = conversion from g to mg

Mass spectral analysis for characterization of phenolics and other phytochemicals

The crude extracts (aqueous, ethanolic, methanolic and hydroalcoholic) of *P. niruri* from different districts were mixed with their corresponding solvents (1:9) and then centrifuged at 13,000 rpm for 10 min. The supernatant obtained was filtered through HEPA filters (0.47 micron membrane filter) and the filtrates were further used for mass spectral analysis of phytochemicals present in *P. niruri* at NIPER (National Institute of Pharmaceutical Education and Research), Mohali, India. Extracts of aqueous, ethanolic, methanolic reaction mixtures were analyzed using ESI–MS (Thermo Scientific, model: LTQ-XL) under positive ionization probe, for the characterization of phenolic compounds present in *P. niruri* between *m/z* ratio (100–800).

Stastistical analysis

All the analysis was carried out in triplicates and the results were averaged to determine the mean, standard error, and standard deviation using MS-Excel. The Metabolomics Standards Initative (MSI) were followed for data processing and analysis.

Results and discussion

Phytochemical characterization of extracts

P. niruri contains various antioxidant and health promoting phytochemicals in view of their health implications. Influence of different extraction solvents on the content of natural antioxidants in extracts have been reported earlier by many researchers.

Total phenolic content (TPC)

Solvents such as methanol, ethanol, acetone, propanol, and ethyl acetate have been commonly used for the extraction of phenolics from fresh products (Tomsone et al., 2012). Phenolics were extracted in different solvents and their contents in various extracts were found to decrease in following order: Methanolic > Hydroalcoholic > Aqueous > Ethanolic (Online Resource 1 and Fig. 1a). Out of all the populations, methanolic extract of Patiala had maximum phenolic content, i.e., 57.77 mg/g, followed by hydroalcoholic extract of Amritsar (41.60 mg/g). Many researchers have reported that the recovery of phenolic compounds from plant materials is influenced by the solubility of the phenolic compounds in the different solvents used for their extraction process, as well as on different concentration of solvents (Tsantili et al., 2011). The TPC of various extracts generally depends on the polarity of solvent used for the preparation of extract. High solubility of phenols in polar solvents provides high concentration of compounds in extract (Mohsen and Ammar, 2008). In one study, it was found that the extraction yield and extraction efficiency of major phenolics of green tea were higher with pure methanol

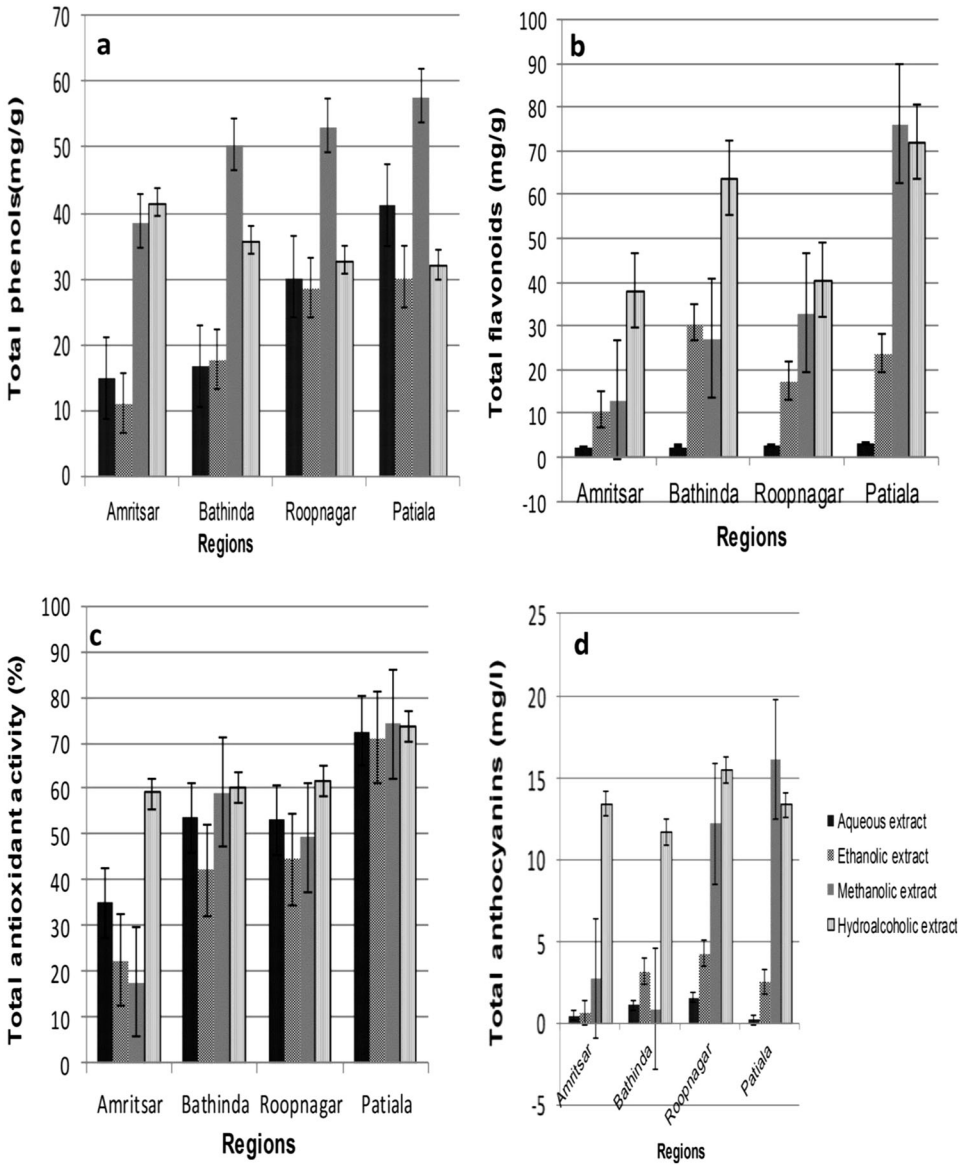

Fig. 1 Distinctive variations reported in different populations of *P. niruri* Linn., **a** total phenolics, **b** total flavonoids, **c** total anthocyanins, and **d** total antioxidants (Data shown are means ± SD of three independent observations and *P*-value $P < 0.05$ was considered significant)

comparing to pure ethanol (Perva-Uzunalic et al., 2006). However, in a similar study by Pinelo et al. (2004), methanol was found to be the best solvent for phenol extraction from pine sawdust. Hence, methanol in extraction medium had a significant effect on the extraction yield of the phenolic compounds as reported in the current study on *P. niruri*.

Total flavonoid content (TFC)

Flavonoids consist of a large group of polyphenolic compounds and are present ubiquitously in plants. Recent interest in flavonoids is due to variety of pharmacological activities exhibited by them (Pandey, 2007). Several mechanisms responsible for the antioxidative properties of flavonoids are scavenging of free radicals, chelation of metal ions, such as iron and copper, and inhibition of enzymes responsible for free radical generation (Benaventa-Garcia et al., 1997). Flavonoid content of various extracts of *P. niruri* from different regions were found to decrease in order: Methanolic > Hydroalcoholic > Ethanolic > Aqueous (Online Resource 1 and Fig. 1b). Extraction was most effective with methanol than ethanol and water as polarity of methanol is highest among all the solvents tested in the study. However, out of all the populations collected, methanolic extract of Patiala exhibited maximum flavonoid content, i.e., 76.22 mg/g. Anwar and Przybylski (2012) reported that highest amount of total flavonoids in flaxseed extracted using pure methanol was highest followed by 80 % ethanol. The concentration of flavonoids in plant extracts depends largely on the polarity of solvents used in the extract preparation (Min and Chun-Zhao, 2005). Thus, the results are in agreement with the previous studies reported.

Table 1 Phytochemicals identified using ESI–MS technique in different *P. niruri* populations of Punjab (novel compounds italicized)

S.No	Compound name	Observed *m/z*	Extracts of				Relative abundance (%)			
			Patiala	Amritsar	Bathinda	Roopnagar	Patiala	Amritsar	Bathinda	Roopnagar
1.	Salicylic acid	138.33 [M]	A	A	M	–	14	16	15	–
				M				10		
2	Coumarin	144.40 $[M+2H]^+$	M	–	–	M	24	–	–	74
3.	Salicylic acid methyl ester	152.07 [M]	–	–	–	–	–	–	–	–
		151.33 $[M-H]^-$	–	E	E	E	–	19	22	12
4.	Protocatechuic acid	155.80 $[M+H]^+$	–	–	A	–	–	–	36	–
		156.13 $[M+2H]^+$	–	–		A	–	–	–	62
5	5-p-coumaric acid	163.60 $[M-H]^-$	E	–	M	–	10	–	23	–
6	Ascorbic acid	176.00 [M]	–	–	A	–	–	–	56	–
		175.00 $[M-H]^-$	A	M	–	–	50	18	–	–
		175.14 $[M-H]^-$	HA				7			
		175.87 $[M-H]^-$								
		174.87 $[M-2H]^-$	–	A	M	–	–	24	40	–
		174.93 [M – 2H]–								
7	Caffeic acid	179.80 $[M-H]^-$	E	–	–	–	22	–	–	–
		181.13 $[M+H]^+$	–	–	HA	–	–	–	4	–
8	Norsecurinine	203.16 [M]	HA	HA	–	–	10	3	–	–
		203.11 [M]								
9	Securinine	204.13 [M]	A	A	E	A	69	62	27	48
		204.14 [M]	E	E	M	E	100	46	100	45
		204.12 [M]	M	M	HA	HA	54	54	59	17
		204.07 [M]	HA	HA			100	100		
		204.20 [M]								
		204.27 [M]								
10	Ethyl caffeate	208.20 [M]	–	–	–	M	–	–	–	58
11	*p*-coumaroyl glycolic acid	222.20 [M]	A,	A	A	–	22	26	75	–
		222.40 [M]		E	M			22	46	
				M	HA			22	7	
				HA				8		
		223.67 $[M+H]^+$	E	–	–	–	30	–	–	–
		220.20 $[M-2H]^-$	–	–	–	A	–	–	54	–
		220.47 $[M-2H]^-$				E			18	
12	Vanillin-4-sulphate $[M-H]^-$	231.18 [M]	HA	–	–		19	–	–	–

Table 1 continued

S.No	Compound name	Observed *m/z*	Extracts of				Relative abundance (%)			
			Patiala	Amritsar	Bathinda	Roopnagar	Patiala	Amritsar	Bathinda	Roopnagar
13	Isopimpinellin	246.73 [M]	–	–	A	A	–	–	46	48
		246.53 [M]								
14	Pinocembrin	256.35 [M]	HA	HA	HA	–	19	52	100	–
		256.37 [M]								
		257.35 $[M+H]^+$	–	–	–	HA	–	–	–	100
16	Caffeic acid 3-sulphate	261.00 $[M+H]^+$	–	–	E	–	–	–	16	–
17	Naringenin	272.93 [M]	–	–	–	A	–	–	–	38
18	Ferulic acid 4-sulphate	274.34 [M]	HA	HA	HA	HA	33	33	38	5
		274.35 [M]								
		274.36 [M]								
		274.37 [M]								
19	Linolenic acid	278.60 [M]	M	–	A	–	40	–	19	–
		278.67 [M]								
20	Cyanidin	288.33 $[M+H]^+$	–	–	–	E	–	–	–	12
21	Catechin	291.30 $[M+H]^+$	–	HA	–	–	–	4	–	–
22	Brevifolin carboxylate	292.53 [M]	–	–	–	M	–	–	–	46
23.	Quercetin	302.81 [M]	–	A	E	–	–	39	18	–
		302.93 [M]		E				50		
		303.00 $[M+H]^+$	A	M	A	E	60	74	28	20
		303.07 $[M+H]^+$	E		M		25		92	
		303.13 $[M+H]^+$								
24.	(±)Gallocatechol/epicatechin-3-gallate	307.38 $[M+H]^+$	–	–	HA	–	–	–	5	–
25.	Myricetin	318.42 [M]	–	–	HA	–	–	–	10	–
26.	Carnosol	330.47 [M]	–	A	–	–	–	13	–	–
		329.34 $[M-H]^-$	–	HA	–	–	–	20	–	–
27.	(5-p-CoQA)	338.27 [M]	M	–	–	–	76	–	–	–
		337.00 $[M-H]^-$	–	–	–	A	–	–	–	24
28.	Phyllnirurin	341.93 $[M-H]^-$	E	–	–	M	70	–	–	66
29.	Hinokinin	354.93 [M]	–	E	E	E	–	48	33	22
		354.60 [M]								
		354.87 [M]								
		355.32 $[M+H]^+$	–	–	–	HA	–	–	–	5
30.	Lariciresinol	361.37 $[M+H]^+$	–	–	HA	–	–	–	17	–

Table 1 continued

S.No	Compound name	Observed *m/z*	Extracts of				Relative abundance (%)			
			Patiala	Amritsar	Bathinda	Roopnagar	Patiala	Amritsar	Bathinda	Roopnagar
31.	Secoisolariciresinol	362.27 $[M-H]^-$	–	–	–	M	–	–	–	92
		364.00 $[M+2H]^+$	E	–	–	–	23	–	–	–
32.	7-hydroxy secoisolariciresinol	374.67 [M]	–	–	M	–	–	–	32	–
33.	Secoisolariciresinol trimethyl ether	376.32 [M]	–	HA	–	–	–	12	–	–
34.	Tectorigenin 4-sulphate	380. 80[M]	A	A, M	M	–	34	34	52	–
		380.87 [M]						22		
		380.93 [M]								
		381.26 $[M+H]^+$	HA	–	–	–	13	–	–	–
35.	Urinatetralin	385.73 $[M+H]^+$	A	–	–	–	20	–	–	–
36	8,5′-diferulic acid	386.13 [M]	–	E	E	A	–	26	28	32
		386.47 [M]				E				12
		386.73 [M]				M				50
		386.60 [M]				HA				3
37.	2,3'-desmethoxy secoisolintetralin	395.33 [M]	HA	–	–	–	8	–	–	–
38.	4-Sinapoyl quinic acid	398.87 [M]	--	–	A	A	–	–	52	100
39.	Lintetralin	400.47 [M]	–	E	–	–	–	18	–	–
40.	Lupenone	424.13 [M]	M	M	–	–	–	–	–	–
		425.20 $[M+H]^+$	–	–	E	–	–	–	–	–
41.	Ellagic acid pentose	434.9 [M]	E	–	–	–	–	–	–	–
		432.73 $[M-2H]^-$		A		–	–	–	–	–
42.	Tricontanal	435.44 [M]	–	–	HA	–	–	–	–	–
43.	Catechin 3-gallate	441.40 $[M-H]^-$	–	–	HA	–	–	–	–	–
44.	Formomonetin-7-O-glucuronide	444.67[M]	A	–	M	–	63	–	–	82
		444.93[M]								
45.	Betulinic acid	456.80 [M]	M	A	E	–	25	40	64	–
		456.87 [M]		E				38		
		454.00 $[M-2H]^-$	–	–	–	A	–	–	–	48
46.	Epigallocatechin 3-gallate	457.35 $[M-H]^-$	HA	HA	HA	HA	52	32	10	40
		457.37 $[M-H]^-$								
		457.39 $[M-H]^-$								
45	VAD	470.33 [M]	M	–	E	–	18	–	24	–
		470.67 [M]			M				48	
		470.80 [M]								

Table 1 continued

S.No	Compound name	Observed *m/z*	Extracts of				Relative abundance (%)			
			Patiala	Amritsar	Bathinda	Roopnagar	Patiala	Amritsar	Bathinda	Roopnagar
		471.35 $[M+H]^+$	–	–	HA	HA	–	–	23	5
		472.53 $[M+2H]^+$	–	–	A	–	–	–	34	–
46.	Ellagic acid acetyl xyloside	476.87 [M]	–	M	–	–	–	–	24	–
47.	Quercetin 3-O-glucuronide/ Miquelianin	478.80 [M]	–	–	–	M	–	–	43	–
49.	Cyanidin-3-O-(-6″-acetyl glucoside)	491.45 [M]	HA	HA	–	–	20	13	–	–
		492.53 $[M+H]^+$	–	–	A	–	–	–	30	–
		490.73 $[M+2H]^+$	–	–	–	A	–	–	–	38
50.	Ligstroside	523.46 $[M-H]^-$	–	E	HA	HA	–	46	27	5
				M				32		
		525.00 $[M+2H]^+$	–	HA	E	E	–	10	100	100
		525.07$[M+2H]^+$								
51.	1-caffeoyl-5-feruloylquinic acid	530.67 [M]	–	–	–	M	–	–	–	50
52.	Cyanidin-3-O-(-6-malonyl glucoside)	536.53 $[M+H]^+$	–	–	M	–	–	–	36	–
53.	Lariciresinol sesquilignan	555.60 $[M-H]^-$	–	A	–	–	–	32	–	–
54.	Secoisolariciresinol-di-O-glucoside	543.80 $[M+H]^+$	–	–	A	–	–	–	32	–
55.	Cyanidin-3-O-(-6″-succinyl glucoside)	548.67 [M]	–	–	–	A	–	–	–	54
56.	Procyanidin dimer B	578.33 [M]	E	E	–	–	32	26	–	–
		578.60 [M]								
57.	Phloretin-2-O-xylosyl glucoside	568.51 [M]	–	–	–	HA	–	–	–	23
		567.07 $[M-H]^-$	M	HA	HA	–	60	35	43	–
		567.48 $[M-H]^-$								
		567. 51$[M-H]^-$								
58.	Naringin	579.47 $[M-H]^-$	–	–	A	–	–	–	40	–
59.	3-Hydroxy phloretin-2′-O-xylosyl glucoside	584.07 [M]	–	–	–	M	–	–	–	73
60.	Kaempferol-3-O-rutinoside	593.07 $[M-H]^-$	E	A	–	E	90	39	–	32
		593.13 $[M-H]^-$		E				35		
		593.20 $[M-H]^-$		M				62		
61.	Delphidin-3-O-sambubioside	598.67 $[M+H]^+$	–	–	A	–	–	–	34	–
62.	Diosmin	607.20 $[M-H]^-$	M	–	–	–	100	–	–	–
63.	Rutin	611.54 $[M+H]^+$	–	–	HA	HA	–	–	63	15
64.	Pectolinarin	621.20 [M]	E	A	E	A	72	100	48	58
		621.51 [M]	HA	E	HA	E	6	100	28	78
		623.73 $[M+2H]^+$	–	–	M	–	–	40	–	–

Table 1 continued

S.No	Compound name	Observed *m/z*	Extracts of				Relative abundance (%)			
			Patiala	Amritsar	Bathinda	Roopnagar	Patiala	Amritsar	Bathinda	Roopnagar
65.	Quercetin-3,4-O-diglucoside	626.41 [M]	M	–	–	–	–	95	–	–
		625.20 $[M-H]^-$	A	M	–	–	–	40	58	–
66.	Corilagin	635.20 $[M-H]^-$	E	–	–	–	30	–	–	–
67.	Malvidin-3-O-(-6'-p-coumaroyl glucoside)	639.5 [M]	M	–	–	–	84	–	–	–
68.	Delphidin-3-O-feruloyl glucoside	642.60 [M]	–	–	A	–	–	–	42	–
69.	Malvidin-3,5-O-diglucoside	655.55 [M]	–	HA	HA	E		23	62	24
		655.58 [M]				HA				18
70.	Luteolin-7-O-(2-apiosyl-6-malonyl)-glucoside	665.40 $[M-H]^-$	–	–	–	A	–	–	–	60
71.	Theaflavin-3-O-gallate	717.20 $[M+H]^+$	M	–	–	–	45	–	–	–
72.	Punicalin	781.60 $[M-H]^-$	–	–	A	–	–	–	36	–

– Not reported; *A* Aqueous, *M* Methanolic, *E* Ethanolic, *HA* Hydroalcoholic

Total antioxidant activity

Antioxidants are substances or nutrients present in our foods that can increase cellular defense and prevent oxidation damage to cellular component of our bodies by maintaining health and preventing diseases such as cancer and coronary heart disease (El Far and Taie, 2009). Both phenolic and flavonoid compounds are potentially responsible for the antioxidant activity in *P. niruri* (Wong et al., 2013). Most frequently used technique for isolation of plant antioxidants is solvent extraction. In case of Patiala district, methanolic extract of *P. niruri* offered highest antioxidant activity of 74.07 %, followed by hydroalcoholic extract with 73.59 %, aqueous extract with 72.5 %, and ethanolic extract with 71.07 % scavenging activity. However, in remaining populations, higher DPPH radical scavenging activity was reported in case of hydroalcoholic extracts. Antioxidant content of extracts was found to decrease in order: Methanolic > Hydroalcoholic > Aqueous > Ethanolic (Online Resource 1 and Fig. 1c). The results are in agreement with an earlier report of Shon et al. (2004) who investigated that hot water and methanol are more efficient to extract antioxidant compounds. However, differences in antioxidant activities of plant extracts could be attributed to different qualitative and quantitative composition of phenolic constituents, ranging from phenolic acids to flavonoids and their derivatives. Furthermore, the antioxidant activity of a plant is not only due to phenolic compounds, but also due to other non-polyphenolic substances such as carotenoids, vitamins, and minerals. Synergistic effect may also take place between different types of antioxidants (Wong et al., 2013).

Total anthocyanin content (TAC)

Solvent extraction is used frequently for the isolation of anthocyanin pigments from plants. However, extraction conditions are also key factors in their overall solubility (Bridgers et al., 2010). Methanolic and hydroalcoholic extracts of *P. niruri* from different regions gave greater anthocyanin content as compared to aqueous and ethanolic extracts (Online Resource 1 and Fig. 1d) wherein, methanolic extract of Patiala region attributed toward maximum anthocyanin content of 16.1 mg/ml followed by hydroalcoholic extract from Roopnagar having 15.46 mg/ml. Anthocyanins are naturally polar compounds, thus their recovery would be more effective in polar solvents. In comparison to water, methanol and ethanol, have similar characteristics to anthocyanins making them better suited for their extraction. In an investigation involving extraction of anthocyanins from industrial purple fleshed sweet potatoes, methanol as solvent performed best as compared to ethanol as solvent (Bridgers et al., 2010). Similarly,

Fig. 2 Hypothetical pathway showing flavonoid biosynthesis in *P. niruri*, where *PAL* Phenyl alanine ammonia lyase, *C4H* Cinnamate 4 hydroxylase, *CHS* Chalcone synthase, *CHI* Chalcone isomerase, *C2H* Cinnamte-2-hydroxylase, *2CβGT* 2-coumarate-O-*β*-glucosyl transferase, *2CβGI* 2-coumarate-O-*β*-glucoside isomerase, *CAGβG* Coumarinic acid glucoside *β*-glucosidase, *C3H* *p*-coumarate 3-hydroxylase, *COMT* Caffeic acid 3-O-methyl transferase, *4CL* 4-coumarate ligase, *CCR* Cinnamoyl CoA reductase, *CAD* Cinnamoyl alcohol dehydrogenase, *PS* Pinoresinol synthetase, *PSS* Piperitol-seasamin synthase, *SDR* Seasamin dihydroseasamin synthase, *PLR* Pinoresinol-lariciresinol synthase, *SDH* Secoisolariciresinol dehydrogenase, *PLS* Pluviatolide synthase, *HS* Hinokinin synthase, *F5H* Ferulate-5-hydroxylase, *HCT* Shikimate-O-hydroxycinnamoyl transferase, *C3′H*, (Coumaroyl-quinate) 3′-monooxygenase, *CHS* Chalcone synthase, *D2′GT* Dihydro chalcone 2′-O-glucosyl transferase, *CHI* Chalcone isomerase, *CYP93C* 2-hydroxy isoflavanone synthase, *HI4OMT* 2,7,4′-trihydroxy isoflavanone 4′-O-methyl transferase, *HID* 2-hydroxy isoflavanone dehydratase, *IF7GT* Isoflavanone 7-O-glucosyl transferase, *FNSI* Flavone synthase, *CYP75A* Flavonoid 3′,5′-hydroxylase, *LMT* Luteolin methyl transferase, *NGT* Naringenin-7-O-glucosyl transferase, *C12RT1* Flavanone-7-Oglucoside 2″-O-*β*-L-rhamnosyl transferase, *FS* Flavone synthetase, *GT/RT* Glucosyl tansferase/Rhamnosyl transferase, DFR, Dihydroflavano 4-reductase, LADO Leucoanthocyanidin deoxygenase, *BZ1* Anthocyanin-3-O-glucosyl transferase, *3MAT1* Anthocyanin 3-O- glucoside-6″-O-malonyl transferase, 5-GT, 5-O-glucosyl transferase, *FLS* Flavonol synthase, *FGT* Flavonol 3-O-glucosyl transferase, *QGT* Quercetin 3-O-glucosyl transferase, *FGRT* Flavonol-3-Oglucoside L-rhamnosyl transferase, *ANS* Anthocyanin synthetase, *LAR* Leucoanthocyanidin-4-reductase, *ANR* Anthocyanidin reductase. Different color codes are used to define different classes of flavonoids and *dotted arrows* are used to indicate biosynthetic steps following multiple reactions. Important metabolic nodes or branch points are highlighted in *black ovals*. (Ralph et al., 1998; Humphreys and Chapple, 2002; Ossipov et al., 2003; Elfahmi et al., 2006; Hemmati, 2007; He et al., 2008; Gosch et al., 2009; Hagarman, 2008; Pandey et al., 2013; Marcotullio et al., 2014; KEGG Pathway database)

Boulekbache-Makhlouf et al. (2013) regarded methanol as the best solvent for extraction of anthocyanins in eggplant.

Mass Spectral Analysis for characterization of phytochemical compounds in different *P. niruri* populations

Both edible and inedible plants contain polyphenolic compounds, which have multiple applications in food, cosmetic and pharmaceutical industries. The antioxidant capacity of phenolic compounds is attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, or metal chelators. These compounds exhibit a wide spectrum of medicinal properties, such as antiallergic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective, and vasodilatory effect in addition to their antioxidant potential (Demiray et al., 2009).

Previously, several studies on *P. niruri* are carried out by the isolation and purification of individual phenolic compounds, but still there is lack of information on the overall composition of the phenolic profile. A systematical investigation of the phenolic compounds in the plants using mass spectrometry is essential to obtain an overall profile of the phenolic compounds (Yang et al., 2012). The MSI are followed for data processing and analysis. In this study 72 phenolic compounds were obtained from different extracts of *P. niruri* from various regions of Punjab (Online Resource 3, 4, 5, 6). These compounds were identified by comparing MS spectral data with those of previous literature reports and by using online databases and computational facilities of Phenol Explorer, Mass Data Bank, and KEGG compounds. Table 1 represents a summary of the phenolic compounds identified in the different extracts of *P. niruri* from various regions of Punjab. Novel compounds identified are provided with their database identifiers in Online Resource 2. The hypothetical biosynthetic pathway leading to flavonoids, lignans, anthocyanins, tannins, hydroxy cinnamic acids and their derivatives, coumarins, chlorogenic acids (CGAs), and phenolic acids detected in *P. niruri* is given in Fig. 2 (Ralph et al., 1998; Humphreys and Chapple, 2002; Ossipov et al., 2003; Elfahmi et al., 2006; Hemmati, 2007; He et al., 2008; Gosch et al., 2009; Hagarman, 2008; Pandey et al., 2013; Marcotullio et al., 2014; KEGG Pathway database).

Flavonoid

Total 20 flavonoids, including flavonols and flavones, including main group compounds were identified in all the studied samples. Peak with an ion at *m/z* 272.93 was identified as naringenin, in aqueous extract of *P. niruri* from Roopnagar. It is a flavanone, abundant in citrus fruits and derived from hydrolysis of glycone form of naringin. It was previously detected in grapefruit (*Citrus paradise*) and orange (*Citrus silences*) (Wilcox et al., 1999). Protonated $[M + H]^+$ catching was reported in hydroalcoholic extract of Amritsar at *m/z* 291.30. Quercetin and its derivatives such as quercetin 3-O-glucuronide or miquelianin and quercetin-3, 4-O-diglucoside were identified in different populations of *P. niruri*. Quercetin was detected at *m/z* 302 in aqueous extract of Amritsar and ethanolic extract of Amritsar and Bathinda. However, protonated quercetin $[M + H]^+$ at *m/z* 303 was detected in aqueous extract of Patiala and Bathinda, in ethanolic extract of Patiala and Roopnagar, while in methanolic extract of Amritsar and Bathinda. Miquelianin was extracted in methanolic extract of Roopnagar at *m/z* 478. Earlier, it was reported in aqueous alcohol extract of African walnut (*Schotia brachypetala*) (Hassaan et al., 2014). Quercetin-3,4-O-diglucoside, previously reported in onions (*Allium cepa*) (Olsson et al., 2010), was detected in methanolic extract of Patiala at *m/z* 626 while its deprotonated ion $[M - H]^-$ was observed in aqueous extract of Patiala and methanolic extract of Amritsar at *m/z* 625. Similarly, protonated $[M + H]^+$ epigallocatechin or Gallo catechol, at *m/z* 307 was noticed in hydroalcoholic extract of Bathinda region. The phytochemicals, catching, quercetin, and epigallocatechin have previously been reported in different species of genus *Phyllanthus* such as *P. amours*, *P. niruri*, and *P.orbiculates* (Pojchaijongdee, 2006). However, myrecetin, a naturally occuring flavonol, widespread among plants such as Bird chilli (*Capsicum frutescens*),black tea (*Camellia sinensis*), papaya *(Carica papaya*), and guava (*Psidium guajava*) (Ong and Khoo, 1997; Miean and Mohamed, 2001), was detected at *m/z* 318 in hydroalcoholic extract of Bathinda. Another compound, identified as tectorigenin-4-sulphateat *m/z* 380 and its protonated ion $[M + H]^+$ at *m/z* 381 was detected in aqueous extract of Patiala and Amritsar, methanolic extract of Amritsar and Bathinda while its protonated ion was observed in MS spectra of hydroalcoholic extract of Patiala. Tectorigenin 4-sulphate has earlier been isolated and identified as metabolite in urinary samples of rats fed on Kakkalide isolated from Kudzu (*Pueraria lobata*) (Bai et al., 2010). However, there is no report citing its presence in plants. Deprotonated $[M - H]^-$ ion of epicatechin-3-gallate at *m/z* 441, was reported in hydroalcoholic extract of Bathinda while it has been previously reported in green tea (*Camellia sinensis*) infusions and also in *P. niruri* (Wan et al., 2004; Narendra et al., 2012). In previous studies, formomonetin 7-O-glucuronide has been identified as metabolite in urinary samples of rats and humans fed on decoction of *Astragali Radix*. Thus, ion peaks at *m/z* 444 in aqueous extract of Patiala and methanolic extracts of Bathinda correspond to formomonetin 7-O-glucuronide. A signal at *m/z* 457 was observed in hydroalcoholic extracts of all the four populations, and was

distinguished to be deprotonated ion of epigallocatechin 3-O-gallate, reported earlier in hairy root cultures of *P. niruri* (Ishimaru et al., 1992). Phloretin was thought to exist in apple (*Malus domestica* Borkh) only, however, it is also present in other species such as *Lithocarpus polystachyus*, dog rose fruit (*Rosa canina*), strawberry (*Fragaria ananassa*), or Cranberry (*Vaccinium macrocarpon*), etc (Gosch et al., 2009). Phloretin glycosides have before been determined in peel and pulp of apple (*Malus domestica* Borkh) (Alonso-Salces et al., 2005). Phloretin 2-O-xylosyl glucosideat *m/z* 568 was identified in hydroalcoholic extract of Roopnagar with its deprotonated $[M-H]^-$ ion peak at *m/z* 567 existed in methanolic extract of Patiala and in hydroalcoholic extract of Amritsar and Bathinda. In addition, *m/z* 584.07 in methanolic extract of Roopnagar corresponds to 3-Hydroxy phloretin-2'-O-xylosyl glucoside. On the other hand, pseudomolecular ion $[M-H]^-$ peak in aqueous extract of Bathinda, point toward naringin, which is the major flavanone glycoside found in citrus fruits such as grapefruit (*Citrus paradisii*) (Wilcox et al., 1999). Deprotonated Kaempferol 3-O-rutinoside $[M-H]^-$ at *m/z* 593 was reported in ethanolic extract of Patiala, Amritsar, and Roopnagar, and in aqueous and methanolic extract of Amritsar. Lam et al. (2007) studied the extract of leaves of *Phyllanthus reticulatus* and characterizedKaempferol-3-O-rutinoside in it along with six more metabolites. Pseudomolecular ion $[M-H]^-$ at m/z 607 was characterized as diosmin. Diosmin is a flavone found in number of citrus fruits such as lemon (*Citrus limon* L.) and hyssop (*Hyssopus officinalis* L.) and in plants of genus *Viccia* (Ivashev et al., 1995; Marin et al., 1998; Del Rio et al., 2004) was also identified in methanolic extract of Patiala. Rutin and corilagin previously reported in *P. niruri* (Bagalkotkar et al., 2006) were also detected, as protonated $[M+H]^+$ rutin at *m/z* 611 in hydroalcoholic extract of Bathinda and Roopnagar, and deprotonated $[M\ H]^-$ corilaginat *m/z* 635, in ethanolic extract of Patiala. Phytochemical, pectolinarin, before isolated from plants, such as, *Cirsium setidens* and *Melampyrum roseum* var. *hirsutum* (Yoo et al., 2008)was identified at *m/z* 621 in ethanolic extracts of all the four populations and in hydroalcoholic extracts of Patiala and Bathinda while in aqueous extract of Amritsar and Roopnagar. Its protonated ion $[M+2H]^+$ at *m/z* 623 was also detected in methanolic extract of Bathinda region. Ion peak at *m/z* 665.40 was identified as luteolin-7-O-(2-apiosyl-6-malonyl)-glucoside $[M-H]^-$ in aqueous extract of Roopnagar. This flavonoid compound has been previously identified in Chinese celery (*A. graveolens* L.) and sweet pepper (*Capsicum annuum* L.) (Marin et al., 2004; Lin et al., 2008). Protonated ion peak of theaflavin-3-O-gallate $[M+H]^+$ was detected in methanolic extract of Patiala. The aflavins are usually formed during fermentation of black tea (*Camellia sinensis*) and was first isolated in 1957 from black tea itself (Wang and Li, 2006). From all the flavonoids identified, 15 compounds including naringenin, catechin, myrecitin, tectorigenin-4-sulphate, catechin-3-gallate, formomonetin7-O-glucuronide, quercetin 3-O-glucuronide, phloretin 2-O-xylosyl glucoside, 3-hydroxy phloretin-2′-O-xylosyl glucoside, Kaempferol 3-O-rutinoside, diosmin, pectolinarin, quercetin 3,4-diglucoside, luteolin 7-O-(-2-apiosyl-6-malonyl)-glucoside, and theflavin-3-O-gallate are novel with respect to *P. niruri*.

Lignans

From all the 10 lignans identified, 5 lignans including lariciresinol, secoisolariciresinol, 7-hydroxy secoisolariciresinol, secoisolariciresinol-di-O-glucoside, and lariciresinol sesquilignan, are novel and identified for the first time in *P. niruri*. However, others have already been known to exist in *P. niruri*. Phyllnirurin at *m/z* 341.13 was observed in ethanolic extract of Patiala and methanolic extract of Roopnagar. Satyanarayana and Venkateswarlu (1991) earlier reported occurence of phyllnirurin in *P. niruri*. Ion peaks at *m/z* 354, detected in ethanolic extract of Amritsar and Bathinda correspond to Hinokinin, whereas protonated hinokinin $[M+H]^+$ was identified in hydroalcoholic extract of Roopnagar. Hinokinin was first isolated from Japanese cypress (*Chamecyparis obtusa*) (Yoshiki and Ishiguro, 1933). It was reported earlier in different species of *Phyllanthus* including *P. niruri* (Calixto et al., 1998). Protonated lariciresinol $[M+H]^+$ at *m/z* 361 was detected in hydroalcoholic extract of Bathinda. Lariciresinol has previously been identified in seasame seeds (*Sesamum indicum*) and vegetables of genus Brassica (Ivon et al., 2005). Ion peaks at *m/z* 362 and 364 were assigned assecoisolariciresinol and protonated secoisolariciresinol $[M+2H]^+$, in methanolic extract of Roopnagar and in ethanolic extract of Patiala. Flaxseeds (*Linum usitatissimum*) are rich source of secoisolariciresinol (Ivon et al., 2005). Another product ion at *m/z* 374.67, point to 7-hydroxy secoisolariciresinol, was present in methanolic extract of Bathinda and detected for the first time in *P. niruri*. It has also been quantified before in Seasame seeds (*Sesamum indicum*) by Smeds et al., (2007). Satyanarayana et al. (1988) reported the presence of lignan, secoisolariciresinol trimethyl ether in *P. niruri* and ion peak corresponding to secoisolariciresinol trimethyl ether at *m/z* 376 was reported in hydroalcoholic extract of Amritsar. Protonated urinatetralin was identified at *m/z* 385.73 in aqueous extract of Patiala. Urinatetralin was earlier isolated from cell suspension cultures of *P. niruri* (Bagalkotkar et al., 2006). Peaks at *m/z* 395.33 and at *m/z* 400.47 were detected as 2, 3′-desmethoxy secoisolintetralin in hydroalcoholic extract of Patiala and lintetralin in ethanolic extract of Amritsar. They have been previously reported in *P. niruri* by Satyanarayana and Venkateswarlu (1991). Protonated

secoisolariciresinol-di-O-glucoside $[M+H]^+$ at *m/z* 543.80 was reported in aqueous extract of Bathinda (Relative Abundance (RA) 32 %) and previously it has been reported in flax(*Linum usitatissimum*), sunflower (*Helianthus anus*), seasame (*Sesamum indicum*), and pumpkin seeds (*Cucurbita pepo* var. pepo) (Strandas et al., 2008). Pseudomolecular ion $[M-H]^-$ at *m/z* 555.60 correspond to Lariciresinol sesquilignan, in aqueous extract of Amritsar, it has previously been reported by Smeds et al. (2007) in seasame seeds (*Sesamum indicum*).

Anthocyanins

Anthocyanins are predominantly found in foods such as cocoa, cereals, fruits, honey, nuts, olive oil, vegetables, and wines (Lila, 2004). To our knowledge, this is the first report citing the presence of anthocyanins in *P. niruri*. Major anthocyanins reported in different extracts include cyanidin, cyanidin-3-O-(-6″-acetyl glucoside), cyanidin-3-O-(-6-malonyl glucoside), cyanidin-3-O-(-6″-succinyl glucoside), procyanidin dimer, delphidin-3-O-sambubioside, malvidin-3-O-(-6′-*p*-coumaroyl glucoside), and malvidin-3,5-O-diglucoside. Cyanidin and its glucosides have been earlier reported in red grapes (*Vitis aestivalis* Michx and *V. vinifera* L), blackberry (*Rubus Watson* and *R. allegheniensis*), and blueberries (*Vaccinium corymbosum* L, *V. darrowi* L and *V. ashei* Reade) genotypes (Cho et al., 2004). However, those reported in *P. niruri* were protonated $[M+H]^+$cyanidin at *m/z* 288.33 in ethanolic extract of Roopnagar, cyanidin-3-(-6″-acetyl glucoside) at *m/z* 491.45 in hydroalcoholic extract from Patiala and Amritsar while its protonated $[M+H]^+$ and deprotonated $[M-H]^-$ ions at *m/z* 492.53 and 490.73 in aqueous extract of Bathinda and Roopnagar. Ion peak at *m/z* 536.53 in methanolic extract of Bathinda corresponds to protonated cyanidin-3-O-(-6-malonyl glucoside) and product ion at *m/z* 548.67 points toward cyanidin-3-O-(-6″-succinyl glucoside) in aqueous extract of Roopnagar. Procyanidin dimer B at *m/z* 578 was identified in ethanolic extract of Patiala and Amritsar. Procyanidins are mainly found in green tea (*Camellia sinensis*) and red wine. Procyanidin dimer B was reported previously in sorghum (*Sorghum biocolor* Moench) and buck wheat (*Fagopyrum esculentum* Moench) (Jeong and Kong, 2004). Protonated ion peak of Delphidin-3-O-sambubioside $[M+H]^+$ was detected at *m/z* 598.67 in aqueous extract of Bathinda. It has been previously reported in roselle or sour tea (*Hibiscus sabdariffa* L.) (Hou et al., 2005) and Cranberry (*Oxycoccus spp.*) (Wei et al., 2011). Ion at *m/z* 639.5 was assigned as Malvidin-3-O-(-6′-*p*-coumaroyl glucoside), in methanolic extract of Patiala. However, peak at *m/z* 655 corresponds to malvidin 3,5-O-diglucoside, reported in hydroalcoholic extracts of Amritsar, Bathinda and Roopnagar and also in ethanolic extract of Roopnagar. Mavidin-3-O-(-6′-*p*-coumaroyl glucoside), i.e., *p*-coumaroylated anthocyanin has been reported earlier in grapes (*Vitis vinifera*) and wine by Calvo et al. (2004), while Malvidin 3,5-O-diglucoside was previously reported in flowers of saffron crocus (*Crocus sativus*), grapes (*Vitis vinifera*), and wine (Flamini, 2013; Lim, 2014).

Hydroxy cinnamic acids and their derivatives

Hydroxy cinnamic acids and their derivatives are widely distributed in different parts of plants such as fruits and vegetables. They play important role in their secondary metabolism and are present either esterified with other hydroxyacids or sugars or in glycosylated form (Bengoechea et al., 1995). Total seven novel metabolites were detected in this category. First is Pseudomolecular ion $[M-H]^-$ of *p*-coumaric acid, at *m/z* 163.60 determined in ethanolic extract of Patiala and methanolic extract of Bathinda. *p*-coumaric acid was previously isolated from wine, vinegar, and barley grain (*Hordeum vulgare*) (Galvez et al., 1994; Zory and Byung-Kee, 2006).Similarly, protonated $[M+H]^+$ (*m/z* 181.13) and deprotonated $[M-H]^-$ (*m/z* 179.80) ions of caffeic acid were present in ethanolic extract of Patiala and hydroalcoholic extract of Bathinda. Niu et al. (2012) earlier reported the presence of pseudomolecular ions or deprotonated ions $[M-H]^-$ of caffeic acid in methanolic extract of *Phyllanthus simplex* Retz. Peak at *m/z* 208.20 corresponds to ethyl caffeate, reported in methanolic extract of Roopnagar and earlier reported in white and red wines, and also isolated from hairy beggarticks (*Bidens pilosa*) (Chiang et al., 2005; Boselli et al., 2009). *p*-coumaroyl glycolic acid at *m/z* 222 was extracted in aqueous extract of Patiala, Amritsar, and Bathinda; in ethanolic extract of Amritsar; and in methanolic and hydroalcoholic extract of Amritsar and Bathinda. Moreover, its pseudomolecular ions at *m/z* 223 $[M+H]^+$ and at 220 $[M-2H]^-$, were also reported in ethanolic extract of Patiala and Roopnagar and in aqueous extract of Roopnagar. *p*-coumaroyl glycolic acid has been previously isolated from cotyledons of lentils (*Lensculinaris* L.) (Duenas et al., 2002). Protonated caffeic acid 3-sulphate $[M+H]^+$ at *m/z* 261 was recognized in ethanolic extract of Bathinda. In addition, peaks at *m/z* 274 were characterized as ferulic acid 4-sulphate in hydroalcoholic extracts of all the four populations. Caffeic acid 3-sulphate and ferulic acid 4-sulphate has not been isolated from any plant, earlier. However, they were reported as metabolites in human urine and plasma samples after consumption of coffee and in urine samples only after ingestion of polyphenol rich juice drink (Stalmach et al., 2009; Borges et al., 2010). Thus, this is the first report citing natural occurrence of these metabolites in *P. niruri*. Product ions at *m/z* 386 points toward 8,5′-diferulic acid present in ethanolic extract of Amritsar and Bathinda

while in all the four extracts, i.e., aqueous, ethanolic, methanolic, and hydroalcoholic of population from Roopnagar. 8,5′-diferulic acid is predominantly present in sugar beet pulp (*Beta vulgaris*), barley (*Hordeum vulgare*), maize bran (*Zea mays*), and rye (*Secale cereale*) (Micard et al., 1997; Andreasen et al., 2000; Hernanz et al., 2001; Bunzel et al., 2004).

Coumarins

Coumarin was first isolated from cumaru or kumaru (*Dipteryx odorata* Willd.) in 1820. Coumarins include a large number of compounds found throughout in plant kingdom such as in essential oils (cinnamom bark oil, lavender oil, etc.) and also in fruits such as bilberry (*Vaccinium myrtillus*), cloudberry (*Rubus chamaemorus*), green tea (*Camellia sinensis*), and other foods such as chicory (*Cichorium intybus*) (Jain and Joshi, 2012). Out of six coumarins identified in different populations of *P. niruri*, only one, brevifolin carboxylate at *m/z* 292.53, identified in methanolic extract of Roopnagar, has been previously reported in *P. niruri* (Calixto et al., 1998). Ion peak at *m/z* 246 was identified as isopimpinellin, in aqueous extract of Roopnagar and Bathinda. Isopimpinellin is a type of furano coumarin, found in healthy celery (*Apium graveolens* var. dulce), parsnip (*Pastinaca sativa*), in fruits of bishop's weed (*Ammi majus* L.), and in rind and pulp of limes (*Citrus limon*) (Kleineret al., 2002). Further, minor peak at *m/z* 434, detected in ethanolic extract of Patiala and aqueous extract of Amritsar was determined as ellagic acid pentose. Ellagic acid glycosides with pentose, hexose, or deoxyhexose as sugar moeity were earlier identified in fruit extracts of *Phyllanthus emblica* (Yang et al., 2012). Peak at *m/z* 476.87 revealed to contain ellagic acid acetyl xyloside in methanolic extract of Amritsar and was previously detected in blueberries (*Vaccinium corymbosum*), blackberries (*Rubus ruticosus*), cranberries (*Vaccinium vitisidaea*), and red raspberries (*Rubus idaeus*) (Diaconeasa et al., 2014). From the reported data, peaks at *m/z* 470.33, 470.67, and 470.80 were identified as valoneic acid dilactone (VAD) in methanolic extract of Patiala as well as in ethanolic and methanolic extract of Bathinda. Its protonated ions $[M+H]^+$ and $[M+2H]^+$ at *m/z* 471 and 472 were also detected in hydroalcoholic extract of Bathinda and Roopnagar and in aqueous extract of Bathinda. VAD was previously determined in leaves of Japanese silverberry (*Elaeagnus umbellata*), mexican heather (*Cuphea hyssopifolia*), and from aqueous extract of leaves of pride of India (*Lagerstroemia speciosa*) (Hideyuki et al., 1999; Unno et al., 2004; Elgindi et al., 2012). *Quasi*-molecular ion $[M-H]^-$ at *m/z* 781 was determined as punicalin in aqueous extract of Bathinda, which is an ellagitannin, before isolated from the the leaves of Indian/tropical almond (*Terminalia catappa* L.) and Pomegranate (*Punica granatum*) husk (Lin et al., 2001; Zhou et al., 2010).

Chlorogenic acids (CGAs)

Chlorogenic acids (CGAs) are cinnamic acid derivatives formed by esterification of acids such as caffeic, ferulic, and *p*-coumaric acids with -(-) quinic acid (Farah et al., 2008). CGAs have been reported for the first time in *P. niruri*. Major ion peak at *m/z* 338.27 was detected as 5-*p*-coumaroyl quinic acid (5-*p*-CoQA) in methanolic extract of Patiala and its deprotonated ion at *m/z* 337.00 was also noticed in aqueous extract of Roopnagar. Green or raw coffee (*Coffee robusta*) is abundant source of 3-, 4- and 5-*p*-CoQA (Farah et al., 2008). It was also isolated from fruit of immature pear (*Pyrus pyrifolia* nakai) (Lee et al., 2013). Similarly, 4-Sinapoyl quinic acid was reported as major metabolite at *m/z* 398.87 in aqueous extract of Bathinda and Roopnagar. It was also detected and characterized earlier in green Robusta coffee beans using LC–MS (Jaiswal et al., 2010). Ion peak at *m/z* 530.67 was reported as 1-caffeoyl-5-feruloylquinic acid present in methanolic extract of Roopnagar. It is previously detected as metabolite of 1,5-dicaffeoylquinic acid in urine and plasma samples of rats, and in *Svensonia hyderobadensis* (Yang et al., 2005; Linga Rao and Savithramma, 2014).

Phenolic acids and their derivatives

Phenolic acids isolated from plants include derivatives of benzeldehyde, ethanone, cinnamic, and benzoic acids, and are among the most widespread class of secondary metabolites (Martens, 2002). Total three phenolic acids were identified and all are novel with respect to *P. niruri*. From the literature, peak at *m/z* 138.33 was identified as salicylic acid, characterized in aqueous extract of Patiala and Amritsar and in methanolic extract of Amritsar and Bathinda. Salicylic acid or 2-hydroxy benzoic acid is found in number of plants such as gumweed (*Grindelia* spp.), medlar (*Mespilus germanica*), poplar (*Populus pseudo-simonii*), Voodoo lily (*Sauromatum guttatum*), and willow bark (*Salix* spp.) (Khadem and Marles, 2010). Peak at *m/z* 152.07 corresponds to salicylic acid methyl ester or methyl salicylate, in ethanolic extract of Amritsar and its deprotonated ion $[M-H]^-$ product at *m/z* 151, in ethanolic extract of Bathinda and Roopnagar. Zhang et al. (2007) reported occurence of methyl salicylate and its glycosides in wintergreen (*Gaultheria yunnanensis*). Protonated ions of protocatechuic acid $[M+H]^+/[M+2H]^+$ at *m/z* 155.80 and 156.13 were detected in aqueous extract of Bathinda and Roopnagar. Protocatechuic acid or 2, 3-dihydroxy benzoic acid has been previously identified in alder (*Alnus spp.*), buckwheat (*Fagopyrum spp.*), danshen (*Salvia*

miltiorrhiza), dog rose (*Rosa canina*), gum-tree (*Eucalyptus grandis*), Japanese pepper (*Zanthoxylum piperitum*), Japanese honeysuckle (*Lonicera japonica*), Korean spruce (*Picea koraiensis*), mulberry (*Morus alba*), medlar (*Mespilus germanica*), Spanish heath (*Erica australis*), shensi (*Picrorhiza kurrooa*), onion and garlic and relatives (*Allium spp*.), sharpleaf galangal (*Alpinia oxyphylla*), and sea buckthorn (*Hippophae rhamnoides*) (Khadem and Marles, 2010).

Triterpenoids

Triterpenoids are organic compounds characterized by basic backbone modified in multiple ways (Petronelli et al., 2009). Two triterpenoids, i.e., lupenone and betulinic acid are identified for the first time in *P. niruri* in this report. Peak at *m/z* 424.13 was assigned as lupenone, in methanolic extract of Patiala and Amritsar along with its protonated ion $[M + H]^+$ at *m/z* 425.30 in ethanolic extractof Bathinda. Lupenone was earlier isolated from *Polypodium vulgare,* a fern widely distributed in Europe, Asia, and North America (Prakash and Prakash, 2012). On the other hand, betulinic acid detected at *m/z* 456 in methanolic extract of Patiala, aqueous extract of Amritsar and in ethanolic extract of Amritsar and Bathinda, was reported earlier in other species of *Phyllanthus* such as *Phyllanthus reticulatus* and *Phyllanthus discoideus* by Hui et al. (1976) and Calixto et al. (1998). Deprotonated ion $[M - H]^-$ of betulinic acid at *m/z* 454 was also detected in aqueous extract of Roopnagar.

Alkaloids

Alkaloids include diverse group of compounds and are characterized by the presence of nitrogen atom. They play an important role in defense mechanism of plants from herbivores and pathogens (Ziegler and Facchini, 2008). Alkaloids isolated from *P. niruri* are securinine, nor securinine, phyllanthine, nirurine, and phyllochrysine (Bagalkotkar et al., 2006). Alkaloids characterized from the MS spectra of extracts of *P. niruri* include securinine, nor securinine, and phyllnirurin. Product ions at *m/z* 203 were identified as nor securinine, in hydroalcoholic extract of Patiala and, Amritsar and those with *m/z* 204 were as securinine, present as major metabolite in all the four extracts from population of Patiala and Amritsar and in ethanolic, methanolic, and hydroalcoholic extract of Bathinda, and also in aqueous, ethanolic, and hydroalcoholic extract of Roopnagar.

Tannins

Tannins are naturally occuring polyphenols, structurally classified into two major groups, i.e., hydrolysable tannins and condensed tannins. Tannins detected from MS spectra of *P. niruri* are corilagin and pinocembrin. Major peaks at *m/z* 256, demonstrated the presence of pinocembrin in hydroalcoholic extracts of all the four populations. Pinocembrin was previously isolated from many plant species such as champoo (*Syzygium samarangense*), damiana (*Turnera diffusa*), edaxia(*Oxytropis falca*), eryngo star thistle (*Centaurea eryngioides*), Liquorice (*Glycyrrhiza glabra L.*), Lychee (*Litchi chinensis*), mountain balm (*Eriodictyon californicum*), mexican origano (*Lippia graveolens*), Prairie clover/indigo bush (*Dalea* elegans), soft hairy rockrose (*Cistus incanus*), salva-de-marajo (*Lippia origanoides*), and small shell ginger (*Alpinia mutica*) (Rasul et al., 2013). Moreover, *qausi* molecular ion $[M - H]^-$ of corilagin at *m/z* 635.20 was detected in ethanolic extract of Patiala. Corilagin was earlier detected in *P. niruri* by Shimizu et al. (1989). However, pinocembrin has been isolated and reported for the first time in *P. niruri*.

Hydroxybenzaldehyde

Hydroxybenzaldehydes are phenolic aldehydes. Deprotonated ion at *m/z* 231.18 was characterized as vanillin 4 sulphate in hydroalcholic extract of Patiala. Suarez et al. (2009) determined vanillin sulphate in plasma samples from humans after consuption of virgin olive oil (*Olea europara*). This is the first report citing the natural source of vanillin sulphate, i.e., *P. niruri*.

Other compounds

Among other phytochemicals detected in *P. niruri*, are tricontanal, ligstroside, carnosol, ascorbic acid, and linolenic acid. Ion peak at *m/z* 176.00 and *quasi*-molecular ions $[M - H]^-/[M - 2H]^-$ at *m/z* 175 were reported as ascorbic acid, present in aqueous extract of Bathinda, Patiala, and Amritsar, in methanolic extract of Amritsar and Bathinda, and in hydroalcoholic extract of Patiala. Ascorbic acid has been earlier reported in leaves of *P. niruri* (Damle et al., 2008). Product ion at *m/z* 278 was detected as linolenic acid in methanolic extract of Patiala and aqueous extract of Bathinda. Linolenic acid is an essential omega-3-fatty acid present in seeds of *P. niruri* (Damle et al., 2008). Peak at *m/z* 330.47 and its deprotonated ion $[M - H]^-$ at *m/z* 329. 34 were identified as carnosol in aqueous and hydroalcoholic extract of Amritsar. Carnosol is an ortho-diphenolic di-terpene, degradation product of carnosic acid, first isolated from sage (*Salvia carnosa*) in 1942. It is also reported in rosemary (*Rosemarinus officinalis*) (Jhonson, 2011) and for the first time in *P. niruri*. Tricontanal was observed at *m/z* 435.44 in hydroalcoholic extract of Bathinda. Major peaks of protonated $[M + 2H]^+$ and deprotonated $[M - H]^-$ ions at *m/z* 523.46 and 525.00 were determined as ligstroside, in

Table 2 Potential therapeutic properties exhibited bynovelphytochemicalsreported in *P. niruri*

S.no.	Novel compound	Previously reported	Therapeutic property	References
1.	1-caffeoyl-5-feruloylquinic acid	*S. hyderobadensis*	Antioxidant activity	Farah and Donangelo, 2006; Linga Rao and Savithramma, 2014
2.	3-hydroxy phloretin-2′-O-xylosyl glucoside	*M. domestica*	Antioxidant, anticancer, estrogenic activity and inhibition of cardiovascular disease	Gosch et al., 2009; Lee et al., 2011
3.	4-sinapoyl quinic acid	*C. canephora*	Antioxidant, antiinflammatory, anticancer and antianxiety	Jaiswal et al., 2010; Niciforovic and Abramovic, 2014
4.	(5-*p*-CoQA)	*P. pyrifolia*	Antioxidant activity	Lee et al., 2013
5.	7-hydroxy secoisolariciresinol	*S. indicum*	Antioxidant, anticancer and antiinflammatory	Moree and Rajesha, 2011
6.	8,5′-diferulic acid	*B. vulgaris, H. vulgare, S. cereale and Z. mays*	Antibacterial, antidiabetic, antifungal, antihypercholestremic, antiinflammatory, antimutagenic and antioxidant	Micard et al., 1997; Andreasen et al., 2000; Hernanz et al., 2001; Bunzel et al., 2004; Boz, 2015
7.	Caffeic acid	*P. simplex*	Antioxidant, Anticancer, antiinflammatory	Prasad et al., 2011
8.	Caffeic acid 3-sulphate	–	–	–
9.	Carnosol	*S. carnosa* and *R. officinalis*	Antiangiogenic, antitumor and strengthening of nerves	Lopez-Jimenez et al., 2011, Jhonson, 2011
10.	Coumarin	*D. odorata*	Used in treatment of asthma and lymphedema	Farinola and Piller, 2005; Jain and Joshi, 2012
11.	Cyanidin	*V. aestivalis, V. vinifera, R. watson, R. allegheniensis, V. corymbosum, V. darrowi, V. ashei*	Antioxidant, Anticancer, antiobesity, antidiabetic, antiinflammatory, antineurodegenerative skin photoprotective, gastroprotective, antineurodegenerative, vasoprotective, ocular and dietary effects	Cho et al., 2004; Galvano et al., 2007
12.	Cyanidin-3-O-(6”-acetyl glucoside)	*V. aestivalis, V. vinifera, R. watson, R. allegheniensis, V. corymbosum, V. darrowi, V. ashei*	Antioxidant activity	Cho et al.,2004; Svarcovaa et al., 2007
13.	Cyanidin-3-O-(-6”-succinyl glucoside)	*V. aestivalis, V. vinifera, R. watson, R. allegheniensis, V. corymbosum, V. darrowi, V. ashei*	Antioxidant activity	Cho et al., 2004; Svarcovaa et al., 2007
14.	Cyanidin-3-O-(-6-malonyl glucoside)	*V. aestivalis, V. vinifera, R. watson, R. allegheniensis, V. corymbosum, V. darrowi, V. ashei*	Antioxidant activity	Cho et al., 2004; Svarcovaa et al.,2007
15.	Delphidin-3-O-feruloyl-glucoside	*P. vulgaris* L.	Antioxidant, antimutagenesis, antiinflammatory and antiangiogenic	Patel et al., 2013
16.	Delphidin-3-O-sambubioside	*H. sabdariffa* L., *Oxycoccus* spp.	Antioxidant activity and protective effect against coronary heart disease	Hou et al., 2005; Wei et al., 2011; Obouayeba et al., 2014
17.	Diosmin	*C. limon* L., *H. officinalis*,	Antidiabetic, anticancer and treatment of lyphedema, hemorrhoids and chronic venous insufficiency	Godeberge, 1994; Pecking, 1995; Tanaka et al., 1997; Marin et al., 1998; Bergan et al., 2001; Del Rio et al., 2004
18.		*R. idaeus*		

Table 2 continued

S.no.	Novel compound	Previously reported	Therapeutic property	References
	Ellagic acid acetyl xyloside		Anticarcinogenic, anti-HBV, antidiabetic and inhibits aldolase reductase	Terashima et al., 1991; Seerama et al., 2005; Li et al., 2005; Patel et al., 2012
19.	Ellagic acid pentose	*P. emblica*	Anticancer, anti-HBV, antidiabetic and inhibits aldolase reductase	Terashima et al., 1991; Seerama et al., 2005; Li et al., 2005; Patel et al., 2012; Yang et al., 2012
20.	Ethyl caffeate	*B. pilosa*	Anticancer	Rocha et al., 2012
21.	Ferulic acid 4-sulphate	–	–	–
22.	Formomonetin-7-O-glucuronide	–	–	–
23.	Isopimpinellin	*Ammimajus* L.	Chemoprotective	Kleiner et al., 2002
24.	Kaempferol 3-O-rutinoside	*P. reticulatis*	Antioxidant, anticancer, antiinflammatory and antimicrobial	Calderon-Montano et al., 2011
25.	Lariciresinol	*S. indicum*, *S. williamsii*	Anticancer and antifungal	Saarinen et al., 2008; Hwang et al., 2011
26.	Lariciresinol sesquilignan	*S. indicum*	Antioxidant and anticancer	Smeds et al., 2007; Yu et al., 2012
27.	Ligstroside	*O. europaea*	Antiatherosclerosis, anticancer, inhibits low-density lipoprotein peroxidation and prevents osteoprosis	Cardoso et al., 2011; Alagna et al., 2012
28.	Lupenone	*P. vulgare*	Stimulates melanogenesis in B16 murine melanoma cells, inhibits α-glucosidase (α-Glu) and protein tyrosine phosphatase 1B (PTP1B) activities, inhibits adipogenic differentiation.	Prakash and Prakash, 2012; Xu et al., 2014
29.	Luteolin-7-O-(2-apiosyl-6-malonyl)-glucoside	*A. graveolens* and *C. annum* L.	Antioxidant activity	Marin et al., 2004; Lin et al., 2008
30.	Malvidin-3,5-O-diglucoside	*C. sativus*	Antiinflammatory	Lim, 2014; Huang et al., 2014
31.	Malvidin-3-O-(-6′-p-coumaroyl glucoside)	*V. vinifera*	Anticarcinogenic	Calvo et al., 2004; Esmaeelian et al., 2007
32.	Myricetin	*C. frutescens, C. sinensis, C. papaya and P. guajava*	Antioxidant, anticarcinogenic, antidiabetic, antiviral, and antiplatelet activity, cytoprotective, and hypoglycemic agent	Miean and Mohamed, 2001; Li and Ding, 2012
33.	Naringenin	*C. paradisi* and *C. sinensis*	Antioxidant, anti-inflammatory agent, anticancer, immunomodulator	Wilcox et al., 1999; Yilma et al., 2013
34.	Naringin	*C. paradisii*	Antioxidant, anti-inflammatory and anti-nociceptive activity	Jourdan et al., 1985; Rout et al., 2013
35.	*p*-coumaric acid	*H. vulgare*, vinegar and wine	Antioxidant, antidiabetic and anticancer	Srivastava et al., 2009; Rocha et al., 2012
36.	*p*-coumaroyl glycolic acid	*L. culnaris*	Antioxidant, antidiabetic and anticancer	Srivastava et al., 2009; Rocha et al., 2012

Table 2 continued

S.no.	Novel compound	Previously reported	Therapeutic property	References
37.	Pectolinarin	*C. setidens* and *M. roseum*	Anti-inflammatory, antidiabetic, anticancer, hepatoprotective	Yoo et al., 2008; Lim et al., 2008; Lu et al., 2014
38.	Phloretin-2-O-xylosyl glucoside	*M. domestica*	Antioxidant, anticancer, estrogenic activity and inhibition of cardiovascular disease	Gosch et al., 2009; Lee et al., 2011
39.	Pinocembrin	*A. mutica, C. eryngioides, C. incanus, D. elegans, E. californicum, G. glabra L., H. gymnococcum, L. chinensis, L. graveolens, L. origano, L. markgravii, O. falca, S. leucanthum, S. samarangense* and *T. diffusa.*	Anticancer, antiinflammatory, antimicrobial, neuroprotective activity	Rasul et al., 2013
40.	Procyanidin dimer B	*F. culentum and S. biocolor*	Antioxidant, anti-hypercholesterolemic effect and anticancer	Jeong and Kong, 2004
41.	Protocatechuic acid	*Alnus* spp., *Allium* spp, *A. oxyphylla*, *E. australis, E. grandis, Fagopyrum spp, H. rhamnoides, L. japonica, M. alba, P. kurrooa*, *P. koraiensis*, *R. canina, Z. piperitum*	Antibacterial, antioxidant, antidiabetic, anticancer, antiulcer, antiageing, antifibrotic, antiviral, anti-inflammatory, anti-analgesic, antiatherosclerotic, hyperlipidemic activity, hepatoprotective, nephroprotective and neurotrophic effects	Khadem and Marles, 2010; Kakkar and Bais, 2014
42.	Punicalin	*T. catappa* L.	Anti-dermatitis and anti-hepatitis	Lin et al., 2001; Lin et al., 1998; Lin et al., 1999
43.	Quercetin 3-O-glucuronide	*S. brachypetala*	Antioxidant activity, prevention of cardiovascular disease	Dzomba and Musekiwa, 2014; Hassaan et al., 2014
44.	Quercetin-3,4-O-diglucoside	*A. cepa*	Antioxidant activity and prevention of cardiovascular disease	Olsson et al., 2010; Dzomba and Musekiwa, 2014
45.	Salicylic acid	*Grindelia spp, M. germanica, P. pseudo-simonii, Salix spp, S. guttatum*	Antiacne, anti-inflammatory, antipyretic, analgesic, antiseptic, antifungal, ichthyosis, keratolytic, psoriasis and seborrheic dermatitis	Khadem and Marles, 2010
46.	Salicylic acid methyl ester	*G. yunnanensis*	Analgesic and rubefacients	Mason et al., 2004; Zhang et al., 2007
47.	Secoisolariciresinol-di-O-glucoside	*C. pepo, H. annus, L. usitatissimum, S. indicum*	Antioxidant, anticancer, anti-inflammatory	Strandas et al., 2008; Moree and Rajesha, 2011
48.	Tectorigenin 4-sulphate	–	Anticancer and hepatic antifibrotic	Lee et al., 2001
49.	Theaflavin-3-O-gallate	*C. sinensis*	Antioxidant, anticancer, antihyperglycaemic, antipathogenic and antiatherosclerosis	Wang and Li, 2006
50.	VAD	*C. hyssopifolia, E. umbellata, L. speciosa*	Antidiabetic	Hideyuki et al., 1999; Unno et al., 2004; Elgindi et al., 2012; Middha et al., 2013
51.	Vanillin-4-sulphate	–	–	–

–Not detected previously in plants

ethanolic extract of Amritsar, Bathinda, and Roopnagar, in methanolic extract of Amritsar, and in hydroalcoholic extract of Amritsar, Bathinda, and Roopnagar. Ligstroside was earlier isolated from olive oil (*Olea europaea*) (Brenes et al., 2000). Tricontanal and tricontanol were isolated from *P. niruri*e arlier also (Bagalkotkar et al., 2006).

Potential therapeutic properties of phytochemicals reported in *P. niruri*

Some of the phenolic compounds reported in MS spectra, showed 100 % relative abundance in extracts from different populations of *P. niruri*. In aqueous and ethanolic extract of Amritsar, pectolinarin had shown RA of 100 % and reported as major flavonoid. It exhibits anticancer, antidiabetic, anti-inflammatory, and hepatoprotective activities (Lim et al., 2008; Lu et al., 2014). Similarly, in methanolic extract of Amritsar, lariciresinol exhibited 100 % RA. Lariciresinol is a new lignan identified in *P. niruri* having anticancer and antifungal activity (Saarinen et al., 2008; Hwang et al., 2011). In hydroalcholic extracts of Amritsar and Patiala, methanolic extract of Bathinda, and ethanolic extract of Patiala, securinine gave similar results, i.e., 100 % RA, whereas in aqueous extract of Bathinda, maximum RA shown by securinine is 68 %. Securinine is one of the major alkaloid present in *P. niruri* and exhibits most of the pharmacological activity demonstrated by the plant. Reported biological activities of securinine include anticancer, antimalarial, antimicrobial, and neuropharmacological activity (Zhang et al., 2011). However, in aqueous extract of Bathinda, *p*-coumaroyl glycolic acid had shown highest RA, i.e., 75 % and it is antidiabetic and anticancer in nature. Ligstroside was reported to have 100 % RA in ethanolic extract of Bathinda and Roopnagar. It possess several activities such as antiatherosclerotic, anticancer, and inhibits low-density lipoprotein peroxidation and prevents osteoprosis (Cardoso et al., 2011; Alagna et al., 2012). Hydroalcoholic extracts of Bathinda and Roopnagar demonstrated 100 % RA in case of pinocenbrin, which display anticancer, anti-inflammatory, antimicrobial, and neuroprotective effect (Rasul et al., 2013). Diosmin, with 100 % RA in methanolic extract of Patiala, was first used as therapeutic agent in 1969 and exhibits several useful properties such as antidiabetic and anticancer. It is also employed in the treatment of lyphedema, hemorrhoids, and chronic venous insufficiency (Godeberge, 1994; Pecking, 1995; Tanaka et al., 1997; Marin et al., 1998; Bergan et al., 2001; Del Rio et al., 2004). 4-Sinapoyl quinic acid and caffeic acid resulted in maximum RA of 100 % in aqueous and methanolic extracts of Roopnagar. 4-Sinapoyl quinic acid and caffeic acid are known for antioxidant, anti-inflammatory, and anticancer properties (Jaiswal et al., 2010; Prasad et al., 2011; Niciforovic and Abramovic, 2014). Table 2, shows potential therapeutic properties exhibited by novel compounds detected in *P. niruri* using ESI–MS.

Identification of discriminative compounds in *P. niruri* populations

Metabolic fingerprinting technique is high-throughput qualitative method for screening of an organism or tissue with the principal aim of sample comparison and distiction analysis. LC–MS (Liquid chromatography–Mass spectral analysis) is the most commonly used technique for metabolic fingerprinting in plant research involving chemotaxonomy, plant biochemistry, food chemistry, and for quality control of medicinally important plants (Safer et al., 2011). It has previously been exploited to compare different species of *Leontopodium* (Safer et al., 2011), for distinction of wild type and transgenic tobacco plants (Choi et al., 2004) and for analysis of alterations in plant secondary metabolites during growth such as in *Angelica sinensis* (Qian et al., 2013). Similarly, metabolic fingerprint analysis was carried out in this study for the discrimination of different *P. niruri* populations. Distinctive compounds for all the four populations are given in Table 3. Some of these compounds are isolated and described for the first time in *P. niruri*. The metabolites detected are only present in particular population and hence discriminating it from others.

Conclusion

Application of ESI–MS in the current study provided useful information in characterization of 51 novel compounds in different classes of phytochemicals, while anthocyanins and chlorogenic acids are the groups detected for the first time in *P. niruri*. However, to confirm the beneficial effects of these extracts, it is necessary to carry out furthur studies on in vivo therapeutic potential and bioavailability. Moreover, exact elucidation of structural homologs of compounds, functional analysis, and mechanism of biosynthesis need to be addressed. High content of medicinally useful metabolites, e.g., caffeic acid, diosmin, lariciresinol, ligstroside, *p*-coumaroyl glycolic acid, pectolinarin, pinocembrin, securinine, and 4-sinapoyl quinic acid in *P. niruri*, make this plant a promising herbal drug for future utilization by companies dealing with natural medicines. The ESI–MS-based metabolomics approach has great potential for discriminating different species and populations. Based on the results, a clear cut distinction of metabolic fingerprints can be deduced between different populations of a plant species. Moreover, taxonomic characterization using morphological and molecular methods is difficult; ESI–MS fingerprinting approach could offer relevant information on species

Table 3 Metabolic fingerprints of distinctive compounds of *P. niruri*

Amritsar			
S.No.	Metabolites detected	RA (%)	Extract
1.	Lariciresinol sesquilignan	32	Aqueous
2.	Ellagic acid acetyl glucoside	24	Ethanolic
3.	Carnosol	20	Aqueous
4.	Lintetralin	16	Ethanolic
5.	Secoisolariciresinol trimethyl ether	10	Hydroalcoholic
Bathinda			
1.	Epicatechin-3-gallate	58	Hydroalcoholic
2.	Naringin	40	Aqueous
3.	Cyanidin 3-O-(-6-malonyl glucoside)	36	Methanolic
4.	Punicalin	36	Aqueous
5.	Delphidin-3-O-sambubioside	34	Aqueous
6.	7-hydroxy secoisolariciresinol	32	Methanolic
7.	Secoisolariciresinol-di-O-glucoside	32	Aqueous
8.	Caffeic acid 3-sulphate	16	Ethanolic
9.	Myricetin	10	Hydroalcoholic
10.	Tricontanal	8	Hydroalcoholic
11.	Gallocatechin	5	Hydroalcoholic
Patiala			
1.	Diosmin	100	Methanolic
2.	Malvidin-3-O-(-6,-p-coumaroyl glucoside)	84	Methanolic
3.	Theaflavin-3-O-gallate	45	Methanolic
4.	Corilagin	32	Ehanolic
5.	Secoisolariciresinol	23	Ethanolic
6.	Urinatetralin	22	Ethanolic
7.	Vanillin-4-sulphate	19	Hydroalcoholic
8.	2,3-Desmethoxy secoisolintetralin	8	Hydroalcoholic
Roopnagar			
1.	3-Hydroxy phloretin-2′-O-xylosyl glucoside	73	Methanolic
2.	Ethyl caffeate	58	Methanolic
3.	Cyanidin-3-O-(-6″-succinyl glucoside)	54	Aqueous
4.	1-Caffeoyl-5-feruloyl quinic acid	50	Methanolic
5.	Brevifolin carboxylate	46	Methanolic
6.	Quercetin-3-O-glucuronide/ Miquelianin	43	Methanolic
7.	Naringenin	38	Aqueous
8.	Cyanidin	12	Ethanolic

relationship and facilitate classification of the species and populations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval : This article does not contain any studies with human participants or animals performed by any of the authors.

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