HPLC–QTOF–MS/MS-based rapid screening of phenolics and triterpenic acids in leaf extracts of *Ocimum* species and their interspecies variation

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**ABSTRACT**

Species of genus *Ocimum* are traditionally used for their medicinal and flavoring properties. These are rich sources of essential oils and found as an ingredient in many Ayurvedic preparations and food products. Phenolics and triterpenic acids are the medicinally active compounds mainly concentrated in the leaves of *Ocimum* species. This study aimed to develop an efficient and reliable analytical method for the rapid screening and characterization of phenolics and triterpenic acids in the leaf extracts of 6 *Ocimum* species using high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (HPLC–ESI–QTOF–MS/MS). A total of 50 compounds were identified and characterized on the basis of their accurate MS and MS/MS information, out of which 23 compounds were confirmed by authentic standards. Identified compounds include 28 flavonoids, 4 propenyl phenol derivatives, 2 triterpenic acids, 11 phenolic acids, and 5 phenolic acid esters. The developed method was applied to study the interspecies variation of identified compounds. Significant variation in the distribution of identified phenolics and triterpenic acids was observed among studied *Ocimum* species. Hence, the established method provides an effective and reliable tool for screening and characterization of phytocannabinoids in *Ocimum* species.

**KEYWORDS**

HPLC–QTOF–MS/MS; *Ocimum* species; phenolics; screening; triterpenic acids

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Introduction

Species of genus *Ocimum* belonging to family Lamiaceae are popularly known as basil. These are native to the tropical and warm temperate regions and extensively found in Asia, Africa, and Central and South America. Several species belonging to genus *Ocimum* are used in traditional medicine or as a spice in Indian traditional cuisine. Essential oils are the volatile liquid of aroma compounds extracted from the leaves and flowering tops of basil. These essential oils are used as flavoring or fragrance agent in foods and beverages and also in the pharmaceutical, perfumery, and cosmetic industries.

The six species of *Ocimum* genus, namely *Ocimum americanum* L. (syn. *Ocimum canum* Sims), *Ocimum basilicum* L., *Ocimum gratissimum* L., *Ocimum kilimandscharicum* Baker ex Gürke, *Ocimum sanctum* L. (syn. *Ocimum tenuiflorum* L.) green and *Ocimum sanctum* L. purple, are commonly found in India. Among these, *O. sanctum* (holy basil) is widely grown in India as a folk medicine and sacred plant, while *O. basilicum* (sweet basil) as a culinary and ornamental herb. The different plant parts such as leaves, roots, and flowering tops of *Ocimum* species are used as a traditional remedy for nausea, flatulence, cold, dysentery, mental fatigue, spasm, rhinitis, arthritis, malaria, diarrhea, skin infections, conjunctivitis, bronchitis, lowering blood sugar level, and healing wounds. The basil tea is consumed for treating coughs, headaches, and kidney malfunction to eliminate toxins and infections of upper respiratory tract.

Principle chemical constituents reported from *Ocimum* species are triterpenic acids and phenolic compounds including phenolic acids, propenyl phenols, and flavonoids. These compounds have been attributed to various pharmacological activities of *Ocimum* species such as anticancer, anti-inflammatory, antiulcer, antimicrobial, cardioprotective, radioprotective, gastroprotective, antioxidiant, and antistress.

*Ocimum* species are characterized by variations in their morphology such as leaf shape, size, and pigmentation, which cause differences in chemical composition and affect the commercial value of this genus. Therefore, an efficient and reliable method is required for rapid screening of phenolics and triterpenic acids in leaf extracts of *Ocimum* species and to study their interspecies variation.

Various analytical methods such as high-performance liquid chromatography (HPLC), high-performance thin layer chromatography (HPTLC), gas chromatography–mass spectrometry (GC–MS), attenuated total reflectance/Fourier transform infrared (ATR/FTIR), FT-Raman, and near infrared (NIR) spectroscopy have been reported for phytochemical analysis of *Ocimum* species. These reported methods suffer from various drawbacks such as low sensitivity, low resolution, high solvent consumption, long analysis time, and need of derivatization.

Recently, liquid chromatography–tandem mass spectrometric (LC–MS/MS) techniques have demonstrated to be more effective for rapid screening and characterization of plant metabolites than above-mentioned methods due to their high sensitivity, selectivity, specificity, and shorter analysis time. So far, there are few LC–MS/MS methods reported using triple quadrupole and ion trap mass spectrometers for the phytochemical analysis of *Ocimum* species but these are mainly focused on the screening of flavonoids. Previously, we have done quantification of targeted 16 marker compounds in the leaf extracts of 6 *Ocimum* species and marked herbal formulations of *O. sanctum* using UHPLC–QqQ–MS/MS.

As the reported LC–MS/MS methods have limited data and also these are based on the application of low-resolution mass analyzers, e.g., triple quadrupole/hybrid linear ion trap triple quadrupole and ion trap mass spectrometers that ensure excellent selectivity and sensitivity for quantitative analyses targeted analytes but give nominal mass and so may not be effective in untargeted metabolite profiling/screening.

On the other hand, high-resolution mass analyzer time-of-flight (TOF) has better accuracy, it provides accurate precursor and product ions information and molecular formula with mass error < 5 ppm, which greatly enhances the metabolite characterization reliability, especially when standard compounds are not available. Therefore, there is a need to develop a versatile high-resolution LC–TOF–MS/MS method for screening and characterization of multiclass phytoconstituents of *Ocimum* species.

Hence, the goal of the present work is to develop an efficient and specific HPLC–ESI–QTOF–MS/MS method for rapid screening and characterization of triterpenic acids and phenolics including flavonoids, propenyl phenol derivatives, phenolic acids, and their esters in leaf extracts of 6 *Ocimum* species, viz. *O. americanum*, *O. basilicum*, *O. gratissimum*, *O. kilimandscharicum*, *O. sanctum* “green,” and *O. sanctum* “purple.” The developed method is also applied to study the interspecies variation of identified compounds.

Experimental procedure

Reagents, chemicals, and plant materials

Acetonitrile, methanol (LC–MS grade), and formic acid (analytical grade) used in the mobile phase and sample preparation throughout the LC–MS analysis were purchased from Fluka, Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water prepared using a Direct-Q 8 UV water purification system (EMD Millipore Corporation, Billerica, MA, USA) was used throughout the analysis. The reference standards (purity ≥ 97%) of caffeic acid, ferulic acid, sinapinic acid, p-coumaric acid, vanillic acid, syringic acid, ursolic acid, isoorientin, orientin, isovertexin, vitexin, apigenin, kaempferol, eugenol, methyl eugenol were purchased from Sigma–Aldrich (St. Louis, MO, USA). The reference standards (purity ≥ 95%) of gallic acid, protocatechuic acid, chlorogenic acid, rosmarinic acid, rutin, kaempferol-3-O-rutinoside, quercetin and luteolin were purchased from Extrasynthese (Z.I Lyon Nord, Genay Cedex, France).

The plant materials (leaves of *O. americanum*, *O. basilicum*, *O. gratissimum*, *O. kilimandscharicum*, *O. sanctum* green, and *O. sanctum* purple) were collected from Nauni, Solan,
Himachal Pradesh, India. Voucher specimens of *O. americanum*-8878 (1), *O. basilicum*-8879 (2), *O. gratissimum*-13422 (3), *O. kilimandscharicum*-8869 (4), *O. sanctum* green-11602 (5), and *O. sanctum* purple-8871 (6) have been deposited in the herbarium (HPL-218-22) of Department of Forest Products, Dr Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India.

**Extraction and sample preparation**

The leaves of selected *Ocimum* species were air dried at room temperature and ground into powder with 40 mesh. Aqueous methanol (80%) was selected for extraction process due to its high efficiency in extracting phenolics and triterpenic acids from plant samples.\(^{[51,52]}\) The dried powder (20 g) of the leaves of each species was extracted with 200 mL of 80% aqueous methanol in an ultrasonic water bath (Fisherbrand FB15067, Fisher Scientific, UK) for 30 min and left for 24 h at room temperature (22–24°C). Three replicates of the extraction process were carried out on each sample. The extract was filtered through Whatman filter paper and concentrated under reduced pressure (20–50 kPa) using a rotary evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at 40°C. Dried residues (1 mg) were weighed accurately, dissolved in 1 mL of methanol, and sonicated using an ultrasonicator (Bandelin SONOREX, Berlin, Germany). The solutions were filtered through 0.22 µm syringe filter (Millex-GV, PVDF, Merck Millipore, Darmstadt, Germany). The filtrates were diluted with acetonitrile to final working concentration for analysis. A mixed standard stock solution (1 mg/mL) of 23 reference standard, and sample solutions were stored at 20°C until use and vortexed before injection.

**Chromatography**

The HPLC analyses were performed on a HPLC instrument of Agilent 1200 series (Agilent Technologies, USA), composed of a quaternary pump (G1311A), online vacuum degasser (G1329A), auto sampler (G1329A), thermostatted column compartment (G1316C), and diode array detector (G1315D). The HPLC separation was accomplished on a Thermo Betasil C8 column (250 mm × 4.6 mm id, 5 µm) operated at 25°C. A gradient elution was achieved using two solvents: 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The 65 min HPLC gradient elution program was as follows: 25–55% (B) from 0–15 min, 55–55% (B) from 15–25 min, 55–65% (B) from 25–30 min, 65–75% (B) from 30–40 min, 75–90% (B) from 40–59 min, 90–25% (B) from 59–65 min, and equilibration time 5 min. The sample injection volume was 4 µL.

**Mass spectrometry**

The MS analyses were performed on a QTOF–MS/MS instrument of Agilent 6520 series, connected with an Agilent 1200 HPLC (Agilent technologies, USA) through a dual ESI interface. Nitrogen was used as drying and collision gas in the ESI source. The ion source parameters were as follow: drying gas flow rate, 12 L/min; heated capillary temperature, 350°C; nebulizer pressure, 45 psi; VCap, fragmentor, skimmer and octopole RF peak voltages set at 3500, 150, 65, and 75 V, respectively. The detection was carried out in positive and negative electrospray ionization mode, and spectra were recorded by MS scanning in the range of m/z 50–1000. The MS/MS analyses were carried out by targeted fragmentation and collision energy was set at 8–40 eV. Mass Hunter software version B.04.00 build 4.0.479.0 (Agilent Technology) was used to control LC–MS/MS system, data acquisition, and processing including the prediction of chemical formula and exact mass calculation.

**Result and discussion**

**Optimization of Conditions for HPLC–ESI–QTOF–MS/MS analysis**

The HPLC conditions were optimized to obtain maximal resolution and signal within a minimal run time. Various chromatographic conditions such as mobile phase composition, flow rate, injection volume, column temperature, and gradient program were studied and optimized for the separation of phenolics and triterpenic acids. Different mobile phase compositions (methanol–water, methanol–0.1% (v/v) formic acid aqueous solution, acetonitrile–water, and acetonitrile–0.1% (v/v) formic acid aqueous solution) were tested in the gradient program at 0.4 mL/min flow rate. A mobile phase composed of 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) at 0.4 mL/min flow rate and 25°C column temperature was found optimal for resolution of the maximum number of peaks in leaf extracts of *Ocimum* species within 65 min. In the MS analysis, both positive and negative ESI modes were tested, and the result showed that all the constituents exhibited good sensitivity in positive mode except phenolic acids and their esters, which showed high sensitivity in negative mode. Therefore, flavonoids, propenyl phenol derivatives, and triterpenic acids were analyzed in positive mode while phenolic acids and their esters were analyzed in negative mode.

**HPLC–ESI–QTOF–MS/MS analysis**

Metabolic profiling of leaf extracts of *Ocimum* species was resulted in the identification of 50 compounds including 28 flavonoids, 4 propenyl phenol derivatives, 2 triterpenic acids, 11 phenolic acids, and 5 phenolic acid esters. The structural identification of each compound was carried out on the basis of their accurate mass, molecular formula, and MS/MS fragmentation by HPLC–ESI–QTOF–MS/MS. Twenty-three compounds were unambiguously identified and characterized by comparing their retention time and fragmentation pattern with authentic standards. Other compounds were tentatively identified by comparing their MS and MS/MS data with available literature. All the identified compounds along with retention time, molecular formulas, m/z calculated and observed, error (Δ ppm), MS/MS data, and their comparative profile for 6 *Ocimum* species are summarized in Table 1a and b.
| Peak no. | t_R (min) | Molecular formula | Calc. m/z [M+H]^+ | Obs. m/z [M+H]^+ | Error (Δ ppm) | CE | Identification | Detection | Class |
|---------|-----------|-------------------|-------------------|------------------|--------------|----|----------------|-----------|-------|
| 1       | 7.5       | C_{21}H_{20}O_{11} | 449.1078          | 449.1079         | 0            | 25 | Isoorientin*    | OA, OG, OK, OS, OTF | Flavonoid C-glycoside |
| 2       | 8         | C_{21}H_{20}O_{10} | 611.1607          | 611.1607         | 0.01          | 11 | Quercetin-3-O-   | OA, OB, OG, OK, OS, OTF | Flavonoid C-glycoside |
| 3       | 8.2       | C_{21}H_{20}O_{11} | 449.1078          | 449.1077         | 0.23          | 25 | Orientin*       | OA, OB, OG, OK, OS, OTF | Flavonoid C-glycoside |
| 4       | 9         | C_{21}H_{20}O_{10} | 433.1129          | 433.1127         | 0.5           | 20 | Isovitexin*     | OA, OG, OK, OS, OTF | Flavonoid C-glycoside |
| 5       | 9.5       | C_{21}H_{20}O_{10} | 433.1129          | 433.1129         | 0.02          | 20 | Vitexin*        | OA, OG, OK, OS, OTF | Flavonoid C-glycoside |
| 6       | 9.7       | C_{21}H_{20}O_{15} | 595.1657          | 595.1657         | 0.09          | 8  | Kaempferol-3-O-  | OA, OB, OG, OK, OS, OTF | Flavonol glycoside |
| 7       | 9.9       | C_{21}H_{20}O_{12} | 465.1028          | 465.1028         | 0.08          | 11 | Quercetin-3-O-   | OA, OB, OG, OK, OS, OTF | Flavonol glycoside |
| 8       | 10.1      | C_{21}H_{20}O_{14} | 579.1708          | 579.1707         | 0.08          | 18 | Apigenin-7-O-    | OA, OG, OK, OS, OTF | Flavone glycoside |
| 9       | 10.2      | C_{21}H_{18}O_{12} | 463.0871          | 463.0872         | 0.16          | 15 | Luteolin-7-O-    | OA, OB, OG, OK, OS, OTF | Flavone glycoside |
| 10      | 10.7      | C_{21}H_{20}O_{15} | 551.1032          | 551.1030         | 0.24          | 15 | Quercetin-3-O-   | OA, OB, OG, OK, OS, OTF | Flavonol glycoside |
| 11      | 11.2      | C_{21}H_{20}O_{11} | 449.1078          | 449.1077         | 0.3           | 15 | Apigenin-7-O-    | OA, OB, OG, OK, OS, OTF | Flavone glycoside |
| 12      | 11.7      | C_{21}H_{20}O_{16} | 433.1129          | 433.1130         | 0.05          | 15 | Apigenin-7-O-    | OA, OB, OG, OK, OS, OTF | Flavone glycoside |
| 13      | 12.2      | C_{21}H_{20}O_{11} | 447.0922          | 447.0922         | 0.02          | 15 | Apigenin-7-O-    | OA, OB, OG, OK, OS, OTF | Flavone glycoside |
| 14      | 12.8      | C_{16}H_{12}O_{2}  | 165.0910          | 165.0909         | 0.54          | 10 | Eugenol*        | OA, OB, OG, OK, OS, OTF | Flavonol phenol derivative |
| 15      | 16.2      | C_{16}H_{16}O_{4}  | 179.0703          | 179.0702         | 0.59          | 15 | Coniferaldehyde  | OA, OB, OG, OK, OS, OTF | Flavone phenol derivative |
| 16      | 16.5      | C_{21}H_{18}O_{6}  | 287.0550          | 287.0551         | 0.35          | 40 | Luteolin*       | OA, OB, OG, OK, OS, OTF | Flavone phenol derivative |
| 17      | 16.9      | C_{30}H_{40}O_{12} | 303.0499          | 303.0500         | 0.12          | 40 | Quercetin*      | OA, OB, OG, OK, OS, OTF | Flavone phenol derivative |
| 18      | 18.3      | C_{13}H_{16}O_{4}  | 331.0812          | 331.0812         | 0.12          | 28 | Isothymusin      | OA, OB, OG, OK, OS, OTF | Flavone phenol derivative |

**Table 1a.** Compounds identified from the leaf extracts of *Ocimum* species in positive ionization mode by HPLC-QTOF-MS/MS.
| C<sub>13</sub>H<sub>16</sub>O<sub>5</sub> | 271.0601 | 271.0601 | 0.13 | 253.0472 (1.6), 243.0652 (4.7), 229.0493 (3.4), 197.0599 (2.9), 187.0398 (2.1), 169.0641 (2.6), 163.0384 (6.5), 153.0177 (100), 145.0280 (15.7), 131.0491 (3.2), 125.0239 (2.1), 119.0491 (46.8), 121.0286 (18.4), 111.0077 (3.8), 97.0292 (2.5), 91.0543 (42.6), 83.0142 (16.6), 68.9974 (23.6), 67.0184 (29.5) | 286.0462 (78.1), 258.0512 (100), 229.0487 (6.4), 153.0168 (5.4) | Flavone |
| C<sub>19</sub>H<sub>18</sub>O<sub>7</sub> | 359.1125 | 359.1125 | 0.4 | 344.0870 (30), 326.0770 (89.4), 315.0856 (5), 298.0820 (100), 283.0754 (2), 270.0867 (3.9), 255.0625 (1.6), 239.0662 (0.7), 227.0672 (1.4), 211.0742 (1.2), 199.0725 (0.5), 183.0290 (17.9), 162.058 (12.3), 147.0429 (11), 136.0145 (1.8), 108.0196 (3) |
| C<sub>24</sub>H<sub>24</sub>O<sub>7</sub> | 381.1292 | 381.1292 | 0.16 | 381.0798 (3), 177.0902 (2), 167.0347 (2), 159.0181 (3), 143.0125 (4), 133.0265 (6), 123.0635 (3), 113.0084 (2), 105.0143 (2), 91.0588 (3), 65.0347 (5) |
| C<sub>28</sub>H<sub>28</sub>O<sub>7</sub> | 405.1360 | 405.1360 | 0.4 | 250.0723 (1.3), 244.0648 (4.29), 228.0643 (0.24), 213.0736 (2.16), 197.0503 (0.68), 182.9919 (6.63), 175.0734 (0.33), 168.0064 (0.21), 154.9947 (2.29), 141.0677 (0.23), 133.0633 (4.7), 121.0145 (0.3), 10.0043 (0.4) |
| C<sub>29</sub>H<sub>30</sub>O<sub>6</sub> | 329.1200 | 329.1200 | 0.01 | 329.0751 (2.8), 296.0658 (69.8), 285.0731 (4.6), 268.0696 (100), 240.0777 (3.6), 205.9837 (2.6), 197.0435 (6), 182.0187 (3.9), 175.1437 (3.2), 169.0591 (2.1), 159.1138 (1.8), 145.0316 (1.7), 136.0142 (7.8), 133.0647 (7.1), 119.0897 (2.3), 108.0181 (8.5), 105.0750 (2) |
| C<sub>31</sub>H<sub>32</sub>O<sub>6</sub> | 359.1125 | 359.1125 | 0.08 | 344.0885 (5.5), 329.0641 (100), 311.0532 (36.6), 298.0817 (6.6), 286.0443 (1.8), 270.0853 (0.49), 255.0661 (0.4), 227.0539 (2.1), 214.0596 (0.3), 197.0071 (4.8), 178.9978 (0.6), 169.0116, 151.002 (0.6), 133.0635 (2.3), 113.0235 (0.5) |
| C<sub>42</sub>H<sub>40</sub>O<sub>6</sub> | 537.3470 | 537.3470 | 0.22 | 439.3551 (100), 425.3745 (6.4), 411.3604 (46.3), 393.3489 (6.6), 371.0990 (14), 338.3395 (8.2), 249.1829 (6.5), 191.1779 (17.8), 158.1526 (7.4) |
| C<sub>45</sub>H<sub>48</sub>O<sub>6</sub> | 579.3760 | 579.3760 | -0.6 | 439.3553 (100), 411.3602 (39.3), 425.3745 (3), 393.3492 (5.7), 347.2470 (4.5), 338.3391 (5.8), 249.1829 (6.5), 203.1777 (42.2), 191.1777 (17.3), 174.1264 (7.1), 158.1524 (6.5) |

OA, O. americanum; OB, O. basilicum; OG, O. gratissimum; OK, O. kilimandscharicum; OS, O. sanctum green; OTF, O. sanctum purple.

*Compounds confirmed by reference standards.*
The HPLC–ESI–QTOF–MS/MS analysis of flavonoids, propenyl phenol derivatives, and triterpenic acids was carried out in positive ionization mode, whereas phenolic acids and their esters were analyzed in negative ionization mode due to higher sensitivity. The base peak chromatograms (BPCs) of the leaf extracts of 6 Ocimum species in positive and negative ionization modes are presented in Figure 1a and b, respectively.

**Identification of flavonoids**

In the present study, 28 flavonoids were identified and characterized including 4 flavonoid C-glycosides, 12 flavones, 5 flavone O-glycosides, 3 flavonols, and 4 flavonol O-glycosides. The structures and fragmentation of flavonoids and their glycosides are shown in Figure 2. Nomenclature of fragment ions is according to Domon and Costello.\[54\]

### Flavonoid C-glycosides

Peaks 1, 3, 4, and 5 were identified as flavonoid C-glycosides, isoorientin, orientin, isovitexin, and vitexin, respectively by cochromatographic and mass spectral analysis with the authentic standards. Isoorientin (luteolin-8-C-glucoside) (peak 1) and orientin (luteolin-8-C-glucoside) (peak 3) showed a similar [M+H]+ ion at m/z 449.1078 and produced similar fragment ions in the CID–MS/MS scan, representing their isomeric structures. They produced similar base peak ions at m/z 329.0658 (\(^0.2X^-\)) by the loss of 120 Da from [M+H]+ ion, which is the characteristic of a hexose substitution in the aglycone moiety.\[54\] (Figure 3). They also generated fragment ion at m/z 431.0958 [M+H–H2O]+ (E1\(^+\)), 413.0869 [M+H–2H2O]+ (E2\(^+\)), 395.0764 [M+H–3H2O]+ (E3\(^+\)), 383.0768 [M+H–5H2O]+, 353.0661 [M+H–7H2O]+, and 299.0538 [M+H–150]+ (\(^0.1X^-\)). Figure 3 shows that the relative intensities of the (E1\(^+\)) and (\(^0.1X^-\)) ions were 16 and
Figure 1. (a) Base peak chromatogram (BPC) of leaf extracts of (A) O. americanum, (B) O. basilicum, (C) O. gratissimum, (D) O. kilimandscharicum, (E) O. sanctum green, (F) O. sanctum purple, and (G) mix reference standards in positive ionization mode. (b) Base peak chromatogram (BPC) of leaf extracts of (A) O. americanum, (B) O. basilicum, (C) O. gratissimum, (D) O. kilimandscharicum, (E) O. sanctum green, (F) O. sanctum purple, and (G) mix reference standards in negative ionization mode.
89% for isoorientin while 36 and 44% for orientin, respectively, at CID energy of 25 eV. These isomers were differentiated based on relative intensities of their characteristic ions. [54]

Isovitexin (apigenin-6-C-glucoside) (peak 4) and vitexin (apigenin-8-C-glucoside) (peak 5) also have isomeric structures, they showed \([M+H]^+\) ion at \(m/z\) 433.1127. Isovitexin produced base peak ion at \(m/z\) 283.0594 \([M+H-150]^+\).
(0.1X+), while vitexin at m/z 313.0707 [M+H-120]+ (0.2X+) at CID energy of 20 eV as reported by Waridel et al.\textsuperscript{[54]} (Figure 3). They also generated fragment ion at m/z 415.1025 [M+H-2H2O]+ (E+), 379.0813 [M+H-3H2O]+, 367.0806 [2,3X-2H2O], 337.0709 [0,4X-2H2O], and 295.0614 [0,2X-H2O].

**Flavones and their O-glycosides**

Peaks 16, 18, 19, 22–27, 29, 31, and 32 were identified as luteolin, isothymusin, apigenin, cirsiliol, cirsimaritin, cirsilineol, nevadensin, acacetin, 5-desmethylsinensetin, salvigenin, gardenin B, and apigenin-7, 4′-dimethyl ether, respectively, in which 16 and 19 were confirmed by comparison with authentic standards. All these are methoxylated flavones except luteolin and apigenin. In CID–MS/MS scan luteolin (m/z 287.0550) (peak 16) and apigenin (m/z 271.0601) (peak 19) produced prominent retro Diels–Alder (RDA) fragment ion at m/z 153.0180 (1,3A+RDA) and other RDA fragment ions 1,3B+, 0,4B+, and 0,4B+-H2O (shown in Figure S1). The 1,3B+, 0,4B+, and 0,4B+-H2O RDA fragment ions are specifically found for the flavones.\textsuperscript{[55]}

Peak 18 (isothymusin) and 22 (cirsiliol) showed a similar [M+H]+ ion at m/z 331.0812 in the MS scan. In CID–MS/MS scan, they generated similar fragment ions at m/z 316.0548 [M+H-15]+ corresponding to loss of methyl radical and at m/z 298.0456 [M+H-33]+ corresponding to loss of methyl radical and water molecule. Figure 4 shows that the relative intensity of [M+H-33]+ was found 27% for isothymusin and 100% for cirsiliol, which have also been observed by Grayer et al. for differentiation of these isomeric flavones.\textsuperscript{[13]}

Isothyrmusin (peak 18) also showed fragment ion at m/z 301.0325 [M+H-30]+ corresponding to loss of two methyl groups and cirsiliol (peak 22) at m/z 270.0504 [M+H-61]+ corresponding to loss of methyl radical, water, and CO.

Peak 23 (cirsimaritin) showed [M+H]+ ion at m/z 315.0864 and produced prominent ions at m/z 282.0510 [M+H-33]+ and at m/z 254.0559 [M+H-61]+ with less abundant ion at m/z 300.0610 [M+H-15]+ (Figure S2).

Peak 24 (cirsilineol) and 25 (nevadensin) showed a similar [M+H]+ ion at m/z 345.0970 indicating their isomeric structures. In CID–MS/MS scan, cirsilineol (peak 24) showed [M+H-15]+ at m/z 330.0718, [M+H-33]+ at m/z 312.0612, and [M+H-61]+ at m/z 284.0662, whereas nevadensin (peak 25) showed [M+H-15]+ at m/z 330.0713, [M+H-33]+ at m/z 315.0482, and [M+H-33]+ at m/z 312.0612 (Figure 4). These fragmentations are in agreement with reported literature.\textsuperscript{[13]}

Peaks 26 at m/z 285.0756 and 32 at m/z 299.0912 were identified as acacetin and apigenin-7, 4′-dimethyl ether, respectively. They are methyl derivatives of apigenin (peak 19). In CID–MS/MS scan, they showed initial loss of methyl radical produced fragment ion at m/z 270.0494 [M+H-15]+ and 284.0667 [M+H-15]+, respectively, and by subsequent loss of CO generated base peak ion at m/z 242.0565 [M+H-43]+ and 256.0723 [M+H-43]+, respectively (Figure S2).

Peaks 27 (5-desmethylsinensetin) and 31 (gardenin B) also have isomeric structures, they showed similar [M+H]+ ion at

Figure 4. CID–MS/MS spectra of [M+H]+ ions of methoxylated flavones.
m/z 359.1124. In the MS/MS analysis, 5-desmethylsinensetin (peak 27) generated fragment ion at m/z 344.0870 [M+H–15]+, 326.0770 [M+H–33]+, and 298.0820 [M+H–61]+, whereas gardenin B (peak 31) generated fragment ion at m/z 344.0885 [M+H–15]+, 329.0641 [M+H–30]+, 311.0532 [M+H–48]+, and 298.0817 [M+H–61]+. The [M+H–48]+ corresponding to loss of two methyl radical and water molecule[13] (Figure 4). Peak 29 (salvigenin) showed [M+H]+ ion at m/z 329.1020 and yielded fragment ions at m/z 314.0765 [M+H–15]+, 296.0658 [M+H–33]+, and 268.0696 [M+H–61]+ as reported earlier[13] (Figure S2).

Peaks 8, 9, 11, 12, and 13 were identified as apigenin-7-O-rutinoside (m/z 579.1707), luteolin-7-O-gluconuride (m/z 463.0872), luteolin-7-O-glucoside (m/z 449.1077), apigenin-7-O-glucoside (m/z 433.1130), and apiggin-7-O-glucuronide (m/z 447.0922). They produced the most prominent (Y1)+ fragment ion at m/z 271.0591 and 287.0539 corresponding to aglycone unit owing to loss of sugar moiety.[55] Apigenin-7-O-rutinoside (peak 8) also generated fragment ion at m/z 433.1110 ([M]+) corresponds to loss of terminal rhamnose unit (146 Da) and at m/z 417.1135 ([Y]+) corresponds to loss of an internal dehydrated glucose moiety (162 Da). The presence of (Y1)+ and (Y2)+ ions indicated peak 8 is O-diglycoside.[55] (Figure S3).

Flavonols and their O-glycosides

Peaks 17, 20, and 21 were identified as quercetin (m/z 303.0500), kaempferide (m/z 301.0708), and kaempferol (m/z 287.0553), respectively, in which peaks 17 and 21 were confirmed by authentic standards. Quercetin (peak 17) and kaempferol (peak 21) generated prominent fragment ion at m/z 153.0168 (13A+ RDA) and other RDA fragment ions 0.2A+ and 0.2B+, which are characteristic ions of flavonols.[55] (Figure S1). Kaempferide (peak 20) is 4’-O-methyl derivative of kaempferol (peak 21), generated fragment ion at m/z 286.0462 by initial loss of methyl radical and at m/z 258.0512 by subsequent loss of CO. Kaempferide also showed characteristic fragment ion at m/z 153.0168 (13A+) by RDA (Figure S1).

Peaks 2, 6, 7, and 10 were identified as quercetin-3-O-rutinoside (m/z 611.1607), kaempferol-3-O-rutinoside (m/z 595.1657), quercetin-3-O-glucoside (m/z 465.1028), and quercetin-3-O-malonylglocuside (m/z 551.1030), respectively, in which peaks 2 and 6 were confirmed by authentic standard. These flavonol O-glycosides produced the base peak (Y1)+ ion at m/z 303.0485 and 287.0539 corresponding to aglycone unit.[55] Quercetin-3-O-rutinoside (peak 2) and kaempferol-3-O-rutinoside (peak 6) are O-diglycoside, they also generated their characteristic (Y1)+ ion at m/z 465.1013 and 449.1077, (Y1)+ ion at m/z 449.103 and 433.1124, respectively.[55] (Figure S4).

Identification of propenyl phenol derivatives

Peaks 14, 15, 28, and 30 were identified as eugenol (m/z 165.0909), coniferaldehyde (m/z 179.0702), methyl eugenol (m/z 179.1068), and methoxy eugenol (m/z 195.1016), respectively, in which peaks 14 and 28 were confirmed by authentic standard. In CID–MS/MS scan eugenol (peak 14), methyl eugenol (peak 28), and methoxy eugenol (peak 30) produced base peak ion at m/z 124.0519, 138.0673, and 154.0626, respectively, due to loss of propenyl radical from their [M+H]+ ions. Eugenol (peak 14) and methoxy eugenol (peak 30) showed fragment ions at m/z 133.0646 and 163.0746, respectively, due to loss of CH3OH. Further loss of CH2O from fragment ion at m/z 163.0746 generated fragment ion at m/z 133.0766 indicating peak 30 is a methoxy derivative of eugenol.[57] Peak 15 (coniferaldehyde) produced base peak ion at m/z 147.0431 corresponding to [M+H–CH3OH]+ and by subsequent loss of CO generated second abundant fragment ion at m/z 119.0486. Coniferaldehyde also yielded [M+H–CH3]+ and [M+H–CO]+ fragments.[57] The structures, fragmentations, and MS/MS spectra of propenyl phenol derivatives are shown in Figure 5.

Identification of triterpenic acids

Two isomeric triterpenic acids, peaks 33 and 34, were identified and characterized as oleanolic and ursolic acid, respectively. These isomeric acids showed similar [M+H]+ at m/z 457.3675 and fragment ions in MS/MS scan but eluted at different retention time, 55.2 and 57.9 min. The identity of ursolic acid (peak 34) at tR 57.9 min was confirmed by authentic standard. The protonated molecular ions of oleanolic (peak 33) and ursolic acid (peak 34) generated base peak ion at m/z 439.3551 due to loss of H2O. They also produced fragment ions at m/z 411.3604 [M+H–COOH]+ and 393.3489 [M+H–COOH–H2O] as reported earlier.[58] The structures, fragmentation, and MS/MS spectra of triterpenic acids are shown in Figure 6.

Identification of phenolic acids and their esters

Analysis of phenolic acids and their esters were carried out in negative ionization mode: 11 phenolic acids and 5 phenolic acid esters were identified and characterized. Peaks 2, 3, 5–8, 10–12, 14, and 15 were identified as gallic acid (m/z 169.0143), chlorogenic acid (m/z 353.0878), protocatechuic acid (m/z 153.0193), caffeic acid (m/z 179.0350), syringic acid (m/z 197.0455), p-hydroxybenzoic acid (m/z 137.0243), sinapinic acid (m/z 223.0611), p-coumaric acid (m/z 163.0401), rosmarinic acid (m/z 359.0772), ferulic acid (m/z 193.0506), and vanillic acid (m/z 167.0350), respectively. All phenolic acids were confirmed by authentic standard except p-hydroxybenzoic acid. In CID–MS/MS scan, gallic acid (peak 2), protocatechuic acid (peak 5), caffeic acid (peak 6), p-hydroxybenzoic acid (peak 8), and p-coumaric acid (peak 11) generated major fragment ion at m/z 125.0258, 109.0280, 135.0450, 93.0355, and 119.0461, respectively, corresponding to [M–H–CO2]–.[51,59] Chlorogenic acid (peak 3) is an ester of caffeic acid and quinic acid, generated base peak ion at m/z 191.0559 corresponds to deprotonated quinic acid by the cleavage of intact caffeoyl quinic acid fragments.[51,59] Rosmarinic acid (peak 12) also a caffeic acid ester, generated major fragment ion at m/z 161.0268 due to loss of water from fragment ion at m/z 179.0381 [M–H–C6H4O3]– corresponds to deprotonated quinic acid.
Figure 5. Structures and CID–MS/MS spectra of [M+H]^+ ions of propenyl phenol derivatives.

Figure 6. Structures and CID–MS/MS spectra of [M+H]^+ ions of triterpenic acids.

Figure 7. Graphical representation of distribution of phenolics and triterpenic acids in the leaf extracts of studied Ocimum species.
cafeic acid moiety.\[^{[51]}\] Syringic acid (peak 7), sinapinic acid (peak 10), ferulic acid (peak 14), and vanillic acid (peak 15) are O-methylated phenolic acids, they showed prominent fragment ion at \(m/z\) 182.0191 \([\text{M–H–CH}_3]^–\), 208.0418 \([\text{M–H–CH}_3]^–\), 134.0391 \([\text{M–H–CH}_2\text{CO}_2]^–\), and 108.0226 \([\text{M–H–CH}_2\text{CO}_2]^–\), respectively.\[^{[51,59]}\]

Peak 1 was identified as galloylglucose \((m/z\) 331.0674\), generated fragment ions at \(m/z\) 271.0462 due to removal of \(\text{C}_6\text{H}_4\text{O}_2\) moiety by cross-ring fragmentation of a glucose molecule, \(m/z\) 169.0117 due to loss of glucose moiety \((\text{C}_6\text{H}_{10}\text{O}_5)\), and \(m/z\) 125.0231 due to decarboxylation of the gallic acid moiety.\[^{[59]}\]

Peaks 4 and 13 were identified as ethyl protocatechuate \((m/z\) 181.0505\) and methyl protocatechuate \((m/z\) 167.0350\), respectively. Ethyl protocatechuate (peak 4) showed fragment ion at \(m/z\) 153.0176 \([\text{M–C}_2\text{H}_4]^–\), 108.0209 \([\text{M–H–C}_2\text{H}_4\text{COOH}]^–\), and methyl protocatechuate (peak 13) at \(m/z\) 152.0090 \([\text{M–H–CH}_3]^–\) and \(m/z\) 108.0193 \([\text{M–H–CH}_2\text{CO}_2]^–\). Peak 9 was identified as methyl gallate \((m/z\) 183.0299\), produced fragment ions at \(m/z\) 168.0054 \([\text{M–H–CH}_3]^–\) and 124.0151 \([\text{M–H–CH}_2\text{CO}_2]^–\).\[^{[60]}\] Peak 16 was identified as ethyl caffeate \((m/z\) 207.0663\), showed base peak ion at \(m/z\) 135.0438 corresponding to \([\text{M–H–C}_2\text{H}_4\text{CO}_2]^–\). The structures, fragmentations, and MS/MS spectra of phenolic acids and their esters are shown in Figures S5–S8.

**Interspecies variation of phenolics and triterpenic acids in Ocimum species**

The LC–MS/MS profile of identified 50 compounds was used to study the interspecies variation of phenolics and triterpenic acids in leaf extracts of 6 *Ocimum* species. Considering the area of extracted ion chromatogram (EIC) of each compound, it was observed that flavonoids were the most abundant class of compounds in *O. sanctum* purple, *O. sanctum* green, and *O. basilicum*, whereas in other species phenolic acid and their esters were the abundant class of compounds. It was also observed that maximum abundance of flavonoids was found in *O. sanctum* purple, propenyl phenol derivatives in *O. americanum*, triterpenic acids in *O. kilimandscharicum*, and phenolic acids and their esters in *O. gratissimum*. Significant variation in the abundance of identified compounds was observed among 6 *Ocimum* species, which is graphically represented in Figure 7. These observations were based on the calculation of averaged relative percent peak area obtained from extracted ion chromatograms (EICs) of all the 50 compounds in triplicate.

**Conclusion**

In summary, the present study described first systematic comparative screening of phenolics and triterpenic acids in leaf extracts of 6 *Ocimum* species using HPLC–ESI–QTOF–MS/MS. A total of 50 compounds including 28 flavonoids, 4 propenyl phenol derivatives, 2 triterpenic acids, 11 phenolic acids, and 5 phenolic acid esters were identified from leaf extracts of 6 *Ocimum* species. They were characterized by their retention behavior, exact mass measurement, molecular formula, MS/MS spectral patterns, and authentic standards. The interspecies variation of identified phenolics and triterpenic acids was studied using LC–MS/MS profile. The results indicated that *O. sanctum* purple is the richest source of flavonoids and *O. kilimandscharicum* is of triterpenic acids, while *O. americanum* is the richest source of propenyl phenol derivatives and *O. gratissimum* is of phenolic acids. This information might be helpful for better swapping of *Ocimum* species. Comparative metabolite profiling could be helpful for consumers in selecting best *Ocimum* species with a better expression of active compounds for their commercial use. Therefore, the developed analytical method is rapid and accurate for the screening and structural characterization of phenolics and triterpenic acids in *Ocimum* species.

**Acknowledgments**

The authors gratefully acknowledge the Sophisticated Analytical Instrument Facility, CSIR-CDRI, Lucknow, where the mass spectrometric studies were carried out. We are thankful to Dr Bhupender Dutt and Dr. Kulkvant Rai Sharma, Department of Forest Products, Dr Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India, for providing plant material. Renu Pandey is thankful to the University Grant Commission, New Delhi, for the award of Junior Research Fellowship. We are also thankful to Ms Pooja Soni (Technical Assistant) for her support (CDRI communication number 9185).

**Funding**

This study was funded by Council of Scientific and Industrial Research, New Delhi, India (grant number BSC-0106).

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