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A Rapid and Highly Sensitive Method for Simultaneous Determination of Bioactive Constituents in Leaf Extracts of Six *Ocimum* Species Using Ultra High Performance Liquid Chromatography-Hybrid Linear Ion Trap Triple Quadrupole Mass Spectrometry

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Abstract

Ocimum species have tremendous value in pharmaceutical, perfumery, food processing and cosmetic industries, also in traditional rituals and medicines. These are rich source of terpenoids and phenolic compounds. Therefore, determination of these bioactive constituents is significant for quality evaluation of *Ocimum* species. In this study, we have developed and validated a rapid and highly sensitive method for simultaneous determination of sixteen bioactive constituents in the leaf extracts of six *Ocimum* species using ultra high performance liquid chromatography-hybrid linear ion trap triple quadrupole mass spectrometry (UHPLC-QqQ_{LIT}-MS/MS). The developed method is applied in leaf extracts of six *Ocimum* species to investigate variations in the content of sixteen bioactive constituents. Quantitative analysis was performed by UHPLC-QqQ_{LIT}-MS/MS operating under multiple reaction monitoring mode in negative electrospray ionization. Chromatographic separation was accomplished on an Acquity UPLC BEH C_{18} column using a gradient elution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The calibration curves of all sixteen analytes showed good linearity ($r^2 \geq 0.9989$) over the concentration range of 0.5-500 ng/mL. The intra- and inter-day precisions and accuracy were within RSDs $\leq$ 1.95% and $\leq$ 1.68%, respectively. Results indicated that ursolic acid and rosmarinic acid were the major constituents in almost all the investigated *Ocimum* species except for *O. americanum*. All the results obtained from this study demonstrated that the developed method was rapid, sensitive and efficient for the quantification of multiple constituents. Therefore, could be reliably utilized for the quality control and authenticity establishment of *Ocimum* species.

Keywords: Multiple reaction monitoring, *Ocimum* species, UHPLC-QqQ_{LIT}-MS/MS.

1. Introduction

The genus *Ocimum* (family Lamiaceae), also known as basil, is a good source of essential oils and traditionally used for its healing properties[1]. It covers more than 200 species of herbs and shrubs with immense medicinal properties.[2] Apart from medicinal value these are used for different purposes which include food preservation, flavoring agents, culinary and ornamental herbs[2]. The essential oils obtained from the leaves and flavoring tops of basil have tremendous value in pharmaceutical, perfumery, food processing and cosmetic industries[1, 2]. It is widely distributed in the tropical regions and abundantly found in Asia, Africa, Central and South America[1, 3]. In India, the most commonly found species are *O. americanum, O. basilicum, O. gratissimum, O. killimandscharicum, O. sanctum green* and *O. sanctum purple*. Among these, *O. sanctum* (holy basil) and *O. basilicum* (sweet basil) are widely explored[3]. *Ocimum* species have wide range of pharmacological activities like antiarthritic, antidiabetic, anti-cataract, anticoagulant, antifertility, antihyperlipidemic, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, antistress, antiulcer, cardioprotective, chemoprotective, hepatoprotective, immunomodulatory, insecticidal, memory enhancing and radioprotective activities[2, 3-7]**.** Phytochemical studies on *Ocimum* species revealed that these are rich source of terpenoids and phenolic compounds including phenolic acids, propenyl phenols, polyphenols such as flavonoids and anthocyanins[3, 5, 6, 8, 9].

Variations in the morphology such as shape, size, pigmentation of leaves and composition of essential oils have been reported from this genus[1]. Differences in chemical composition usually cause different pharmacological activities and affecting the commercial value of this genus. Therefore, development of an efficient method that will allow the discrimination of *Ocimum* species in terms of distribution of bioactive phenolic acids, flavonoids, propenyl phenol and

terpenoid in leaf extracts of different *Ocimum* species is of high importance for the establishment of quality parameters.

In the present study, bioactive constituents including phenolic acids, flavonoids, their glycosides, propenyl phenols and terpenoids were selected for quantitative determination. These compounds have been reported to exhibit multiple pharmacological activities such as anticancer[10-12], anti-HIV[13], anti-inflammatory[14], antimicrobial[6, 14], antioxidant[6, 7] and antistress activities[4]. The simultaneous quantitative determination of multiple components is a suitable method for species discrimination. The present proposed study is aimed to evaluate the chemical variations and explore best suited species among six *Ocimum* species for therapeutic potential and commercial use.

Literature survey revealed that, a variety of analytical methods including HPLC, HPTLC, ATR/FT-IR; FT-Raman; NIR, GC-MS, LC-MS[15-24] have been developed for identification and determination of phenolic acids, flavonoids, propenyl phenol and terpenoid in *Ocimum* species. Compared to the above mentioned methods, UHPLC-ESI-MS/MS method in MRM acquisition mode is a more powerful approach, to rapidly quantify multi-components in complex matrix due to its rapid separation power, greater sensitivity and high specificity[25]. Although, we have previously done quantification of phenolic acids, flavonoids, propenyl phenol and terpenoid in the leaf extract of *O. sanctum* and its marketed herbal formulations[26], but quantification of these bioactive constituents have still not been carried out in six *Ocimum* species.

In this communication, the previously developed UHPLC-ESI-MS/MS method is modified and validated for simultaneous determination of sixteen bioactive constituents including seven phenolic acids, seven flavonoids, one propenyl phenol and one terpenoid in the leaf extracts of *O. americanum, O. basilicum, O. gratissimum, O. killimandscharicum, O. sanctum* green and *O.*

sanctum purple by ultra high performance liquid chromatography-hybrid linear ion trap triple quadrupole mass spectrometry (UHPLC-QqQ_{LIT}-MS/MS). This method was applied to investigate content variations of sixteen bioactive constituents among six *Ocimum* species.

2. Experimental

2.1 Reagents, chemicals and plant materials

Acetonitrile (LC-MS grade) and formic acid (analytical grade) purchased from Fluka, Sigma-Aldrich (St. Louis, MO, USA) were used in mobile phase and sample preparation throughout the LC-MS analysis. Ultra pure water, obtained from Direct-Q system (Millipore, Milford, MA, USA), was used throughout the analysis. The analytical standards (purity ≥ 97%) caffeic acid, ferulic acid, sinapinic acid, ursolic acid, apigenin and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The analytical standards of (purity ≥ 95%) gallic acid, protocatechuic acid, chlorogenic acid, rosmarinic acid, quercetin-3, 4'-diglucoside, rutin, kaempferol-3-O-rutinoside, quercetin, luteolin, eugenol and andrographolide (IS) were purchased from Extrasynthese (Z.I Lyon Nord, Genay Cedex, France). The structures of these analytes and internal standard are shown in **Fig. 1.**

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 in early October, 2013. Voucher specimens number 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 Department of Forest Products, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India.

2.2 Extraction and sample preparation

The leaves of selected *Ocimum* species were washed thoroughly under running tap water, air-dried at room temperature and ground into powder with 40 mesh. The dried powder (20g) of the leaves of each species was weighed precisely and sonicated with 200 mL of aqueous methanol (80%) for 30 min at room temperature using ultrasonic water bath (53 KHz) and left for 24 hours at room temperature (maintained at 22-24°C). Three replicates of the extraction process were carried out on each individual sample. The solution was filtered through Whatman filter paper and evaporated to dryness under reduced pressure using rotary evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at 40°C. Dried residues (1 mg) were weighed accurately, dissolved in 1 mL of acetonitrile and sonicated using ultrasonicator (Bandelin SONOREX, Berlin). 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. 30 ng/mL of internal standard (IS), andrographolide was spiked into final working solution, vortexed for 30 s and 1 µL aliquot was injected into the UHPLC-MS/MS system for analysis.

2.3 Preparation of standard solutions

A mixed standard stock solution (1 mg/mL) of selected analytes was prepared in acetonitrile. The working standard solutions were prepared by diluting the mixed standard solution with acetonitrile to a series of concentrations within the ranges from 0.5 to 500 ng/mL used for plotting calibration curve. The IS andrographolide was spiked to each concentration at a final concentration of 30 ng/mL. The standard stock and working solutions were stored at -20°C until use and vortexed prior to injection.

2.4 Instrumentation and analytical conditions

The UHPLC-ESI-MS/MS analysis was performed on Waters Acquity UPLC^{TM} system (Waters, Milford, MA, USA) interfaced with hybrid linear ion trap triple-quadrupole mass spectrometer

(API 4000 QTRAP™ MS/MS system from AB Sciex, Concord, ON, Canada) equipped with electrospray (Turbo V) ion source. The Waters Acquity UPLC™ system was equipped with a binary solvent manager, sample manager, column oven and photodiode array detector (PDA). AB Sciex Analyst software version 1.5.1 was used to control the LC-MS/MS system and for data acquisition and processing. All the statistical calculations related to quantitative analysis were performed using Graph Pad Prism software version 5.

2.4.1 UPLC conditions

The chromatographic separation of selected analytes and internal standard was achieved on an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm id, 1.7 µm) at a column temperature of 50°C. Analysis was completed with gradient elution of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as mobile phase at a flow rate of 0.3 mL/min. The 13 min UPLC gradient system was as follows: 0-2 min, 10-10% B; 2-3 min, 10-20% B; 3-4.5 min, 20-20% B; 4.5-5.2 min, 20-24% B; 5.2-6 min 24-24% B; 6-7.5 min, 24-40% B; 7.5-7.8 min, 40-50% B; 7.8-8.5 min 50-70% B; 8.5-9 min, 70-70% B; 9-9.3 min 70-95% B; 9.3-11.3 min, 95-95% B; 11.3-12.3 min 95-10% B; 12.3-13 min 10-10% B. The sample injection volume was 1µL.

2.4.2 MS conditions

All the analytes with internal standard (IS) were detected in negative electrospray ionization using precursor ion scan and mass spectra were recorded in the range of *m/z* 100-1000 at a cycle time of 9s with a step size of 0.1 Da. Nitrogen was used as the nebulizer (GS1), heater (GS2), and curtain gas (CUR) as well as the collision activated dissociation gas (CAD). Simultaneous quantitation of analytes was carried out using multiple reaction monitoring (MRM) acquisition mode at unit resolution. Optimization of MRM conditions was carried out by infusing 50 ng/mL solutions of the analytes and internal standard dissolved in acetonitrile at a flow rate of 10

μL/min using a Harvard '22' syringe pump (Harvard Apparatus, South Natick, MA, USA). The transitions and optimized compound dependent MRM parameters: declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) for each analyte and internal standard are listed in **Table S1** (Supporting information). The dwell time for all analytes was set at 200 ms. Optimized source parameters were as follows: ion spray voltage set at -4200 V, curtain gas, nebulizer gas and heater gas set at 20, 20 and 20 psi, respectively with a source temperature of 550°C. The collision activated dissociation gas was set at medium and the interface heater was on.

3. Results and discussion

3.1 Optimization of LC conditions

In order to achieve a rapid and efficient analysis, a short chromatographic column Acquity UPLC BEH C18 column (50 mm×2.1 mm id, 1.7μm) was employed in the UPLC system. Different mobile phase systems (water–methanol, water–acetonitrile, 0.1% formic acid in water–methanol, 0.1% formic acid in water– acetonitrile, 0.1% formic acid in water– 0.1% formic acid in methanol and 0.1% formic acid in water– 0.1% formic acid in acetonitrile) using different compositions of solvents in gradient elution at different flow rates (0.2, 0.3, 0.35, 0.4 and 0.5 mL/min) as well as column temperatures (25, 30, 35, 40 and 50°C) were examined and compared in order to obtain better chromatographic behavior and appropriate ionization. It was found that 0.1% formic acid in water– 0.1% formic acid in acetonitrile was better than others. Furthermore, different concentrations of formic acid (0.1%, 0.2%, 0.3%, and 0.4% v/v) were added into the mobile phase to improve the peak shape and restrain the peak tailing. Finally, 0.1% formic acid in water– 0.1% formic acid in acetonitrile was chosen as the eluting solvent system at a flow rate

of 0.3 mL/min with the column temperature of 50°C to give the acceptable separation and ionization within a run time of 13 min.

3.2 Optimization of MS conditions

Preliminary, each targeted analyte was infused into the mass spectrometer and MS spectra were studied in both positive and negative ionization modes. During tuning (Q1 scan) it was observed that all analytes exhibited good signal sensitivity in negative ionization mode. Then, the compound dependent MRM parameters: DP, EP, CE and CXP were optimized for each targeted analyte by injecting the individual standard solution into the mass spectrometer to achieve the most abundant, specific and stable MRM transition shown in **Table S1** (Supporting information). The source parameters including the curtain gas, GS1, GS2 and ion source temperature were further optimized in order to get the highest abundance of precursor-to-product ions. The optimized compound dependant parameters and source parameters were combined and finally the optimized UHPLC-ESI-MS/MS method in MRM acquisition mode was applied to quantify sixteen bioactive constituents in the six *Ocimum* species using andrographolide as an internal standard. UHPLC-MRM extracted ion chromatogram of analytes and internal standard is shown in **Fig. 2.**

3.3 Identification of targeted analytes

The targeted analytes in the samples were unambiguously identified by the comparison of their retention times and MS/MS spectra with those of the authentic standard solution. The MS spectra generated for all the targeted compounds by ESI-MS in the negative ion mode gave the deprotonated molecule $[M-H]^-$. The MS/MS spectra and fragmentations of the sixteen bioactive constituents and internal standard andrographolide are shown in **Fig.** S**1 (a), (b) and (c)** (Supporting information)**.** The predominant product ion of each targeted compound was selected

for MRM transition. The predominant product ion in the MS/MS spectra of the $[M-H]^-$ ions of gallic acid, *m/z* 169 $[M-H]^-$, protocatechuic acid, *m/z* 153 $[M-H]^-$ and caffeic acid *m/z* 179 $[M-H]^-$, was generated due to the loss of CO_2 molecule, providing an anion of $[M-H-CO_2]^-$[27]. Chlorogenic acid, *m/z* 353 $M-H]^-$ is an ester of caffeic acid and quinic acid, generated the predominant product ion at *m/z* 191 due to loss of $C_9H_6O_3$ moiety from deprotonated molecular ion by the cleavage of intact caffeoyl and quinic acid fragments [28]. Ferulic acid, *m/z* 193 $[M-H]^-$ generated the predominant product ion at *m/z* 134 corresponding to $[M-H-CH3-COO]^-$ and sinapinic acid, *m/z* 223 $[M-H]^-$ at *m/z* 149 corresponding to $[M-H-2CH3-COO]^-$[29]. Rosmarinic acid, *m/z* 359 $[M-H]^-$ is a caffeic acid ester, generated the predominant product ion at *m/z* 161 due to loss of water from fragment ion at *m/z* 179 $[M-H-C_9H_8O_4]^-$, corresponds to deprotonated caffeic acid moiety[28].

Quercetin-3, 4'-diglucoside, *m/z* 625 $[M-H]^-$, rutin, *m/z* 609 $[M-H]^-$, and kaempferol-3-*O*-rutinoside, *m/z* 593 $[M-H]^-$ yielded predominant product ion at *m/z* 463, *m/z* 301 and *m/z* 285, respectively due to *O*-glycosidic cleavage[30]. Quercetin, luteolin and apigenin generated predominant product ion at *m/z* 151, *m/z* 133 and *m/z* 117 respectively by retro diels-alder (RDA) reaction[30, 31]. Kaempferol, *m/z* 285 $[M-H]^-$ yielded product ion at *m/z* 239 corresponding to $[M-H_2O-CO]^-$[31, 32]. Eugenol, *m/z* 163 $[M-H]^-$, yielded predominant product ion at *m/z* 148 due to loss of methyl radical[33]. In the MS/MS spectra of ursolic acid no dominant product ions were formed. Hence the CE in Q2 was set to a low value (9 eV) to minimize fragmentation and the parent ion isolated in Q1 was passed through Q2 without fragmentation. In Q3, the same ion was monitored[26, 34]. The internal standard andrographolide, *m/z* 349 $[M-H]^-$, yielded fragment ion at *m/z* 287 by subsequent loss of water and carbon dioxide[35].

3.4 Analytical method validation

The proposed UHPLC-ESI-MS/MS method for quantitative analysis was validated according to the guidelines of International Conference on Harmonization (ICH, Q2R1) by determining linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and solution stability.

3.4.1 Linearity, LOD and LOQ

The stock solution was diluted with LC-MS grade acetonitrile to provide a series of concentrations in the range of 0.5-500 ng/mL for the construction of calibration curves. The linearity of calibration was performed by the analytes-to-IS peak area ratios versus the nominal concentration and the calibration curves were constructed with a weight **$(1/x^2)$** factor by least-squares linear regression. The applied calibration model for all curves was **y = a x + b**, where **y** = peak area ratio (analyte/IS), **x** = concentration of the analyte, **a** = slope of the curve and **b** = intercept. The LOD and LOQ were determined based on calibration curve method by following equations: **LOD = 3.3 Sy.x/Sa** and **LOQ = 10 Sy.x/Sa**, where **Sy.x** = the residual standard deviation of a regression line and **Sa** = the slope of calibration curve. The results are listed in **Table 1**. All the calibration curves indicated good linearity with correlation coefficients (r^2) from 0.9989 to 1.0000 within the test ranges. The LOD for each analyte varied from 0.041-0.357 ng/mL and LOQ from 0.124-1.082 ng/mL.

3.4.2 Precision, stability and recovery

The intra-day and inter-day variations, which were chosen to determine the precision of the developed method, were investigated by determining sixteen analytes with IS in six replicates during a single day and by duplicating the experiments on three consecutive days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD).The overall intra-day and inter-day precision were not more than

1.95%. Stability of sample solutions stored at room temperature was investigated by replicate injections of the sample solution at 0, 2, 4, 8, 12 and 24 h. The RSD values of stability of the sixteen analytes ≤ 2.91%. The results of precision and stability are shown in **Table 1.**

A recovery test was applied to evaluate the accuracy of this method. The test was performed by adding known amounts of the sixteen analytical standards at low (50% of the known amounts), medium (the same as the known amounts) and high (150% of the known amounts) levels into samples. The spiked samples were then analyzed at each level with the proposed method in triplicate and average recoveries were determined. The analytical method developed had good accuracy with overall recovery in the range from 95.10-103.04% (RSD ≤ 1.68%). Recovery results are shown in **Table 2**.

3.5 Quantitative analysis of samples

The developed UHPLC-ESI-MS/MS method was successfully applied for simultaneous quantitative determination of sixteen bioactive constituents in the leaf extracts of six *Ocimum* species. The contents of sixteen bioactive constituents are summarized in **Table 3** and graphical representations of results are shown in **Fig. 3 (a)** and **(b)**, where significant content variations of sixteen bioactive constituents is visible among six *Ocimum* species. Quantitative analysis indicated that ursolic acid with content range of 373.4-14100 µg/g and rosmarinic acid with content range of 1653.3-10966.7µg/g were the major constituents in all the analyzed *Ocimum* species except for *O. americanum*. Rosmarinic acid (10966.7 µg/g) and rutin (10900.0 µg/g) were the predominant constituents in *O. americanum.*

The total content of seven phenolic acids were found highest (14182.4 µg/g) in *O. americanum* and lowest (4700.6 µg/g) in *O. sanctum* green, similarly total content of seven flavonoids were found highest (12722.3 µg/g) in *O. americanum* and lowest (2367.6 µg/g) in *O.*

killimandscharicum. The content of terpenoid (ursolic acid) found highest (14100 µg/g) in *O. killimandscharicum* and lowest (373.4 µg/g) in *O. americanum*, similarly content of propenyl phenol (eugenol) was found highest (206.7 µg/g) in *O. killimandscharicum* and lowest (34.0 µg/g) in *O. basilicum*.

The overall quantitative analysis results indicated that *O. killimandscharicum* contain maximum amount of ursolic acid and eugenol, the major bioactive constituents and showing the highest total content (28090.6 µg/g) of sixteen bioactive constituents compared to other samples. *O. killimandscharicum* is the less explored species of this genus. This information could be helpful for better swapping of *Ocimum* species.

4. Conclusion

The present study involved development and validation of a rapid, sensitive and reliable analytical method for simultaneous determination of sixteen bioactive constituents in leaf extracts of six *Ocimum* species using UHPLC-QqQ_{LIT}-MS/MS in MRM acquisition mode. The developed method was successfully applied in leaf extracts of six *Ocimum* species to investigate variations in the content of sixteen bioactive constituents. The sensitivity, linearity and precision of this method meet international regulations. Results indicated that ursolic acid and rosmarinic acid were the major constituents in almost all the investigated *Ocimum* species except for *O. americanum*. It was also found that *O. killimandscharicum* contain maximum amount of ursolic acid and eugenol and showing the highest total content of sixteen bioactive constituents compared to other samples. The comparative analysis of contents of phenolics, flavonoids, propenyl phenol and terpenoid in leaf extracts of six *Ocimum* species will help consumers to select one of them according to their requirement. All the results obtained from this study demonstrated that the developed method is rapid, sensitive, accurate and precise for the

quantification of multiple bioactive constituents in *Ocimum* species, therefore could be a well-acceptable strategy to compare and evaluate the quality of *Ocimum* species.

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Figure captions

Figure 1. Chemical structures of investigated analytes and internal standard.

Figure 2. UHPLC-MRM extracted ion chromatogram of analytes and internal standard.

Figure 3. (a). Graphical representation of distribution of sixteen bioactive constituents in leaf extracts of six *Ocimum* species. **(b)**. Total content (μg/g) of phenolic acids, flavonoids, propenyl phenol and terpenoid in leaf extracts of six *Ocimum* species.

Table 1. Regression equation, correlation coefficients, linearity ranges, limits of detection (LOD), limit of quantitation (LOQ), intra-day, inter-day precision and stability for sixteen analytes.

Analyte	Regression Equation	r2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/ml)	Precision RSD (%) Intra-day (n=6)	Inter-day (n=6)	Stability RSD (%) (n=6)
Gallic acid	22.58x-0.094	0.9997	10-500	0.216	0.653	1.61	1.71	1.55
Protocatechuic acid	21.15x+0.564	0.9995	1-200	0.107	0.326	1.59	1.95	2.28
Chlorogenic acid	6.870x- 0.283	0.9999	10-500	0.150	0.455	1.06	1.49	2.10
Caffeic acid	23.43x-0.219	0.9999	10-500	0.093	0.280	1.28	1.52	2.79
Quercetin-3, 4'-diglucoside	17.72x-1.105	0.9994	10-500	0.298	0.903	1.70	1.92	1.98
Ferulic acid	5.956+0.484	1.0000	10-500	0.086	0.260	1.37	1.81	1.86
Rutin	2.426x+0.441	0.9992	10-500	0.357	1.082	1.71	1.93	2.91
Sinapinic acid	3.739x+0.030	0.9997	10-500	0.197	0.596	1.62	1.90	2.68
Kaempferol-3-O-rutinoside	23.39x-0.438	0.9995	1-200	0.114	0.347	1.28	1.59	1.78
Rosmarinic acid	32.78x+1.085	0.9994	1-200	0.123	0.372	1.69	1.91	2.52
Quercetin	29.58x+0.694	0.9996	1-200	0.104	0.315	1.49	1.58	2.31
Luteolin	8.548x-0.225	0.9995	10-500	0.284	0.861	1.45	1.91	2.02
Apigenin	57.20x-0.430	0.9989	0.5-100	0.094	0.284	1.31	1.68	2.29
Kaempferol	18.17x+0.211	0.9999	1-200	0.041	0.124	1.25	1.59	2.21
Eugenol	0.2039x+0.028	0.9995	10-500	0.274	0.832	1.03	1.54	2.28
Ursolic acid	95.75x+5.267	0.9999	10-500	0.141	0.426	0.89	1.21	2.01

Table 2. Recovery (n=3) of sixteen analytes.

Analytes	Original (ng/mL)	Spiked (ng/mL)	Observed (ng/mL)	Recovery (%)	Mean	RSD (%)
Gallic acid	11.00	5.50	16.67	101.03		
		11.00	22.91	104.14	102.21	1.65
		16.50	27.90	101.45		
Protocatechuic acid	17.60	8.80	25.85	97.92		
		17.60	34.76	98.75	97.89	0.89
		26.40	42.69	97.01		
Chlorogenic acid	24.10	12.05	35.87	99.23		
		24.10	47.83	99.22	99.84	1.07
		36.15	60.90	101.08		
Caffeic acid	73.00	36.50	109.87	100.34		
		73.00	148.39	101.64	101.04	0.65
		109.50	184.57	101.13		
Quercetin-3,4'-diglucoside	9.27	4.64	13.85	99.60		
		9.27	18.63	100.50	100.98	1.65
		13.91	23.83	102.83		
Ferulic acid	13.40	6.70	18.94	94.23		
		13.40	25.67	95.78	95.55	1.29
		20.10	32.38	96.66		
Rutin	55.20	27.60	83.00	100.24		
		55.20	114.21	103.45	101.72	1.59
		82.80	140.01	101.46		
Sinapinic acid	7.10	3.55	10.85	101.92		
		7.10	14.75	103.89	102.50	1.18
		10.65	18.05	101.69		
Kaempferol-3-O-rutinoside	9.65	4.83	14.69	101.49		
		9.65	19.45	100.78	101.05	0.38
		14.48	24.34	100.87		
Rosmarinic acid	236.00	118.00	365.47	103.24		

		236.00	480.31	101.76	102.15	0.93
		354.00	598.59	101.46		
Quercetin	2.03	1.02	2.90	95.24		
		2.03	3.82	94.11	95.10	0.98
		3.05	4.87	95.96		
Luteolin	19.30	9.65	29.80	102.94		
		19.30	39.15	101.42	101.50	1.38
		28.95	48.32	100.15		
Apigenin	3.70	1.85	5.63	101.39		
		3.70	7.36	99.43	99.91	1.30
		5.55	9.15	98.92		
Kaempferol	9.79	4.90	14.20	96.70		
		9.79	19.26	98.34	98.09	1.32
		14.69	24.29	99.24		
Eugenol	2.58	1.29	3.94	101.89		
		2.58	5.27	102.13	101.03	1.68
		3.87	6.39	99.07		
Ursolic acid	208.00	104.00	316.60	101.47		
		208.00	431.89	103.82	103.04	1.31
		312.00	539.87	103.82		

Table 3. The content (µg/g) of sixteen bioactive constituents in the leaf extracts of six *Ocimum* species.

Analytes (µg/g)	Leaf extracts					
	O. americanum	***O. basilicum***	***O. gratissimum***	***O. killimandscharicum***	***O. sanctum green***	***O. sanctum purple***
Phenolic acids						
Gallic acid	396.7	255.3	366.7	220.7	282.0	315.7
Protocatechuic acid	730.0	610.0	586.7	683.3	593.3	830.0
Chlorogenic acid	460.0	180.0	803.3	463.3	1113.3	320.7
Caffeic acid	1080.0	920.0	2433.3	1413.3	390.0	1006.7
Ferulic acid	336.7	546.7	446.7	315.3	446.7	356.7
Sinapinic acid	212.3	239.0	236.7	253.7	222.0	233.3
Rosmarinic acid	10966.7	7600.0	7866.7	8066.7	1653.3	8000.0
Total	14182.4	10351.0	12740.1	11416.3	4700.6	11063.1
Flavonoids						
Quercetin-3, 4'-diglucoside	301.7	310.3	309.0	305.3	312.3	304.3
Rutin	10900.0	1653.0	920.0	693.3	74.3	173.3
Kaempferol-3-O-rutinoside	370.0	296.7	321.7	252.3	206.7	222.0
Quercetin	39.3	47.3	67.7	43.3	39.7	40.7
Luteolin	643.3	683.3	643.4	696.7	1116.7	1530.0
Apigenin	94.7	134.0	123.3	119.0	700.0	443.3
Kaempferol	373.3	301.3	326.3	256.7	211.0	226.3
Total	12722.3	3426.2	2711.4	2367.6	2660.7	2939.9
Propenyl phenol						
Eugenol	145.7	34.0	44.5	206.7	94.3	43.0
Terpenoid						
Ursolic acid	373.4	8033.3	6933.3	14100.0	1473.3	4800.0

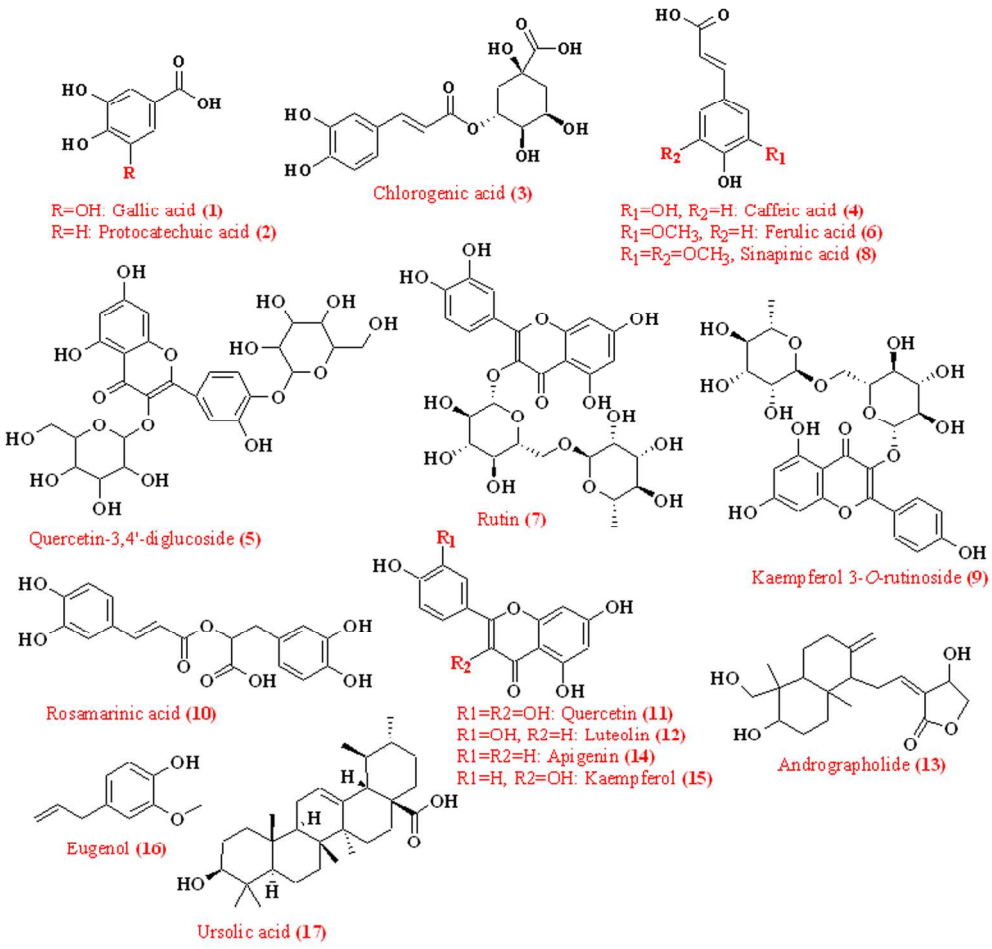

Figure 1. Chemical structures of investigated analytes and internal standard.
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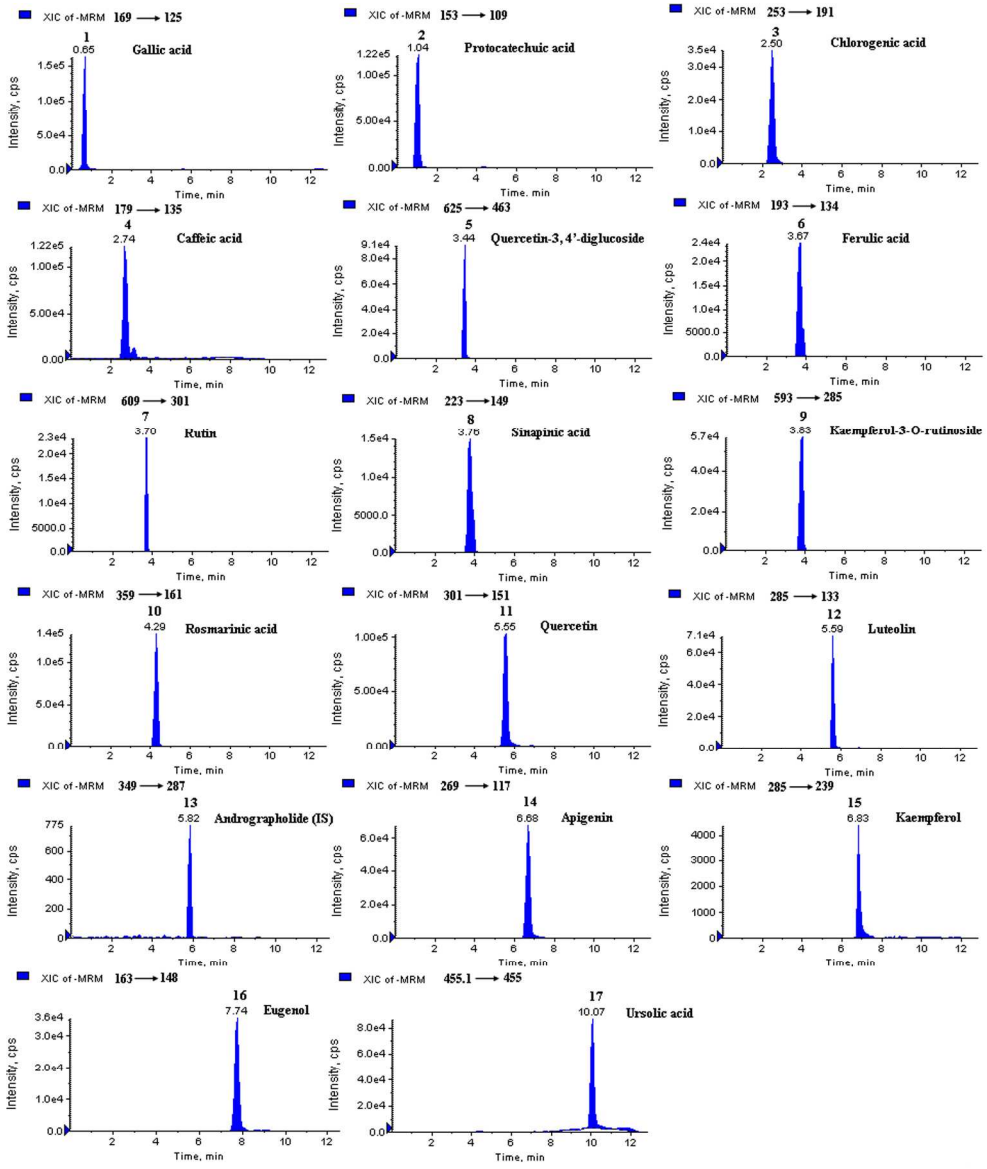

Figure 2. UHPLC-MRM extracted ion chromatogram of analytes and internal standard.
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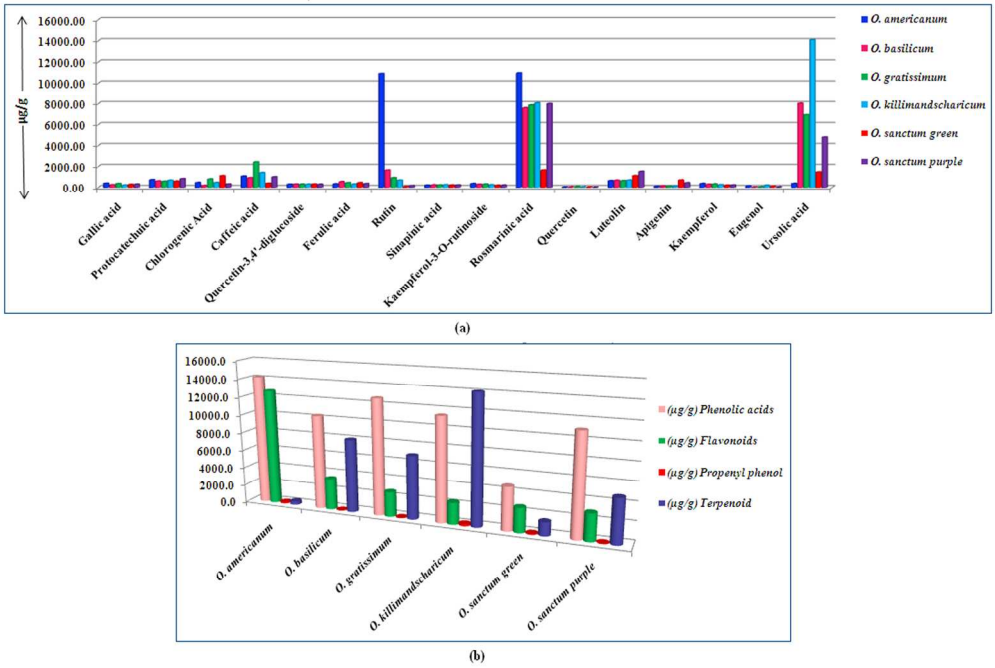

Figure 3. (a). Graphical representation of distribution of sixteen bioactive constituents in leaf extracts of six Ocimum species. (b). Total content (µg/g) of phenolic acids, flavonoids, propenyl phenol and terpenoid in leaf extracts of six Ocimum species.
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Graphical abstract:

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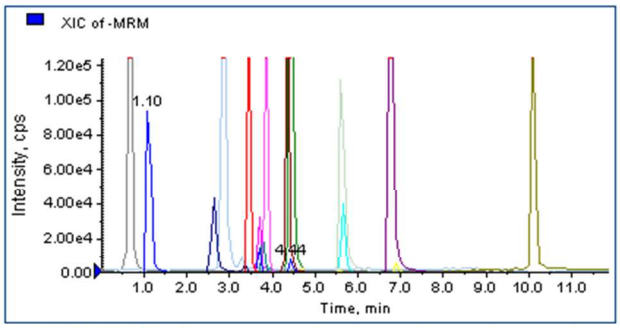
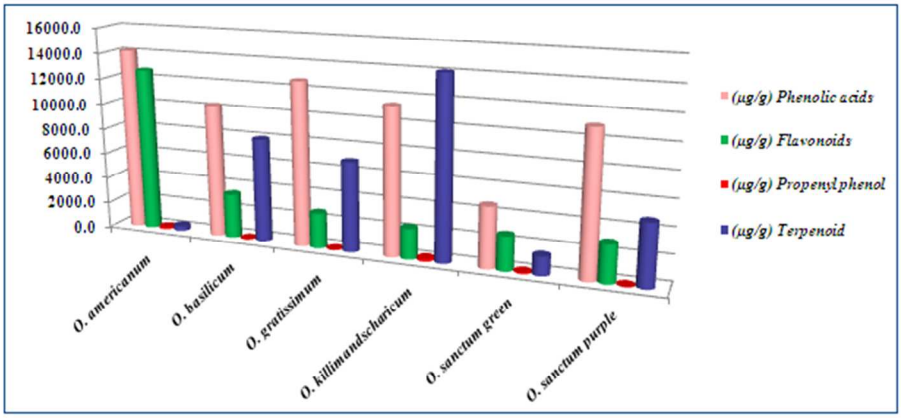
UHPLC-ESI-MS/MS analysis



UHPLC-MRM chromatogram of analytes and internal standard

Aqueous methanolic extract of leaf



Total content (µg/g) of phenolic acids, flavonoids, propenyl phenol and tepenoid in leaf extracts of six *Ocimum* species.