

Identification of Anthocyanin, Flavonoids, Triterpenoids and Phosphatidylcholines from *Rhododendron arboreum* by Using LC-ESI-MS/MS Analysis

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Abstract : *Rhododendron arboreum* (Sm.) is one of the most frequently distributed an evergreen small tree species at 1500-3300 m in Nepal. The flowers of *Rhododendron* are a promising source of bioactive constituents and could be used as functional food and edible colorants. The purpose of this study was to reveal the components of *R. arboreum* flowers by LC-MS/MS. Twelve compounds were identified by LC-ESI-MS/MS from the different crude fractions prepared by liquid-liquid extraction process. The MS/MS fragmentation patterns of each compound were determined and proposed. The isolated compounds belong to flavonoids, anthocyanins, triterpenoids and phosphatidylcholines. The major anthocyanin identified was cinnamtannin. The identified flavonoids are quercetin-3-*O*-glucoside, quercetin-3-*O*-pentoside, kaempferol-3-*O*-pentoside, quercetin and kaempferol. Ursolic acid represents triterpenoid derivative. 1-linoleoyl-sn-glycero-3-phosphoethanolamine, 1-linolenoyl-sn-glycero-3-phosphocholine, 1-linoleoyl-sn-glycero-3-phosphocholine, 1-Palmitoyl-sn-glycero-3-phosphocholine and 1-oleoyl-sn-glycero-3-phosphocholine belong to phosphatidylcholines. These phosphatidylcholines have been first time reported from the genus *Rhododendron*.

Keywords : *Rhododendron arboreum*, LC-ESI-MS/MS, Flavonoids, Anthocyanins and Phosphatidylcholines

Introduction

About 1000 species of *Rhododendron* are known to world while there are 31 species known in Nepal.¹ *Rhododendron arboreum* (Sm.) is one of the most frequently distributed species at 1500-3300 m. *R. arboreum*, an evergreen small tree, is one of the most popular species due to its beautiful bright red flower. It has been designated as the national flower of Nepal. The flowers bloom in a huge quantity at March/April once a year in the mountains of Nepal. The flowers have a sweet-sour taste and are used by local people for preparing jellies, local brews, pickles, chut-

ney, and jam. *Rhododendrons* have been used in Asian, North American and European traditional medicine mainly against inflammation, pain, skin ailments, common cold and gastro-intestinal disorders.^{2,3} The flowers of *R. arboreum* are one of the major components in the Ayurvedic formulations such as Asoka Aristha and Rohitaka.^{4,6}

The flowers are a promising source of bioactive constituents and could be used as functional food and edible colorants. The use of HPLC coupled with MS is extremely valuable as a screening tool for study and analysis of metabolites of complex matrix. LC-MS analysis of the genus *Rhododendron* revealed that anthocyanins and flavonoids are the major constituents of flowers. Besides, the phenolics and triterpenoids constituents are also reported.⁷⁻⁹ The major anthocyanidins are cyanidin, peonidin, delphinidin, petunidin, malvidin, and pelargonidin derivatives. Most of these present in *Rhododendron* as 3-monoglycosides or 3,5-diglycosides. The main flavonoids of *Rhododendron* petals are quercetin, kaempferol, and myricetin, though many others exist. The profiling of *R. arboreum* using UPLC-DAD-ESI-QTOF-MS is reported by Bhatt et al. 2022.¹⁰ Constituents including anthocyanins, phenolic acids, and flavonoids were identified in the flower extract.¹¹ The HPLC-DAD-ESI-MS² study showed the composition

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of anthocyanin and flavonoids among the petals of the different *Rhododendron* species are markedly different. It is reasonable to assume that qualitative and quantitative changes in these pigment compositions of petals are responsible for the variety of flower colors observed in *Rhododendron*.¹²⁻¹⁴ Proanthocyanins, flavonoid-*O*-glycosides and hydroxycinnamates were extracted and analyzed from the leaves of different *Rhododendron* species by LC-ion trap mass spectrometer.^{15,16} Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids in biological membranes. The relative abundance of PC and PE regulates lipid, lipoprotein and energy metabolism.¹⁷ These phosphatidylcholines have been not reported from the genus *Rhododendron*.

In the present study, we describe an application of HPLC-ESI-MS/MS with ion trap detector to determine the structures of different metabolites present in *Rhododendron* extracts. Phytochemical investigations of *Rhododendron* flowers by LC-ESI-MS/MS revealed that anthocyanins, flavonoids, triterpenoids and phosphatidylcholines are the major constituents. The MS/MS fragmentation patterns of compounds for its identification are discussed in this paper.

Experimental

Collection of samples

Rhododendron arboreum (Sm.) was collected from the central hilly region (Phulchoki), Nepal at the heights of 1500 to 2000 m in the months of April 2022. Plant materials were authenticated by comparison with voucher specimen deposited at National Herbarium and Plant Laboratories, Godawari, Lalitpur, Nepal.

Chemicals and equipments

Analytical grade solvents (Fisher Scientific) were used for extraction and HPLC grade solvents (Aldrich-Sigma, USA) were used for chromatographic analysis. The separation and analysis of metabolites were performed on a hyphenated HPLC-ESI-MS/MS. The HPLC system employed consisted of an Analytical HPLC (Thermo/Dionex) coupled to amazon speed ion trap low-resolution mass spectrometer (Bruker Daltonics). Nitrogen was used as the nebulizing gas at a pressure of 20 psi and a flow rate of 6 L/min. The heated capillary and the voltage were maintained at 180°C and 4.5 KV, respectively. Full scan MS of the compounds were measured from m/z 100 to 3000 amu. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas. The collision energy was set at 100%. MS data were acquired in the positive and negative ionization mode. MS/MS data were acquired in full-scan mode and auto-MSⁿ mode was used to acquire the fragments. The data was acquired in compass hystar and trap control. The acquired data were analysed with Bruker compass data analysis (4.2).

Chromatographic separations were carried out on Agilent

porosil EC-18 MS column (2.7 μ m x 1.8 mm x 50 mm). The mobile phase comprised two solvents, namely, 0.1% Formic acid in ACN (Solvent A) and 0.1% Formic acid in H₂O (solvent B). In a gradient program which was initially 95:5 (B:A) and changed to 80:20 (B:A) at 1 min, 70:30 (B:A) at 2 min, 60:40 (B:A) at 3 min, 50:50 (B:A) at 8 min, 40:60 (B:A) at 10 min, 20:80 (B:A) at 13 min, 10:90 (B:A) at 16 min, and finally 95:5 (B:A) at 23 min. The flow rate was 0.3 mL/min, and the sample injection volume was 10 μ L.

Extraction and fractionation

Air dried *R. arboreum* flowers (1.5 Kg) were crushed into the powder before extraction. These powdered samples were extracted by methanol by percolation to get the crude methanolic extract (141.65 g, ME). The crude extract was suspended in Milli-Q water and then further successively fractionated in different solvents by liquid-liquid extraction process to obtain 12.30 g hexane (Hex), 7.66 g dichloromethane (DCM), 12.48 g ethyl acetate (ETY), 16.22 g *n*-butanol (BU) and 85.01 g water (H₂O) extracts.

Results

LC-ESI MS conditions and analysis

Metabolite profiles of *R. arboreum* were qualitatively analyzed using LC-ESI-MS/MS approach. The compounds were characterized on the basis of their retention times, and their MS spectra. All the data discussed here have been analyzed in the both positive and negative ionization mode in an ion trap mass spectrometer. The precursor ions of compounds are fragmented into daughter ions and are characteristic to the compounds and used for the complete or partial identification of the compound structure. LC-ESI-MS/MS based identification of the metabolites was assured using proposed fragmentation pattern at low resolution and a high resolution spectrometer would have been more suitable for the further studies.

For further qualitative analysis, the crude methanolic extract was separated by liquid-liquid extraction process as mentioned in the experimental section. The chromatograms of *R. arboreum* crude extract and different fractions are presented in Figure 1. There are significant differences in metabolites for the different fractions (Supplementary Figure S1-S38). The compounds peaks at m/z 163 [M+H]⁺, 478 [M+H]⁺, 518 [M]⁺ and 520 [M]⁺ eluted at 1.67, 14.77, 16.05 and 17.44 min respectively, were common to all fractions. The methanolic extract showed characteristic intense peaks for flavonoids at m/z 449 [M+H]⁺, 433 [M+H]⁺, 303 [M+H]⁺ and 287 [M+H]⁺ at 5.56, 5.92, 6.84 and 8.05 min, respectively.

The numbers of metabolites detected in ESI⁻ mode are considerably different than in ESI⁺ mode (Supplementary Figure S39-S66). The chromatograms in negative ionization mode are shown in supplementary figures (Figure S67). The complementary [M-H]^{-ve} peaks at m/z 501 (9.3 min), m/z 697 (12.73 min), m/z 542 (13.80 min), m/z

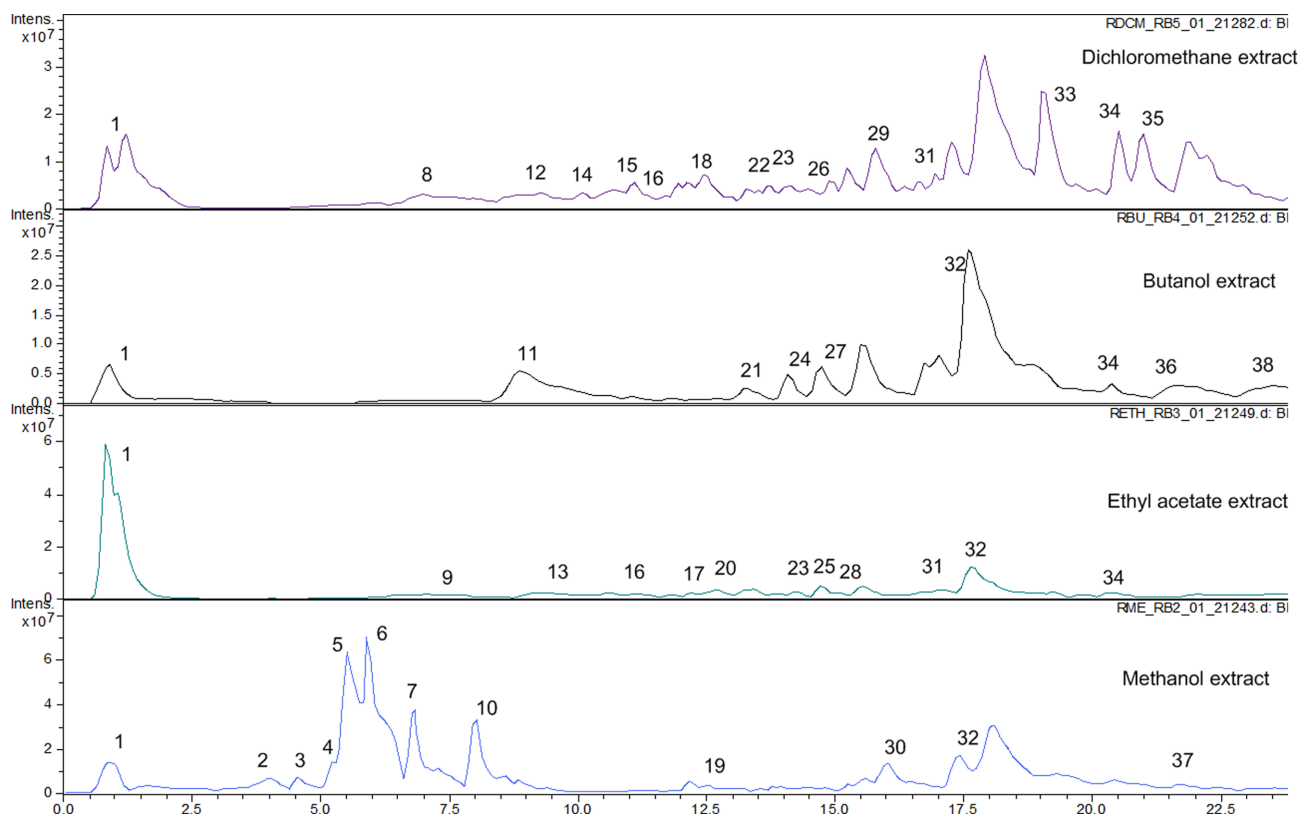


Figure 1. Stack chromatograms of methanol, ethyl acetate, butanol and dichloromethane extracts of *Rhododendron arboreum* in ESI+ mode.

476 (14.77 min) and m/z 556 (15.30 min) in ethyl acetate, dichloromethane and butanol fractions further confirms the calculated molecular weight (Supplementary Table S1). $[2M+H]^+$ adducts formed during ESI ionization at m/z 1397 (12.73 min), m/z 955 (14.77 min), and m/z 1269 (15.11 min) assist to confirm the calculated molecular weight.

Total twelve compounds were identified from different fractions as shown in Table 1. The identified compounds belong to flavonoids, anthocyanins, triterpenoids and phosphatidylcholines derivatives and their corresponding chemical structures are shown in Figure 2. The anthocyanin identified was cinnamtannin (3) (procyanidin trimer). The main flavonoids of *Rhododendron* petals are quercetin-3-*O*-glucoside (4), quercetin-3-*O*-pentoside (5), kaempferol-3-*O*-pentoside (6), quercetin (7) and kaempferol (10). 1-linoleoyl-sn-glycero-3-phosphoethanolamine (27), 1-linolenoyl-sn-glycero-3-phosphocholine (30), 1-linoleoyl-sn-glycero-3-phosphocholine (32), 1-Palmitoyl-sn-glycero-3-phosphocholine (36) and 1-Oleoyl-sn-glycero-3-phosphocholine (38) belong to phosphatidylcholines. Ursolic acid (37) is triterpenoid.

Identification of compounds

All the data discussed here, have been analyzed in the positive mode in the MS/MS system. In an ion trap mass spectrometer, the precursor ions of compounds are frag-

mented into daughter ions and neutral losses. The tandem MS spectra and their compound structures with proposed fragmentation schemes are shown for these compounds. The fragmentation is characteristic to the compounds and can be used for the complete or partial identification of the compound structure.

Mass fragmentation of cinnamtannin

A peak at t_R 4.61 min with m/z 865 was speculated to be $[C_{45}H_{37}O_{18}]^+$ for cinnamtannin (3). It relates to A type procyanidin trimer which has already been reported from *Rhododendron*.¹⁸

Mass fragmentation of quercetin derivatives

Quercetin-3-*O*-glycoside (4) at t_R 5.24 min were assigned to the peaks with $[C_{21}H_{22}O_{12}]^+$ and m/z 465 $[M+H]^+$. A intense peak at t_R 5.56 min with m/z 449 $[M+H]^+$ and $[C_{21}H_{21}O_{11}]^+$ was assigned as quercetin-3-*O*-pentoside (5) in the methanolic fraction. Quercetin (7) m/z at 303, was eluted at 6.84 min. Quercetin derivatives were identified based on their common characteristic molecular ion peaks. The compounds produced secondary peak of m/z 303 by cleavage of glycosyl moiety in MS². The peaks at m/z 285 $[M+H-H_2O]$ and m/z 257 $[M+H-H_2O-CO]$ are common characteristics fragments of flavonoids. The peak at m/z 165 is $^{0.2}A^+$ ring cleavage (Figure 3).¹⁹

Table 1. Chromatographic and mass spectrometric data of *R. arboreum* in ESI⁺ mode

Ret. time	Mol. wt	Measured mol. Wt.	Product ions and percentage intensity	Identification of compounds	ME	ETY	BU	DCM
			2M+H					
1	1.67	162	163	-	145 (100)	-	+	+
2	4.08	354	355	-	163 (100), 145 (8)	-	+	
3	4.61	864	865	-	865 (100)	Cinnamtannin	+	
4	5.24	464	465	-	303 (100), 257(20), 165 (20)	Quercetin-3- <i>O</i> -glycoside	+	
5	5.56	448	449	-	303(100), 257 (15), 165 (15)	Quercetin-3- <i>O</i> -pentoside	+	
6	5.92	432	433	-	287(100), 241(12), 165 (10)	Kaempferol-3- <i>O</i> -pentoside	+	
7	6.84	302	303	-	303 (100), 257(22), 165 (20)	Quercetin	+	
8	7.01	290	291	-	273 (100), 227 (22), 145 (20)	-		+
9	7.04	502	503	-	455 (100), 187 (15)	-	+	
10	8.05	286	287	-	287 (100), 241(12), 165 (10)	Kaempferol	+	
11	8.89	268	269	-	267 (30), 213 (100), 199 (22), 159 (20)	-		+
12	9.30	502	503	-	455 (100), 467(55), 421(55)	-		+
13	9.33	452	453	-	435 (40) 407 (100)	-	+	
14	10.10	314	315	-	300 (100), 241(5), 199 (5)	-		+
15	11.10	284	285	-	217 (65), 235 (100)	-		+
16	11.28	516	517	-	471(100), 499 (85)	-	+	+
17	12.23	345	346	-	311(100), 265 (20)	-	+	
18	12.50	278	279	-	261(100), 243 (52), 165 (45)	-		+
19	12.56	315	316	-	297(100), 123 (10)	-	+	
20	12.73	698	699	1397	700 (100), 411(45), 243(40)	-	+	
21	13.25	452	453	-	435 (35), 407 (100)	-		+
22	13.30	618	619	-	601(10), 445 (45), 362 (100), 258 (75)	-		+
23	13.80	543	544	-	382 (100) 363 (15)	-	+	+
24	14.13	578	579	-	561(40), 337 (100)	-		+
25	14.28	531	532	-	497 (10), 453(15), 353(100), 261(50)	-	+	
26	14.50	350	351	-	277 (100), 179 (55)	-		+
27	14.77	477	478	955	460 (100), 417 (8), 337 (75), 306 (15)	1-Linoleoyl-sn-glycero-3-phosphoethanolamine	+	+
28	15.11	634	635	1269	453 (100), 407 (70), 636 (70)	-	+	
29	15.30	557	558	-	396 (100)	-		+
30	16.05	518	518	-	500 (100), 184(65)	1-Linolenoyl-sn-glycero-3-phosphocholine	+	+
31	17.0	384	385	-	368 (40), 256 (20), 130 (100)	-	+	+
32	17.44	520	520	-	502 (40), 184 (100)	1-linoleoyl-sn-glycero-3-phosphocholine	+	+
33	19.10	354	355	-	263(100), 245 (85), 337 (70)	-		+
34	20.39	312	313	-	312 (75), 257 (100)	-	+	+
35	21.0	356	357	-	339 (100), 265 (99)	-		+
36	21.65	496	496	-	478 (100), 184 (60)	1-Palmitoyl-sn-glycero-3-phosphocholine		+
37	21.83	456	457	-	439 (100), 411(30)	Ursolic acid	+	+
38	23.65	522	522	-	504 (100), 184 (60)	1-Oleoyl-sn-glycero-3-phosphocholine		+

Note: ME (methanolic extract), ETY (ethyl acetate), BU (*n*-butanol), DCM (dichloromethane)

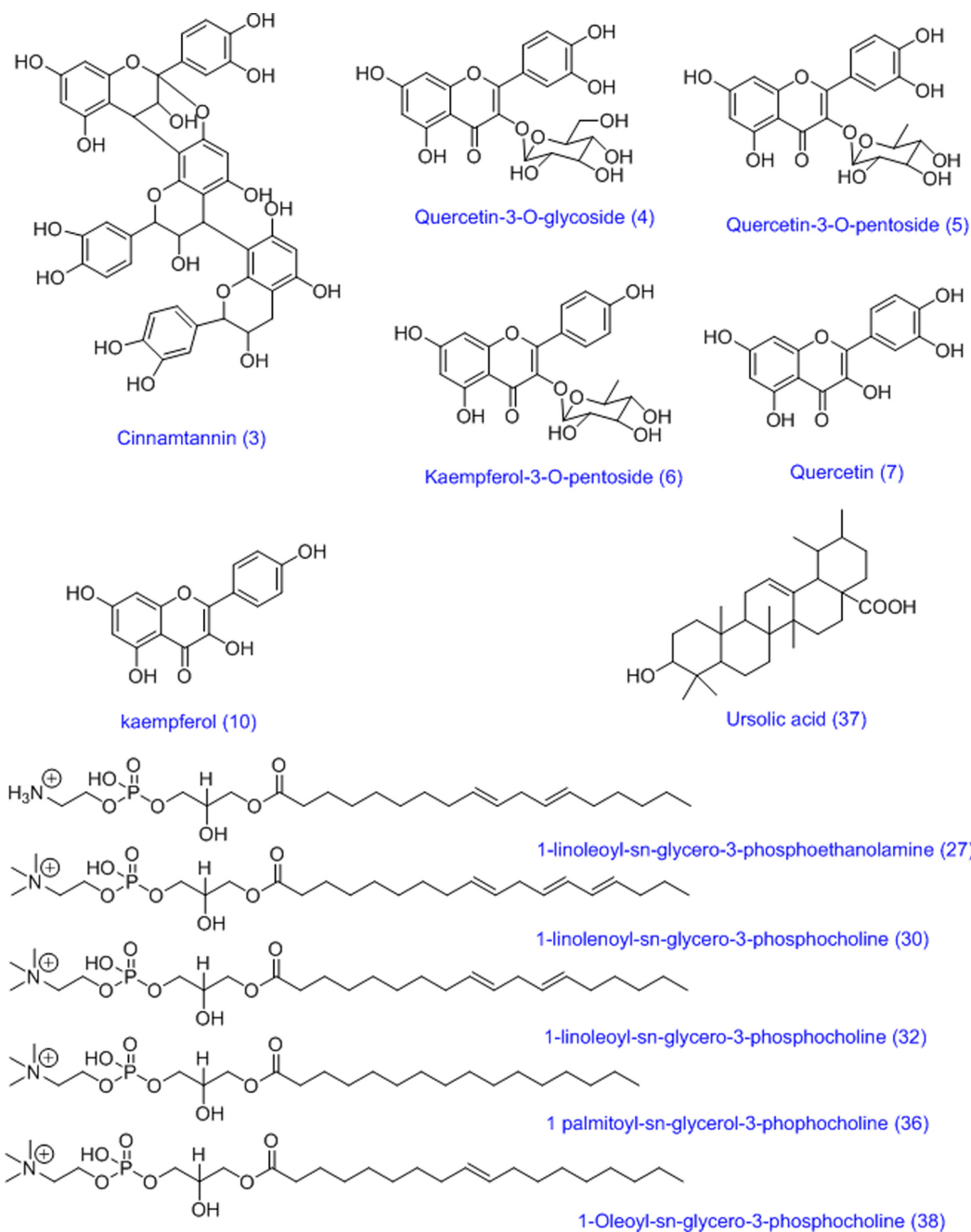


Figure 2. Structures identified from petals of *R. arboretum* by LC-ESI-MS/MS.

Mass fragmentation of kaempferol derivatives

Another intense peak m/z 433 $[M+H]^+$ for $[C_{21}H_{21}O_{10}]^+$ in the methanolic extract at 5.92 min was assigned to kaempferol-3-*O*-pentoside (6). Kaempferol (10) at t_R 8.05 min were assigned to the peaks with $[C_{15}H_{11}O_6]^+$ and m/z 287 $[M+H]^+$. The MS² fragmentation pattern showed peak at m/z 287, by cleavage of pentose moiety, m/z 241 $[M+H-H_2O-CO]$ by loss of water and carbonyl group and m/z 165 $^{0.2}A^+$ cleavage (Figure 4).^{19,20}

Mass fragmentation of 1-linoleoyl-sn-glycero-3-phosphoethanolamine (27)

1-Linoleoyl-sn-glycero-3-phosphoethanolamine, m/z 478 $[M+H]^+$ was eluted at 14.77 min. The MS² spectra showed peaks at m/z 460 $[M+H-H_2O]^+$. The peaks at m/z 417 and m/z 337 are due to consecutive loss of C_2H_6NO and $C_2H_8NO_4P$ (Figure 5).²¹

Mass fragmentation of phosphocholine derivatives

1-Linolenoyl-sn-glycero-3-phosphocholine (30), m/z 518

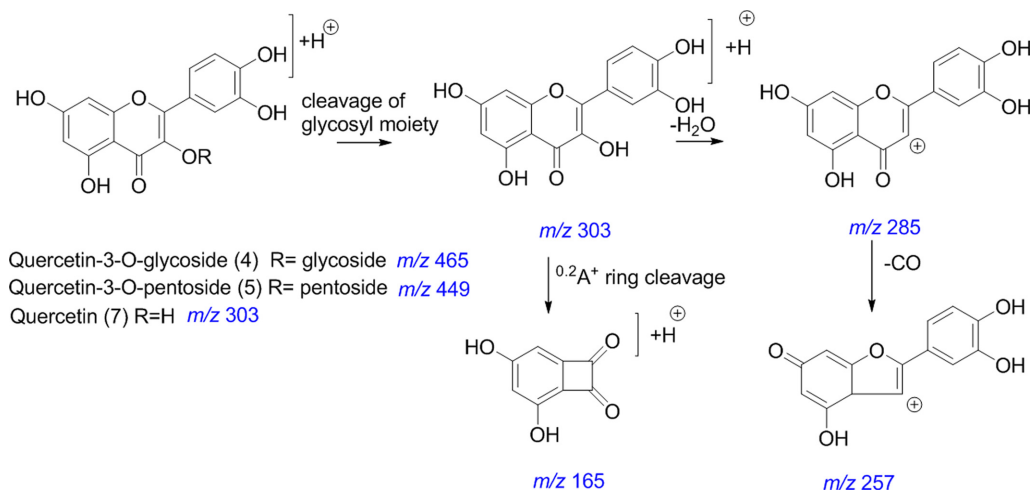


Figure 3. Scheme for mass fragmentation of quercetin derivatives.

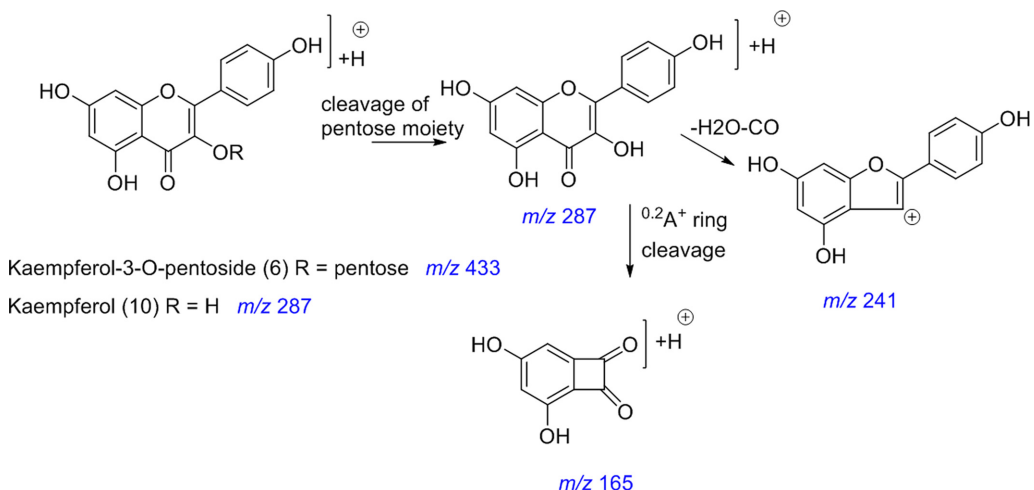


Figure 4. Scheme for mass fragmentation of kaempferol derivatives.

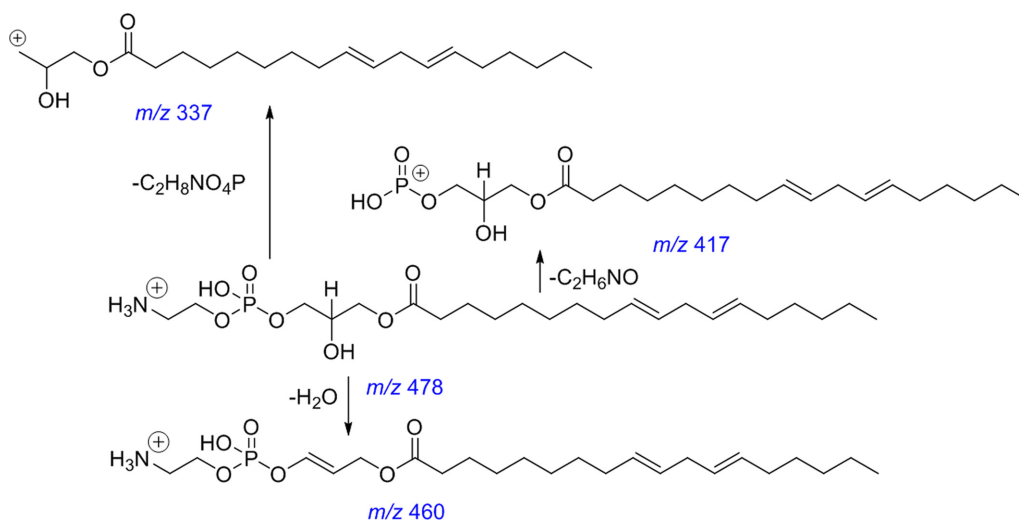


Figure 5. Scheme for mass fragmentation of 1-linoleoyl-sn-glycero-3-phosphoethanolamine.

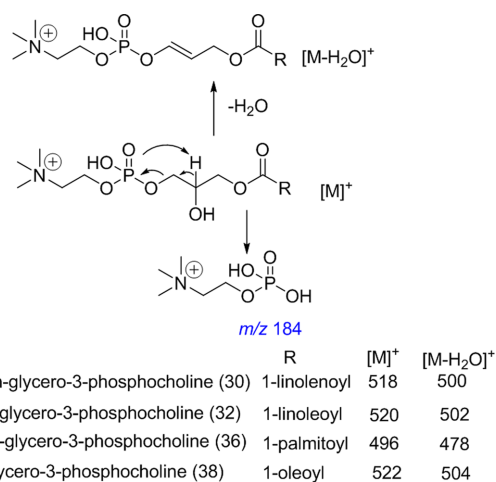


Figure 6. Scheme for mass fragmentation of phosphocholine derivatives.

[M]⁺, 1-Linoleoyl-sn-glycero-3-phosphocholine (32), *m/z* 520 [M]⁺, 1-Palmitoyl-sn-glycero-3-phosphocholine (36) *m/z* 496 [M]⁺ and 1-Oleoyl-sn-glycero-3-phosphocholine (38), *m/z* 522 [M]⁺ were eluted at 16.05 min, 17.44 min, 21.65 min and 23.65 min, respectively. Phosphocholine contains a quaternary nitrogen with a fixed positive charge and form positive ions by electrospray ionization (ESI). The [M]⁺ ions of phosphocholine yield a prominent ion at *m/z* 184, representing a phosphocholine ion. Mass peaks at *m/z* 500, *m/z* 502, *m/z* 478 and *m/z* 504 by loss of water molecule in 30, 32, 36 and 38 respectively. The fragmentation pattern to these phosphocholine derivatives were similar and its mass difference is speculated due to unsaturation and carbon chain (Figure 6).^{21,22}

Mass fragmentation of ursolic acid (37)

A peak at *t_R* 21.83 min with *m/z* 457 [M+H]⁺ was assigned to ursolic acid. The peak at *m/z* 439 in MS² spectra is due to loss of water molecule and peak at *m/z* 411 was observed due to loss of CO₂ moiety (Figure 7).²³

Discussions

Regarding these compounds, the flavonoids are commonly used as the natural food pigments. In humans, these compounds are related to a large range of health benefits arising from their bioactive properties, such as anti-inflammatory, anticancer, antiaging, cardio-protective, neuroprotective, immunomodulatory, antidiabetic, antibacterial, antiparasitic, and antiviral properties. Furthermore, glycosylation of flavonoid compounds increases their solubility in water, which results into increase bioavailability to improve their pharmaceutical properties. Quercetin-3-*O*-glucoside has been shown to possess anti-proliferative, hyperglycemia and antioxidant activities.²⁴ Phosphatidyl-

choline (PC) and phosphatidylethanolamine (PE) represent a major lipid of the outer envelope membrane and act as signaling molecules in a wide range of physiological and pathological events including inflammation, reproduction, nervous and vascular system development and carcinogenesis. The relative abundance of PC and PE regulates the size and dynamics of lipid droplets in mitochondria and changes in the PC and PE content of various tissues are implicated in metabolic disorders such as atherosclerosis, insulin resistance and obesity.²⁵ The flowers of *Rhododendron* thus showed a promising source of bioactive constituents.

Conclusion

Flavonoid and anthocyanins have been reported by different authors. These classes of compounds should be further studied for development of colour pigments. Phosphatidylcholines compounds have not been previously reported from *Rhododendron* species. Further isolation by column chromatography and identification by various NMR techniques will support for detail verification.

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