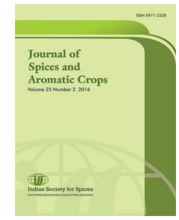
Journal of Spices and Aromatic Crops
Vol. 25 (2) : 123–132 (2016)
www.indianspicesociety.in/josac/index.php/josac

Indian Society for Spices



Phenolic profiling of *Piper* species by Liquid Chromatography-Mass Spectrometry

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Received 21 August 2015; Revised 25 January 2016; Accepted 01 February 2016

Abstract

In the present study, phenolic compounds from four *Piper* species *viz*., *P. nigrum* (Black pepper), *P. longum, P. chaba* and *P. colubrinum* were identified by LC-MS analysis. The identified phenolic compounds mainly include phenolic acids and flavonoids. Thirteen compounds were identified in black pepper which mainly included hydroxybenzoic acids (syringic acid, protocatechuic acid etc.), hydroxycinnamic acids (caffeic acid, ferulic acid and 4-coumaric acid) and flavonoids (luteolin-8-C-glucoside and apigenin). Among the six compounds identified in *P. longum,* three belonged to hydroxybenzoic acid and the other three belonged to hydroxycinnamic acid category. Seven compounds were identified from *P. chaba* which comprised of hydroxybenzoic acids, phenolic aldehydes and hydroxycinnamic acids. In *P. colubrinum,* ten compounds were identified and majority were flavonoids like kaempferol-5-glucoside and apigenin-7-galactoside. Hydroxybenzoic acids like protocatechuic acid and phenolic aldehyde like vanillin were also identified in *P.colubrinum.* Salicylic acid, a monohydroxybenzoic acid was identified in all the four species.

Keywords: *Piper nigrum*, *Piper longum, Piper chaba, Piper colubrinum*, phenolic compounds, LC-MS

Introduction

Phenolics are the most abundant secondary metabolite in plant kingdom and are responsible for their defense mechanisms under different environmental stress conditions such as infection, UV irradiation, wounding etc. (Bennet & Wallsgrove 1994; Dixon & Paiva 1995). Plant phenolics ranges from simple molecules such as phenolic acids to highly polymerized substances such as tannins. Flavonoids are the largest group among the natural phenolic compounds. The physical, chemical and biological properties of phenolics are determined by their structure (Jain *et al.* 2013; Kartsova & Alekseeva 2008). Recent studies revealed that phenolic compounds from plants exert several health promoting functions like reducing the risks of cancer, heart and neurodegenerative diseases (Indap *et al.* 2006; Vita 2005). The free-radical scavenging capability and consequent antioxidant properties of the phenolic compounds play an important role in protecting our body from oxidative stress and other biological effects which forms the root causes of these chronic diseases (Rimbach & De Pascual-Teresa 2005). Because of these health-promoting effects, phenolics from various plant sources have been reported in recent years. So our study focused on identification of these medicinally important phenolic compounds from four important *Piper* species *viz., P. nigrum, P. longum, P. chaba* and *P. colubrinum*.

Piper nigrum (Black pepper) is valued for its pungency and aroma. The alkaloid piperine is responsible for pungency whereas aroma is contributed by essential oil constituents. Black pepper is well known for various medicinal properties like antioxidant, antimicrobial, anticancer, analgesic, antipyretic and also for enhancement of bioavailability of drugs (Karsha & Lakshmi 2010; Vijayakumar *et al.* 2004; Lee *et al.* 1984; Bano *et al.* 1991; Pingili *et al.* 2012). *Piper longum* (Long pepper) and *Piper chaba* are the two important spices forms the ingredients in many traditional formulations. Their antioxidant activity, anti-tumour effect, antimicrobial activity and efficacy against respiratory tract disorders, diarrhoea and rheumatic pain were studied (Rahman *et al.* 2011; Srinivasa *et al.* 2001; Sunila & Kuttan 2004; Samudram *et al.* 2009). *Piper colubrinum*, a distant relative of *Piper nigrum* is native to Northern part of South America and is immune to *Phytophthora capsici* and *Radopholus similis* (Ravindran & Remashree 1998).

Even though phenolic compounds from black pepper were reported by researchers, individual phenolic compounds were not explored much from other three *Piper* species. Salicylic acid, caffeic acid, protocatechuic acid, coumaric acid, ferulic acid, vanillic acid, syringic acid, gentisic acid, guaiacol, 4-hydroxybenzoic acid, synapic acid, kaempferol-3-O-β-glucoside, quercetin-3-O-β-galactoside, rhamnetin-O-triglucoside etc. were the phenolic compounds already reported in black pepper (Jagella & Grosch 1999; Variyar & Bandyopadhyay 1994; Swain *et al.* 1985; Parmar *et al.* 1997; Chatterjee *et al.* 2007). Gentisic acid, syringic acid, melilotic acid, protocatechuic acid, ferulic, rosamarinic acid and apigenin were the only phenolics known to have been reported in *P.longum* (Mammen & Daniel 2014; Chandra *et al.* 2015). As per avaliable literature, protocatechuic acid, caffeic acid, luteolin, quercetin, rosamarinic acid and apigenin were the only phenolic compounds identified from *P.chaba* fruits (Chandra *et al.* 2015). Likewise, phenolic acids like protocatechuic acid, ferulic acid, rosamarinic acid, vanillic acid and flavonoids like luteolin, kaempferol, quercetin and apigenin were identified from *P. colubrinum* fruits by Chandra *et al.* (2015). So, present study aimed to identify phenolic compounds from *P. nigrum, P. longum, P. chaba* and *P. colubrinum* by Liquid Chromatography Mass Spectrometry (LC-MS) analysis.

Materials and methods

Collection of samples

Matured and dried fruits/berries of high yielding black pepper (*P. nigrum*) variety Panniyur-1, *P. longum*, *P. chaba* and *P. colubrinum* were collected from ICAR-Indian Institute of Spices Research (ICAR-IISR) Experimental Farm, Peruvannamuzhi (Kerala, India) during the period of 2012-14 and used for the study.

Determination of total phenol content

All the samples were powdered and extracted with methanol. Methanolic extracts were then used for estimation of total phenolic content by Folin-Ciocalteau method (Malick & Singh 1980). In alkaline medium, phenolics reacts with phosphomolybdic acid component of Folin-Ciocalteau reagent and produce molybdenum blue and the resultant blue colour was measured at 650 nm. Mean value of three replications was taken for total phenolic content of selected samples. Data were combined and analyzed by analysis of variance (ANOVA). The ANOVA was performed with the MSTATC software (version 1.41). Significant difference ($p<0.05$) was estimated by Duncan's multiple range test (DMRT) using 'RANGE' procedure.

Extraction of phenolic compounds

Matured and dried berries/fruits of above selected *Piper* species were powdered and weighed (1g each) and subjected to acid digestion with HCl (2N) for 20 minutes on a water bath (100^0C). After cooling, the contents were filtered and the filtrate was extracted thrice with diethyl ether. The pooled ether layer was then extracted with anhydrous sodium carbonate (5%) solution. The pH of the collected sodium carbonate layer was adjusted to 3.0 using H_2SO_4 (5%). The acidified fraction was re-extracted with diethyl ether. After complete evaporation of diethyl ether, the residue was dissolved in methanol (1 mL) and stored in amber colored bottle at 4^0C until analysis (Bate-Smith 1954).

Liquid Chromatography Mass Spectrometry (LC-MS) analysis

LC-MS analysis was performed using Agilent 1290 Infinity UHPLC system coupled with an Agilent 6530 Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (MS/MS) with JetStream ESI ion source. The chromatographic separation of phenolic compounds was performed by injecting 1 μL of filtered sample in UHPLC system equipped with Zorbax Eclipse plus C-18 column (3.0 mm X 150 mm; particle size of 1.8 μm) at 40^0C. The mobile phase used for separation was 10 mM ammonium acetate in water (solvent A) and methanol (solvent B) in gradient mode with a flow rate of 0.5 mL min^{-1} and the chromatogram was recorded at 254 nm using UV-Visible detector. The separated compounds were then ionized using JetStream electron spray ionization (ESI) ion source in a negative ionization mode with a capillary voltage of 3.5 KV and fragment voltage of 160 V. The drying gas used was high purity nitrogen with a flow rate of 7.0 L min^{-1} and temperature of 350^0C. The nebulizer pressure was set at 20 psig. The sheath gas (N_2) flow rate was maintained at 11 L min^{-1} at a temperature of 400^0C. The mass analysis was performed in the range of 90-1700 m/z. The Q-TOF data obtained by MS and MS/MS was analysed using Mass Hunter software. Molecular Feature Extraction (MFE) algorithm and Metlin data base were used to identify the compounds and deduct their empirical formula. The prediction of the structure of individual compounds were based on the empirical formula generated, Molecular Structure Correlator (MSC) software tool (MS/MS fragmentation) and also based on literature survey. The mass accuracy of LC-MS data was <2ppm in most of the cases.

High Performance Liquid Chromatography (HPLC) analysis

HPLC analysis was performed using Shimadzu High Performance Liquid Chromatography (HPLC) equipped with SPD-10A UV-Visible detector. The column used was reverse phase C-18 with a size of 4.6 × 250 mm. Mobile phase was Acetic acid/water 5:95 (solvent A) and acetonitrile/water 40:60 (solvent B) with a flow rate of 1 mL min^{-1}. The measurement was taken at 280 nm after injecting 20 μL samples. Gradient programme: 100% A- 0 to 50 min; 100% B-50 to 55 min; 100% A- 55 to 60 min. HPLC analysis was carried out to confirm the compounds identified in LC-MS, with available standards (10 mg mL^{-1} methanol).

Results and discussion

Total phenol content of selected samples

The total phenol content estimated by Folin-Ciocalteau method varied from 0.42 to 5.1% (Fig. 1). It was found to be very high in *P. colubrinum* whereas low in black pepper (Panniyur-1), *P. longum* and *P. chaba* samples. Total phenol in

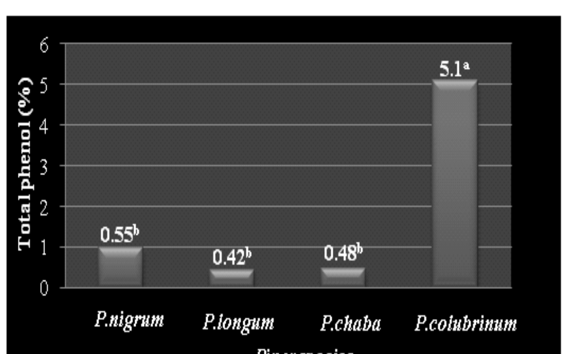

Fig. 1. Total phenol content of selected *Piper* species

dried berries of Panniyur-1 along with 25 different black pepper cultivars collected from Peruvannamuzhi and Panniyur (Kerala) were reported as 0.55 mg% by Zachariah *et al.* (2010). Total phenolic content (0.30 to 0.63%) were also reported for Panniyur-1 dried berries collected from eleven locations of India by Sruthi *et al.* (2013). Rami *et al.* (2013) reported total phenolic content of leaf, root and fruit of *P. longum* as 24.27± 0.008, 1.26±0.013 and 0.04±0.010 mg g^{-1}, respectively.

LC-MS analysis of Phenolic extracts

LC-MS analysis was performed for extracts and the possible phenolic compounds were identified based on their MS, MS/MS spectra, monoisotopic mass, generated empirical formula, predicted structures, Molecular Feature Extraction (MFE) algorithm, Metlin data base and also by literature survey (Figs. 2 to 5).

As illustrated in Table 1, thirteen compounds were identified in black pepper (Panniyur-1) extracts and which includes hydroxybenzoic acid, hydroxycinnamic acid and flavonoid category. Apart from this, other phenolic compounds like guaiacol and 4-hydroxymandelic acid were also identified from this extract. The compound 4-hydroxymandelic acid was detected in *P. nigrum* for the first time.

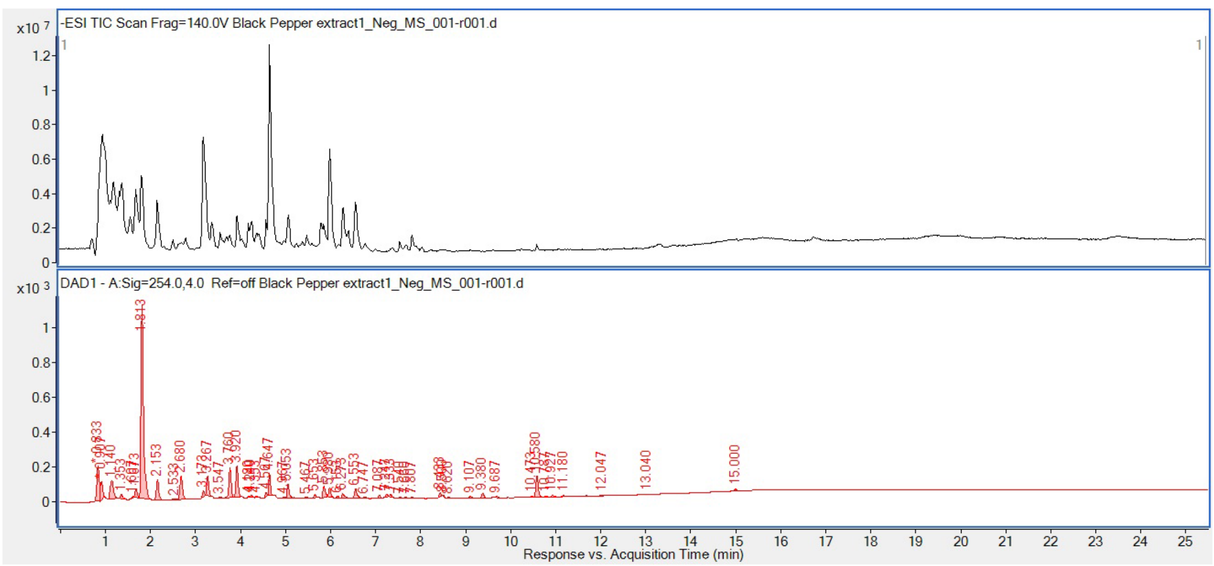

Fig. 2. LC-MS analysis: Overlaid chromatogram of UV at 254 nm and TIC for Panniyur-1 black pepper extract

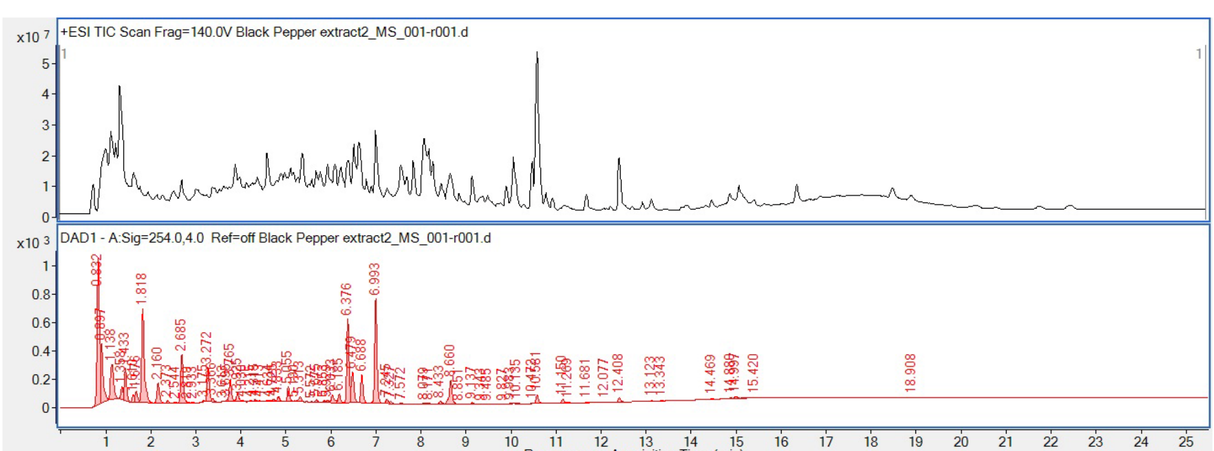

Fig. 3. LC-MS analysis: Overlaid chromatogram of UV at 254 nm and TIC for *P. longum* extract

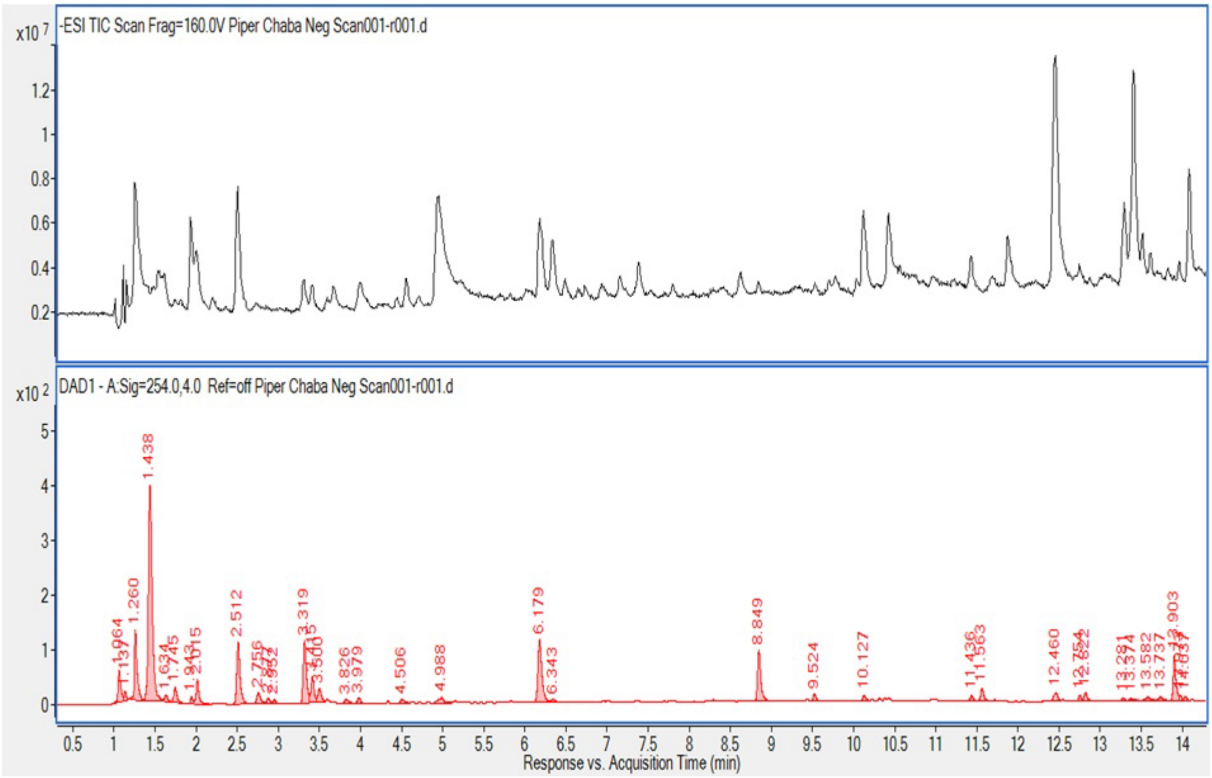

Fig. 4. LC-MS analysis: Overlaid chromatogram of UV at 254 nm and TIC for *P. chaba* extract

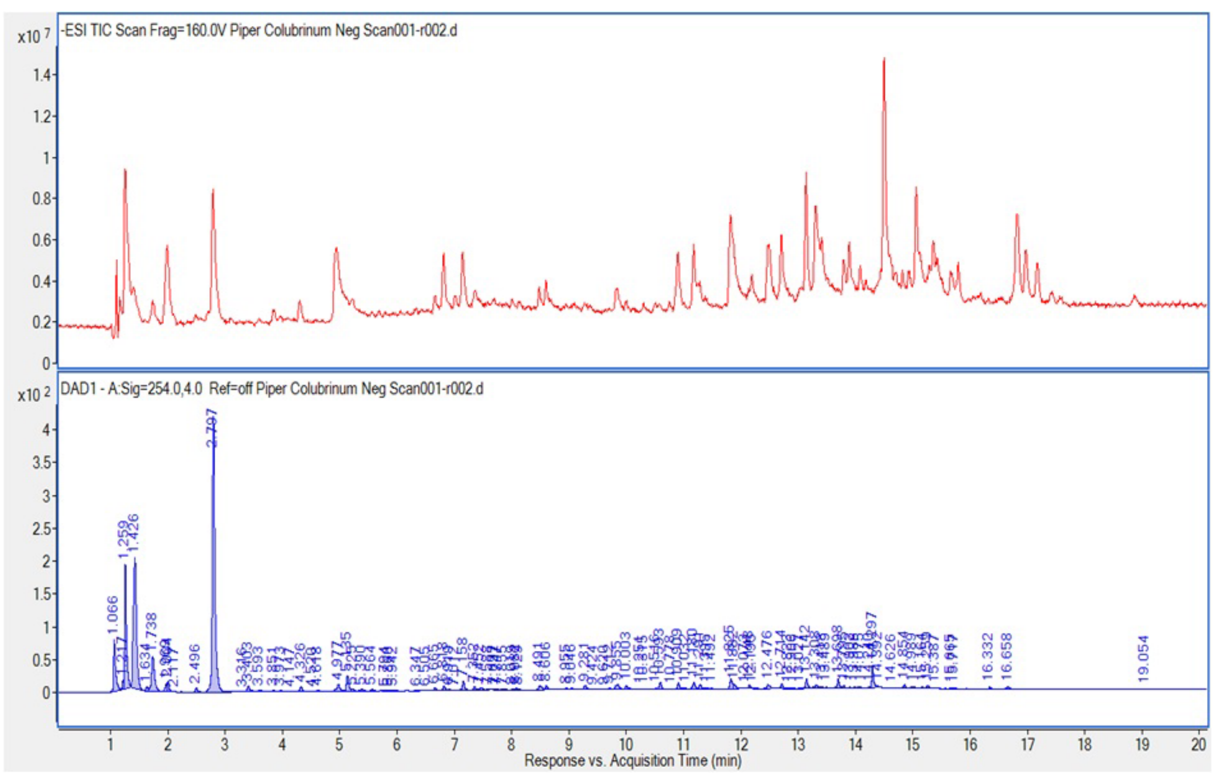

Fig. 5. LC-MS analysis: Overlaid chromatogram of UV at 254 nm and TIC for *P. colubrinum* extract

Table 1. Phenolic compounds identified from *P. nigrum* and *P. longum* by LC-MS

Compounds	$(M-H)^-$ (Da)	Monoisotopic mass (Da)	Ion Formula	Molecular Formula
P. nigrum (Panniyur-1)				
Guaiacol	123.0453	124.0524	$C_7H_7O_2$	$C_7H_8O_2$
Salicylic acid	137.0243	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
4-Hydroxybenzoic acid	137.0245	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
Gentisic acid	153.0194	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Protocatechuic acid	153.0195	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
4-Coumaric acid	163.0401	164.0473	$C_9H_7O_3$	$C_9H_8O_3$
Vanillic acid	167.0349	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
4-Hydroxymandelic acid	167.0351	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
Caffeic acid	179.0352	180.0423	$C_9H_7O_4$	$C_9H_8O_4$
Ferulic acid	193.051	194.0579	$C_{10}H_9O_4$	$C_{10}H_{10}O_4$
Syringic acid	197.0456	198.0528	$C_9H_9O_5$	$C_9H_{10}O_5$
Apigenin	269.0459	270.0528	$C_{15}H_9O_5$	$C_{15}H_{10}O_5$
Luteolin-8-C-glucoside	447.0936	448.1006	$C_{21}H_{19}O_{11}$	$C_{21}H_{20}O_{11}$
P. longum				
Salicylic acid	137.0243	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
2-pyrocatechuic acid	153.0194	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Gentisic acid	153.0195	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
4-Coumaric acid	163.0402	164.0473	$C_9H_7O_3$	$C_9H_8O_3$
Caffeic acid	179.0353	180.0423	$C_9H_7O_4$	$C_9H_8O_4$
Ferulic acid	193.0509	194.0579	$C_{10}H_9O_4$	$C_{10}H_{10}O_4$

Even though luteolin-8-C glucoside was detected for the first time in Panniyur-1 extract, luteolin 6-C-a-D-glucopyranoside-8-C-R-L-arabinopyranoside, luteolin 7-O-[2-(a-D-apiofuranosyl)-a-D-glucopyranoside-8-C-R-L-arabinopyranoside and luteolin 7-O-[2-(a-D-apiofuranosyl)-4-(a-D-glucopyranosyl) were reported by Al-Shahwany (2014). Salicylic acid, caffeic acid, protocatechuic acid, coumaric acid, ferulic acid, vanillic acid, syringic acid, gentisic acid, guaiacol, apigenin and 4-hydroxybenzoic acid were already reported in black pepper (Swain *et al.* 1985; Jagella & Grosch 1999; Chatterjee *et al.* 2007; Variyar & Bandyopadhyay 1994; Parmar *et al.* 1997; Chandra *et al.* 2015).

Out of six phenolic constituents identified in *P. longum*, gentisic acid was reported earlier by Mammen & Daniel (2014). They had also reported syringic acid, melilotic acid and apigenin from *P. longum* and revealed that no other flavonoid or phenolic acids were identified from any part of this plant. Later, Chandra *et al.* (2015) identified protocatechuic acid, ferulic acid, rosamarinic acid and apigenin from *P. longum* fruits. They have also reported caffeic acid (below detection limit) from this sample. Thus, in this study, salicylic acid, 4-coumaric acid and 2-pyrocatechuic acid have been reported for the first time in *P. longum* (Table 1).

Seven phenolic compounds were identified from *P. chaba* (Table 2) and except protocatechuic and caffeic acids, the remaining compounds were not reported earlier. Chandra *et al.* (2015) identified protocatechuic acid, caffeic acid, luteolin and quercetin from the fruit, ferulic and vanillic acids from the leaf, rosamarinic acid and apigenin from both fruit and leaf of *P. chaba.*

Table 2. Phenolic compounds identified from *P. chaba* and *P. colubrinum* by LC-MS

Compounds	$(M\text{-}H)^-$ (Da)	Monoisotopic mass (Da)	Ion Formula	Molecular Formula
P. chaba				
Salicylaldehyde	121.0297	122.0368	$C_7H_5O_2$	$C_7H_6O_2$
Salicylic acid	137.0247	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
Protocatechuic acid	153.0196	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Caffeic aldehyde	163.0404	164.0473	$C_9H_7O_3$	$C_9H_8O_3$
5-Methoxysalicylic acid	167.0353	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
Caffeic acid	179.0352	180.0423	$C_9H_7O_4$	$C_9H_8O_4$
Ferulic acid	193.0510	194.0579	$C_{10}H_9O_4$	$C_{10}H_{10}O_4$
P. colubrinum				
2-Pyrocatechuic acid	153.0199	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Salicylic acid	137.0247	138.0317	$C_7H_5O_3$	$C_7H_6O_3$
Protocatechuic acid	153.0194	154.0266	$C_7H_5O_4$	$C_7H_6O_4$
Kaempferol-5-glucoside	447.0934	448.1006	$C_{21}H_{19}O_{11}$	$C_{21}H_{20}O_{11}$
5-Methoxysalicylic acid	167.0355	168.0423	$C_8H_7O_4$	$C_8H_8O_4$
Apigenin-7-galactoside	431.0986	432.1056	$C_{21}H_{19}O_{10}$	$C_{21}H_{20}O_{10}$
5,7,2′,5′-Tetrahydroxy flavanone	287.0564	288.0634	$C_{15}H_{11}O_6$	$C_{15}H_{12}O_6$
Kaempferide-3-glucoside	461.1090	462.1162	$C_{22}H_{21}O_{11}$	$C_{22}H_{22}O_{11}$
Vanillin	151.0404	152.0473	$C_8H_7O_3$	$C_8H_8O_3$
Scutellarein-4′-methyl ether	299.0565	300.0634	$C_{16}H_{11}O_6$	$C_{16}H_{12}O_6$

Table 3. Common phenolic compounds in the selected *Piper* species

Compound	Species
Salicylic acid	All four species
Ferulic acid & Caffeic acid	*P. nigrum*, *P. longum* & *P. chaba*
Protocatechuic acid	*P. nigrum, P. chaba* & *P. colubrinum*
4-Coumaric acid & Gentisic acid	*P. nigrum* & *P. longum*
2-Pyrocatechuic acid	*P. longum* & *P. colubrinum*
5-Methoxysalicylic acid	*P. chaba* & *P. colubrinum*

Table 4. Phenolic compounds specific to each *Piper* species

P. nigrum	*P. longum*	*P. chaba*	*P. colubrinum*
4-Hydroxymandelic acid	—	Salicylaldehyde	Vanillin
4-Hydroxybenzoic acid		Caffeic aldehyde	Apigenin-7-galactoside
Vanillic acid			Kaempferol-5-glucoside
Syringic acid			Kaempferide-3-glucoside
Guaiacol			Scutellarein-4′-methyl ether
Luteolin-8-C glucoside			5,7,2′,5′-Tetrahydroxy flavanone
Apigenin			

Total of ten phenolic compounds were identified from *P. colubrinum* fruits (Table 2). Phenolic acids like protocatechuic acid, ferulic acid, rosamarinic acid, vanillic acid and flavonoids like luteolin, kaempferol, quercetin and apigenin were identified from *P. colubrinum* fruit by Chandra *et al.* (2015). They have also reported caffeic acid in below detection limit from this sample. Except these compounds identified by above researchers, all other phenolics listed for *P. colubrinum* fruit, in Table 2, is not reported earlier. Chandra *et al.* (2015) also identified protocatechuic acid, ferulic acid, caffeic acid, rosamarinic acid, vanillic acid and flavonoids like luteolin, kaempferol and apigenin from leaf of *P. colubrinum*. Leela & Pillai (2008) had reported two flavones *viz*., 5,4'-dihydroxy-7-methoxy-flavone and 5, 3'-4'-trihydroxy-7-methoxy-flavone from chloroform extract of *P. colubrinum* leaves.

The hydroxybenzoic acid, hydroxycinnamic acid, phenolic aldehydes and flavonoids were the main group of phenolic compounds identified from the selected *Piper* species. The hydroxybenzoic acid category includes salicylic acid, 4-hydroxybenzoic acid, 2-pyrocatechuic acid, protocatechuic acid, gentisic acid, vanillic acid and syringic acid. Salicylic acid, a monohydroxy benzoic acid was detected in all four phenolic extracts.

Ferulic acid, caffeic acid and 4-coumaric acid were the hydroxycinnamic acids identified in the selected phenolic extracts. Caffeic acid and ferulic acid were identified in black pepper, *P. longum* and *P. chaba* extracts whereas 4-coumaric acid was identified in black pepper and *P. longum*. However, the hydroxycinnamic acids were not detected in *P. colubrinum.*

The flavonoid aglycones and flavonoid glycosides were included in the flavonoid group of compounds identified from the selected *Piper* species. The luteolin-8-C glucoside (flavone glycoside) and apigenin (flavone) were identified in black pepper whereas apigenin-7-galactoside, (flavone glycoside), kaempferol-5-glucoside and kaempferide-3-glucoside (flavonol glycosides), scutellarein-4'-methyl ether and 5,7,2',5'-tetrahydroxy flavanone were identified in *P. colubrinum*. However, flavonoid compounds were not detected in *P. longum* and *P. chaba* extracts.

The identified phenolic aldehydes include caffeic aldehyde (*P. chaba*) and vanillin (*P. colubrinum*). The 4-hydroxymandelic acid, guaiacol, 5-methoxysalicylic acid and salicylaldehyde were the other phenolic compounds identified. Among these identified compounds, Table 3 illustrates common phenolic compounds and Table 4 illustrates those specific to each species.

HPLC analysis of phenolic extracts

HPLC analysis was performed to confirm the phenolic compounds identified by LC-MS, with authentic standards. The compounds in the extracts were confirmed by comparing their retention time with those of standards and also by peak enrichment. Thus, HPLC clearly established the presence of salicylic acid, 4-hydroxybenzoic acid, syringic acid, ferulic acid, 4-coumaric and caffeic acid in black pepper, salicylic acid, 4-coumaric acid, caffeic acid and ferulic acid in *P. longum,* salicylic acid, caffeic acid and ferulic acid in *P.chaba* and salicylic acid in *P. colubrinum.*

Even though LC-MS-Q-TOF showed the presence of those phenolic compounds, their molecular structure can be established further by additional techniques like NMR and IR. Some of these identified compounds are under explored for their medicinal values or well studied compounds are still unknown for certain medicinal properties. Thus, these findings may be a lead to researchers in the pharmacological field to extend their work on this natural phytochemicals.

Acknowledgements

Authors are grateful to DST (INSPIRE), New Delhi for funding, Director, ICAR-Indian Institute of Spices Research, Kozhikode for providing all the facilities and Agilent Technologies India Ltd, Bangalore for LC-MS analysis.

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