Trends in Phytochemical Research (TPR)

Journal Homepage: https://tpr.shahrood.iau.ir/

Original Research Article

Anti-melanogenic, antioxidant potentialities, and metabolome classification of six *Ocimum* species: Metabolomics and *in-silico* approaches

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ABSTRACT

This investigation was designed to evaluate the anti-tyrosinase and antioxidant potentialities of leaves and inflorescences of six *Ocimum* species. Currently, cosmaceutical and nutraceutical industries are looking for sources that are substitutes for synthetic agents which are natural or plant-derived bioactive components which are non-toxic skin lightening agents and anti-browning agents for fruits and vegetables. Both leaves and inflorescences of all the *Ocimum* species demonstrated anti-tyrosinase activities in a dose-dependent manner. GC/MS revealed seven phenolic compounds viz., *p*-arbutin, quinic acid, ferulic acid, 1,2,4-benzene triol, gallic acid, vanillic acid, and *p*-coumaric acid present in the studied plant parts and exhibited anti-tyrosinase activity with significant IC_{50} values. Metabolomic and chemometric strategies deciphered metabolome classification of the studied species. Seven identified phenolic compounds showing anti-tyrosinase activity were further subjected to *in silico* analysis to explore their binding mechanism with tyrosinase enzyme, and were found to interact with the targeted enzyme with high binding affinity.

ARTICLE HISTORY

Received: 13 January 2023
Revised: 12 March 2023
Accepted: 27 March 2023
ePublished: 29 March 2023

KEYWORDS

Anti-melanogenic
Antioxidant
Metabolome
Ocimum
p-Arbutin
Quinic acid

dor: 20.1001.1.25883623.2023.7.1.2.5

1. Introduction

Undesirable browning during food processing and preparation is one of the critical concerns for food technologists. Browning of fruits, vegetables and other foods may cause deterioration in appearance, taste, color, smell, and other organoleptic and nutritional properties of food (Friedman et al., 1996). Browning of food is a chemical reaction, triggered by the enzymatic oxidation of phenolic compounds in foods. In this reaction, quinones are produced as an intermediate product, which changes rapidly and forms brown polymers called melanins (Martinez and Whitaker, 1995). On the other hand, the coloration in eyes, skin, and hair of human body are regulated by the distribution of melanin pigments (Hearing and Tsukamto, 1991). Although melanin protects our skin from broad wavelengths of UV radiation of sunrays and scavenges free radicals produced in skin (Brown, 2001), but its overproduction or accumulation in an abnormal amount in epidermal layers of human body may lead to many skin disorders such as lentigines, melasma, freckles, abnormal pigmentation, periorbital hyperpigmentation, degeneration of neurons linked with Parkinsonism and melanoma or skin cancer risk (Cestari et al., 2014; Dorga and Sarangal, 2014; Nouveau et al., 2016).

The whole process of melanin synthesis is known as melanogenesis. Tyrosinase (EC1.14.18.1) is the main phenolic oxidase enzyme under the metallo-protein group. This group has two copper atoms which are located on its active site. In the biochemical process of melanin biosynthesis, the monophenol, L-tyrosine gets hydroxylated to an orthophenol, named 3,4-dihydroxyphenylanaline (DOPA) by tyrosinase, and subsequently oxidizes DOPA into dopaquinone (orthoquinone) under the influence of molecular oxygen and enzymes such as cresolase and catecholase,

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respectively. Furthermore, dopaquinone goes through some catalytic reaction and melanin is formed as an end-product of this process (Sánchez-Ferrer et al., 1995; Chang, 2009).

Reactive Oxygen Species (ROS) are originated from cellular metabolisms. Huge amount of reactive oxygen species may contribute to oxidative stress causing oxidation of biomolecules such as amino acids, polysaccharides, nucleic acids and fatty acids and may expedite to cellular death (Ragu et al., 2007). As a result, it may induce several stress related disorders, cardiovascular diseases, and different types of cancer (Kunsch and Medford, 1995). Our skin being the most unprotected part of our body rapidly goes through several types of oxidative stresses. When our skin remains continuously exposed to direct solar UV radiation that results in hyper-pigmentation and untimely aging (Kim et al., 2008; Heo et al., 2009).

As tyrosinase is the key controlling enzyme for melanin synthesis, much prominence in bio-prospecting must be given to plant extracts and their fractionated compounds or products that may have potential anti-tyrosinase activity, and those can be used especially in cosmetic and food industries with growing demand. Currently, different pharmaceutical, cosmaceutical and nutraceutical industries are looking for sources that are alternative of synthetic agents, either natural or plant-derived, and safer bioactive components such as antioxidants and non-toxic skin lightening agents, like tyrosinase inhibitory agents (Pillaiyar et al., 2017). Thus, the development of tyrosinase inhibitors with antioxidant, anti-aging, anti-wrinkle and anti-melanogenic potential have become more tempting issues in current scientific study and research. There are several synthetic agents or compounds to control the metabolism of pigmentation such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) which could be promoters of carcinogenesis (Barlow, 1990) . So it is worthy to find more active but safer medicines from natural sources viz. plants. Nowadays, pharmaceutical companies are mostly showing their interest in finding and developing active chemical substances from which synthetic analogues can be prepared and developed. To a great extent these can be much more reproducible, patentable, safe and economically viable (Svoboda and Hampson, 1999; Newman, 2008). In the last few decades, skin-lightening agents predominantly have been controlling the whole world's cosmetic industry. It has been reported that globally 15% of the population invested their money for purchasing these skin-lightening agents (Pillaiyar et al., 2017).

Natural products which inhibit melanin synthesis are one of the popular useable products in case of cosmetic applications for skin-whitening agents and in food processing industries for anti-browning preparations of food substances. On the other hand, oxidative stress is a major cause for modulation of dermal alterations such as UV exposure and aging of skin. Antioxidants such as ascorbic acid, flavonoids, and polyphenolic substances inhibit oxidative stress and avert or ameliorate skin aging. Antioxidant properties of plant extracts contribute to anti-wrinkle and anti-aging property, as well (Masaki, 2010).

In the fields of phytochemistry, pharmacognosy and ethnopharmacology, specific identification of collected medicinal plants plays immense importance to take a plant-derived compound from local traditional healers to the pharmacy. Lamiaceae is a big family which has high numbers of medicinal plants (Amirahmadi et al., 2022). Several species of this family are aromatic plants that are highly useful in traditional medicine and in pharmaceutical and food industries because of their immense biological activities (Nieto et al., 2017). They are used as stimulants for blood circulation and digestion, to strengthen the central nervous system, as expectorant, anti-spasmodic, antiseptic, diuretic, carminative and tonic (Nieto et al., 2017; Popovic-Djordjevic et al., 2018). The most popular and familiar plants in the family Lamiaceae are oregano (*Origanum vulgare*), Sage (*Salvia officinalis*), thyme (*Thymus vulgaris*), rosemary (*Salvia rosmarinus*) etc. (Nieto et al., 2017). *Ocimum* is another widely pre-eminent herb of the Lamiaceae family.

Ocimum (Lamiaceae) comprises of nearly 150 species of herbs and shrubs, widely distributed throughout the world especially native to Asia, Africa, South and Central America. Different *Ocimum* species collectively called basil are the most common and esteemed culinary. The word basil emanates from basilikos meaning royal. Basils are mainly used as fresh, frozen and dried leaves, essential oils, and basils are consumed around the world. Enzyme inhibitors have the potentiality to prevent several diseases by preventing the reaction of the enzyme with substrate and further the product formation. Modern nutraceutical, pharmaceutical, and cosmaceutical industries are in search of plant-derived, safer bioactive drugs or components for anti-browning agents for food substances and skin lightening agents (Mohammadhosseini et al., 2021, 2022). The extracts of leafy parts and florescences of six *Ocimum* species were assessed for antioxidant properties and activities against the tyrosinase enzyme, and very high activities were found in controlling the melanogenesis.

Natural enzyme inhibitors have great specificity and potency with few or no adverse effects, and possess minimum lethal outcome. Metabolites, separated and identified from medicinal plants and its extracts and fractions have pronounced enzyme inhibition effect (Rauf et al., 2017). Therefore, the present work aims to screen and compare the anti-tyrosinase and antioxidant activities of leaves and florescences of six *Ocimum* species. The use of tulsi (*Ocimum* sp.) in daily rituals is a great witness in Ayurveda for providing solutions to modern problems (Afolabi et al., 2007). The metabolome classification and chemoprofiling of the florescences and leaves of the selected species of *Ocimum* were also determined using GC/MS based metabolomics and chemometric tools. Further correlation analysis drove us to identify the potent plant metabolites against anti-tyrosinase activity. Furthermore, molecular docking simulates underlying mechanism behind tyrosinase inhibition.

2. Experimental

2.1. Reagents and chemicals

DPPH (α,α-diphenyl-β-picrylhydrazyl), ammonium molybdate, ascorbic acid were procured from MERCK, sodium nitrite, aluminium chloride, catechin, vanillic acid, 1,2,4-benzenetriol, *p*-coumaric acid, ribitol (adonitol), methoxyamine hydrochloride, MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) with 1% TMCS (trimethylchlorosilane), fatty acid methyl esters (FAME) were purchased from SIGMA-ALDRICH, folin-ciocalteau, gallic acid, sodium nitroprusside, sulphanilamide, naphthylethylenediaminedihydrochloride (NEDD), nitro blue tetrazolium (NBT), ethylenediamine tetra acetic acid (EDTA), methionine, riboflavin, L-tyrosine, tyrosinase (polyphenol oxidase) ex. mushroom, *p*-arbutin, quinic acid, ferulic acid were obtained from Sisco Research Laboratory (SRL) Pvt. Ltd. IMC ShriTulsi Drop (20 mL) was procured from Herby House Pvt. Ltd. For GC/MS analysis HPLC grade solvents were used.

2.2. Collection and authentication of plant materials

The leaves and florescences of six *Ocimum* species belonging to Lamiaceae family, viz., *Ocimum tenuiflorum* L. (green basil), *Ocimum tenuiflorum* L. (purple basil), *Ocimum americanum* L., *Ocimum gratissimum* L. and *Ocimum kilimandscharicum* Guerke were collected from the medicinal plant garden of Narendrapur Ramakrishna Mission, Kolkata, West Bengal, India. Collection of leaves were done during the month of June to August (monsoon) and of inflorescences during the month of November to February (winter season), 2018. Plant materials of *Ocimum basilicum* L. were procured from the Agri Horticultural Society of India, Kolkata, India. The plant species were authenticated by BSI, India. Taxonomically green basil (*O. tenuiflorum*) and purple basil (*O. tenuiflorum*) were found identical, so they were depicted in the same species name as *Ocimum tenuiflorum* L. These plant specimens were deposited with voucher specimen numbers of 20101 to 20106 at Calcutta University Herbarium (CUH), Kolkata, West Bengal, India (Table 1, Fig. 1).

2.3. Preparation of hydro-methanolic extract of plant materials

Collected leaves and inflorescences were cleaned properly in tap water and after that washed with double distilled water for removing the dirt and suspended particulate matters. The leaves and florescence parts of *Ocimum* sps. were freeze ground individually with mortar and pestle using liquid N_2. The fine dust was then suspended with 70% methanol and heated at 60-70 °C for 3 hrs in a heated water bath with occasional shaking. After cooling it down to room temperature, extracts were cold-centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant collected were pooled carefully and then concentrated upon evaporation using rotary evaporator under controlled temperature and pressure. The residual samples or the crude extracts were preserved at -20 °C refrigerator before further experiments. A mixture of 70% methanolic extracts of six experimental species of *Ocimum* were prepared (mixed in 1:1:1:1:1:1 ratio) to compare the mixture's bioactivity with commercially available *Ocimum* drop (ShriTulsi) manufactured by International Herbal Corporation PVT. LTD. This marketed *Ocimum* drop was a combination of five species of tulsi as mentioned in the product's label. This market-available mixed *Ocimum* liquid was dissolved in 100% methanol.

2.4. Preparation of samples for chemoprofiling among *Ocimum* species

10 mg of crude extracts of both leaves and inflorescences of each of the species of *Ocimum* were dissolved separately in methanol: water (1:1) solution. 20 µL of ribitol [prepared 2 mg/10 mL solution in water (HPLC)] was added as an IS [Internal Standard] in each extract. 100 µL of each sample aliquot was divided into 4 EP [eppendorff tubes] tubes and dried upon evaporation under reduced temperature in a boiling waterbath. The pellet obtained was re-constituted in 5 µL of methoxyamine hydrochloride (prepared in pyridine @ 200 mg/ 10 mL) and the mixture was vortexed occasionally for 1 hour 30 min at 30 °C. Subsequently into the solution, 45 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) was given, and the mixture was shaken and incubated at 37 °C for another half an hour. Through this process the volatility of metabolites had been increased. 1 µL of fatty acid methyl esters (FAME) markers [a mixture of Internal Retention Index (IRI)] was added into the mixture. FAME was prepared by dissolving fatty acid methyl esters (C_8-C_{26} linear chain length) into chloroform (HPLC). C_8-C_{16} were prepared at a concentration of 8 mg /10 mL and C_{18}-C_{26} were prepared at a concentration of 4 mg/10 mL.

2.5. GC/MS analysis of the samples

For chromatographic analysis and identification of metabolites, the methods of Kind et al., 2009 was followed after a few modifications (Das et al., 2016). DB-5 MS capillary column (Agilent J&W GC columns, USA) with a dimension of 30 m length × 0.25 mm diameter × 0.25 µm film was used. Injection was designed in sandwich-style with rapid needle pace to avoid formation of viscous. The run process was accomplished for 37.5 min with 10 min hold until cooling the machine, under the following oven temperature program: oven ramp 60 °C (1-min hold) to 325 °C at 10 °C/min. The injection temperature was adjusted at 250 °C, the MS transferline at 290 °C, and the ion source at 230 °C. Helium was the carrier gas used which was streamed at a constant stream rate of 0.723 mL/min (carrier linear velocity 31.141 cm/sec). Split ratio (1:5) was used while 1 µL of samples were injected into the GC injection port. Before analysis, the method was finely tuned using standards of fatty acid methyl ester available on the Fiehn GC-MS Metabolomics library (2008) by following users' guide. AMDIS was used for simplification (deconvolution) of chromatographical and mass spectroscopical results and to detect

Table 1
Plant materials collected during monsoon and winter seasons, their scientific names, and accession numbers.

Name of *Ocimum* species	Accession No.	Collected parts	Collection time
Ocimum tenuiflorum L. (Green basil)	20102	Leaves	Rainy season (June-August)
		Inflorescence	Winter season (November-February)
Ocimum tenuiflorum L. (Purple basil)	20104	Leaves	Rainy season (June-August)
		Inflorescence	Winter season (November-February)
Ocimum basilicum L.	20103	Leaves	Rainy season (June-August)
		Inflorescence	Winter season (November-February)
Ocimum americanum L.	20106	Leaves	Rainy season (June-August)
		Inflorescence	Winter season (November-February)
Ocimum kilimandscharicum Guerke	20101	Leaves	Rainy season (June-August)
		Inflorescence	Winter season (November-February)
Ocimum gratissimum L.	20105	Leaves	Rainy season (June-August)
		Inflorescence	Winter season (November-February)

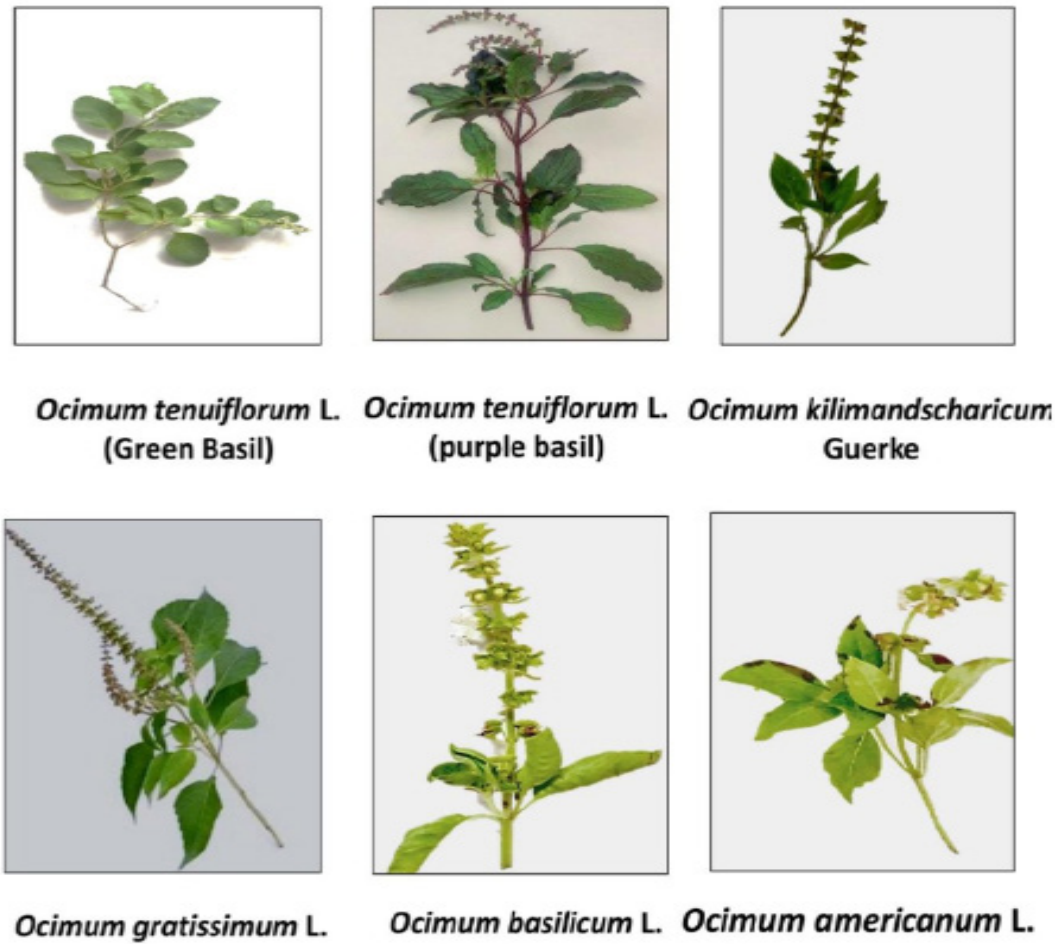


Fig. 1. Morphological variations among the different species of *Ocimum* under experimental study.

metabolite peaks. Air water check, auto-tuning and tune evaluation of MSD were done at least once or twice in a week. All samples were analyzed in a randomised manner. Identification of compounds were done by equating the Retention Time [RT], Retention Index, and also by matching the fragmentation patterns of the mass spectra with the entries of metabolites in Agilent Fiehn GC/MS Metabolomics library (2008) (Agilent Technologies INC., Wilmington, USA) using Metabolite database-Automated Mass Spectral Deconvolution and Identification System (AMDIS) and also by using Agilent Retention Time Locking [RTL] method. RT of several metabolites was also authenticated with RT of standard compounds and thereafter identification of the metabolites were confirmed.

2.6. Multivariate data analysis for metabolome classification of *Ocimum* species

Chemometric analyses were conducted, in an attempt to classify the studied *Ocimum* species based on GC/MS detected polar metabolites of both leaves and inflorescences. Before normalization, half of the limit of detection had been used in place of the missing values of the datasets (Fiehn, 2006). The peak areas of the detected metabolites had been regularised (normalised) with the mass of the crude extract taken for GC/MS analysis and peak areas of the IS and thus the ratios of relative responses (RRR) of all the metabolites had been considered. The RRRs of all the identified components were assumed as the independent variables.

For multivariate analysis (MVA), the regularised (normalised) data were transformed into .csv and the data were imported to Metaboanalyst 5.0. PCA and PLS-DA were executed for all the studied *Ocimum* species and for both leaf and inflorescence samples. R^2 (goodness of fit) [0.98889 (for leaves) and 0.99313 (for inflorescence)] and Q^2 (goodness of prediction) [0.82644 (for leaves) and 0.93542 (for inflorescence)] values were checked for validation of these models. The important variables responsible for metabolome classification among the species were selected from the VIP (variable importance in projection) scores. One way ANOVA and Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$) were implemented by the regularised data. Generalized logarithm transformed dataset was also subjected to generate dendograms.

2.7. Inhibition of mushroom tyrosinase activity

Mushroom tyrosinase inhibition was accomplished using the methodology of Vanni et al. 1990 and Kang et al., 2004 with some minor modifications. In this assay as a substrate L-tyrosine was used. The reaction mixture consisted of 1.5 mM L-tyrosine, 100 µL of plant extract and 140 µL (0.05 mM) of sodium-phosphate buffer (pH 6.8). Then 20 µL of tyrosinase polyphenol oxidase enzyme (source: mushroom) (1250 unit/mL) was put in the reaction mix and the reaction mixture was incubated at 37 °C for half an hour. The optical density (OD) value was estimated at 492 nm in UV - VIS spectrophotometer (Labman Scientific Instruments PVT. LTD, Chennai, India). *p*-arbutin was taken as a positive standard. For calculating the percentage of inhibition activity equation has been used as:

$$\% \text{ inhibition} = (A - S) / A \times 100 \quad \text{(Eqn. 1)}$$

Where A represents the OD value of the control and S represents the OD value of the test sample. From triplicate measurements all data were stated as means ± SD and they were analysed by ANOVA. The value of $p < 0.05$ was reflected as significant value of the data.

2.8. Measurement of antioxidant capacity

2.8.1. Assay for DPPH scavenging activity

The anti-radical efficiency of *Ocimum* extracts was estimated by DPPH assay following the method reported by Braca et al. (2001), on the basis of the stable free radical DPPH hydrogen donor activity. The reaction blend contained 50 µL of plant extract of different concentrations and 1.5 mL of DPPH solution [0.004% of α,α-diphenyl-β-picrylhydrazyl prepared in methanol] The reaction mixture was incubated at normal room temperature for 30 min. Experimental samples showed changes in colour from mauve to yellow after 30 min. The optical density value was recorded at 517 nm. The blank set consisted of samples in methanol only and control set contained solution of DPPH. The percentage of activity for reduction of DPPH by plant extracts was measured as:

$$\text{Scavenging activity (\%)} = (C - S / C) \times 100 \quad \text{(Eqn. 2)}$$

Where C defined as OD value of control and S defined as OD value of sample mixture.

2.8.2. Assay for $O_2^{\cdot-}$ superoxide scavenging activity

Determination of superoxide anion anti-radical activity was done according to the method of Banerjee and De (2013). In this system, the capacity for the inhibition of the photochemical reduction of NBT (nitrobluetetrazolium) was measured (Beauchamp and Fridovich, 1971). Superoxide radicals ($O_2^{\cdot-}$) were generated by illumination of riboflavin containing solution. One mL sample solution of plant extract was added to a mixture of 50 mM sodium phosphate buffer (pH-7.8), riboflavin (2 µM), EDTA (100 µM), methionine (13 mM) and NBT solution (45 µM). Blue formazan produced under illumination with a fluorescent lamp was measured spectrophotometrically at 560 nm wavelength after 10 min incubation. The whole reaction was conducted inside an enclosed box lined with aluminium foil for proper illumination of the experimental sets. Blank set was placed in the dark. For calculating the % inhibition of superoxide generation, the absorbance values of control set and those of the reaction mixture containing plant extract were compared.

2.8.3. Determination of TAC (Total antioxidant capacity)

The assay was followed using the method of Prieto et al., 1999. In this assay, Mo(VI) is reduced to Mo(V) by the plant sample and subsequently formed a green phosphate/Mo(V) complex at acidic pH. 150 µL of plant extract was added to 1.5 mL of reagent solution. 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM

ammonium molybdate were mixed with each other for preparation of reagent solution. The reaction mixture was incubated at 95 °C for 1 hour 30 min. After cooled down at room temperature, the optical density value of the reaction mixture was read at 695 nm against a blank set. The antioxidant capacity was reflected as ascorbic acid (mg) equivalent per mg of plant extract.

2.8.4. Nitric oxide (NO) anti-radical activity

The study was done by following the method of Sumanout et. al., 2004. Nitric oxide (NO) generated from sodium nitroprusside was detected by Griess reagent at 546 nm in presence or absence of plant extract. 100 µL of sodium nitroprusside (100 mM) dissolved in PBS (pH-7.4) and 900 µL of different concentrations of plant crude extracts were prepared in PBS. These mixtures were incubated at 25 °C for 2 hours 30 min. NO released was detected by addition of (1 mL) Griess reagent. Griess reagent was prepared by mixing 1% sulphanilamide and 0.1% NEDD (*N*-1-naphthyleth ylenediaminedihydrochloride) each in phosphoric acid (2.5%); The OD value of the chromophore was recorded spectrophotometrically at 546 nm. This chromophore was formed due to diazotization of nitrite with sulphanilamide and its subsequent coupling with *N*-1-naphthylethylenediaminedihydrochloride.

2.8.5. Estimation of TPC

Folin-Ciocalteau method had been used for the estimation of total phenol content (TPC) (Sadasivam and Manikam, 1992) of individual plant extracts. 250 µL Folin-Ciocalteau was mixed to 1.5 mL of each plant extract. After 3 min incubation at room temperature, 2 mL of Na_2CO_3 (20%) solution was mixed thoroughly and then the mixture was placed in heated water bath for 1 min. After cooling the OD value of the mixture was recorded at 690 nm against blank. TPC was estimated on the basis of mg/mg gallic acid equivalents in mg/mg (mg GAE/mg) of crude extract.

2.8.6. Estimation of TFC

The total flavonoid content (TFC) of individual plant extract was measured following Kim et al. (2003). 500 µL of plant extract was mixed with 2 mL of double distilled water and 150 µL of $NaNO_2$ solution (5%). After 5 min incubation, 150 µL of $AlCl_3$ (10%) was put in this mixture. Again after 1 min, 1 mL of (1 M) sodium hydroxide and 1.2 mL of double distilled water were added to form a 5 mL reaction mixture. OD value of the mixture (pink colour) was estimated at 510 nm against blank. Total flavonoid content (TFC) was evaluated on the basis of mg/mg catechin equivalents in mg/mg (mg CE/mg) of crude extract.

2.9. Statistical analysis

All the assays were executed thrice. Using MS Excel 2010, mathematical calculations such as means ± standard deviation (SD) from replicates within the experiments and analyses were done. For GC/MS analyses, after standardizing the peak areas of the metabolites, the RRR of all the detected compounds were measured by extract dry weight and by the peak areas of the IS (adonitol) (Oms-Oliu et al., 2011). The metabolome classification of the studied *Ocimum* species was statistically evaluated by Tukey's method to relate between the studied species.

2.10. *In-silico* Approaches

Recently in drug designing, innovation and expansion, *in-silico* technologies play an important role chiefly due to their rapid productivity, cost-effectiveness and labour saving characteristics (Ekins et al., 2007). *In-silico* approaches have been used for detection and identification of new natural compounds as drug targets, for comparing between the *in vitro* and *in vivo* counterparts in response to the bioactivities. Most significantly, *in silico* methods are a rational addition of meticulous in vitro experiments to shorten elaborative screening via high throughput methods (Colquitt et al., 2011).

Gibbs free energy is an important parameter that determines the ligand-protein interaction. Gibbs free energy is measured in terms of binding energy in AutoDockVina tools. AutoDockVina is an *in-silico* docking tool specialized in molecular docking studies. Table 3 summarizes the list of various phytochemicals and their inhibitory potential against tyrosinase enzyme. Briefly, the phytochemicals (ligands) are downloaded from Pubchem as .sdf file, and were subsequently converted into .pdb file by an online tool, OpenBabel. Likewise, the protein (PDB ID: 4OUA) is downloaded from Protein Data Bank as .pdb file as a co-crystal complex between the latent and active form of mushroom tyrosinase. The protein was further prepared for docking by removal of water, hetero-atoms and ligands, and latent domain (544 amino acids chain) in the protein crystal by Discovery Studio visualizer, while preserving the active domain of tyrosinase (382 amino acids), whereas, the addition of polar hydrogen, Kollman and Gasteiger charges were done by AutoDockVina. All the ligands and proteins were converted into AutoDockVina compatible .pdbqt files. The docking parameters for each ligand-protein interaction includes a grid dimension of 21.303, 50.422 and 39.872 for x-, y- and z-coordinates. Likewise, the grid size includes 54, 54 and 56 for x-, y- and z-coordinates.

3. Results and Discussion

3.1. Chemoprofiling of *Ocimum* species

116 metabolites comprising 16 amino acids, 32 organic acids, 20 phenols, 6 fatty acids, 26 sugars and polyols,1 inorganic acid and 106 metabolites containing 17 amino acids, 20 organic acids, 16 phenols, 8 fatty acids, 30 sugars and polyols, 1 inorganic acid were identified from leaves and inflorescences respectively using Agilent Fiehn Metabolomics library. For correct and appropriate identification, the MS of the derivatized products of the polar metabolites with locked retention time were considered. Semi-quantitative comparison of

Table 2

IC_{50} values of *Ocimum* sp. and standard phenolic compounds. ns: not significant; nd: not detected.

Plant parts used	Plant Samples	Enzyme inhibitory activity	Antioxidant activities					
		Tyrosinase inhibition IC_{50} value ± SD (mg/mL)	DPPH inhibition IC_{50} value ± SD (mg/mL)	Superoxide radical scavenging activity IC_{50} value ± SD (mg/mL)	Nitric Oxide radical scavenging activity IC_{50} value ± SD (mg/mL)	TAC AAE ≡ µg/mg	TPC GAE ≡ µg/mg	TFC CE ≡ µg/mg
Leaves	*Ocimum tenuiflorum* (Green basil)	28.26 ± 1.707	0.19 ± 0.01	0.79 ± 0.01	12.15 ± 0.18	0.02 ± 0.04	123.80 ± 11.03	12.22 ± 3.62
	Ocimum gratissimum	5.86 ± 0.09	0.24 ± 0.01	1.12 ± 0.05	6.53 ± 0.07	0.04 ± 0.01	131.19 ± 22.26	10.60 ± 2.80
	Ocimum kilimandscharicum	7.27 ± 0.16	0.1 ± 0.01	0.51 ± 0.00	6.46 ± 0.04	0.05 ± 0.01	170.44 ± 15.90	25.37 ± 4.97
	Ocimum americanum	6.13 ± 0.36	0.04± 0.00	0.15 ± 0.00	3.75 ± 0.02	0.07 ± 0.03	251.21 ± 35.69	43.88 ± 6.33
	Ocimum tenuiflorum (Purple basil)	3.06 ± 0.08	0.1 ± 0.00	0.25 ± 0.00	12.67 ± 0.14	0.05 ± 0.04	267.10 ± 38.04	32.11 ± 12.56
	Ocimum basilicum	8.71 ± 0.66	0.04 ± 0.01	0.08 ± 0.09	1.66 ± 0.01	0.07 ± 0.04	207.70 ± 15.14	55.07 ± 8.07
	Mixed *Ocimum* extracts	3.55 ± 0.2	0.1 ± 0.00	0.11 ± 0.00	nd	0.32 ± 0.02	nd	nd
	Shri Tulsi (Commercial drug)	19.59 ± 0.15	ns	168.00 ± 32.50	nd	6.27E-05± 0.00	nd	nd
Inflorescences	*Ocimum tenuiflorum* (Green basil)	6.97 ± 1.13	0.22 ± 0.00	0.29 ± 0.01	7.64 ± 0.09	0.07 ± 0.04	134.37 ± 21.65	14.31 ± 3.64
	Ocimum gratissimum	10.63 ± 0.36	0.2 ± 0.00	0.19 ± 0.02	21.94 ± 0.04	0.06 ± 0.02	278.93 ± 26.34	33.55 ± 13.01
	Ocimum kilimandscharicum	1.73 ± 0.44	0.19 ± 0.00	0.14 ± 0.00	17.50 ± 0.02	0.06 ± 0.03	215.60 ± 22.41	34.45 ± 8.21
	Ocimum americanum	7.03 ± 0.28	0.22 ± 0.00	0.44 ± 0.01	12.56 ± 0.05	0.04 ± 0.01	254.40 ± 12.92	41.99 ± 11.76
	Ocimum tenuiflorum (Purple basil)	7.62 ± 0.61	0.32 ± 0.00	0.47 ± 0.01	15.97 ± 0.30	0.07 ± 0.04	157.99 ± 23.58	26.16 ± 3.88
	Ocimum basilicum	1.78 ± 0.01	0.13 ± 0.00	0.09 ± 0.00	18.22 ± 0.01	0.08 ± 0.05	284.04 ± 15.24	43.86 ± 15.31

the identified metabolites based on RRR is presented in SM1 (leaves) and SM2 (inflorescences).

3.2. Multivariate Analysis (MVA) for different species of *Ocimum*

Principal component analysis (PCA) is a technique of multivariate analysis that analyses a data table based on which observations are described by several inter-correlated quantitative dependent variables. It's goal is to represent the important information in a more amenable, lower-dimension form without losing too much information (Mishra et al., 2017). In metabolomics study a large data table is generated from which only the most important information can be extracted by compressing the data and by analysing the structure of the observations and the variables. MVA describes the data sets in a meaningful simplified but robust manner. The leaves and inflorescences of the studied *Ocimum* showed clear discrimination among the species based on their metabolite profiling. Scores scatter plot of PCA model revealed clear discrimination among the species both in case of leaves (Fig. 2) and inflorescences (Fig. 3). High R^2 [0.98889 (for leaves) and 0.99313 (for inflorescence)] for PLS-DA and Q^2 [0.82644 (for leaves) and 0.93542 (for inflorescence)] for PLS-DA values validated the model. The ANOVA p values ≤ 0.05 also proved the discrimination of species distinctly. Among the 116 leaf metabolites, VIP scores plot revealed 25 top metabolites (Fig. 4A). The top 25 metabolites detected were mentioned in Supplementary Material (SM 3) which were the most important variables accountable for leaf metabolic discrimination of *Ocimum* species. On the other hand, among the detected inflorescence metabolites, VIP scores plot identified top 25 metabolites (Fig. 4B) (SM 4) which were found as the most important metabolites responsible for inflorescence metabolite discrimination among all the studied *Ocimum* species. Fig. 5A and Fig. 5B respectively show the level of differences of the 25 metabolites among the studied species both in leaves and inflorescences where X axis represents the different species and Y axis represents the RRR ± SE [Standard Error].
Dendograms were prepared with the help of Euclidean distance measures and ward clustering algorithms. Based on metabolite profiling of leaves and inflorescences, all the studied species showed distinct clusters as shown in Fig. 6.

3.3. Tyrosinase inhibitory activity

The crude 70% methanolic extracts of six species of *Ocimum* leaves and inflorescences were subjected to assays against the enzyme tyrosinase. Both leaves and inflorescences showed activity against the tyrosinase enzyme in a dose dependent mode. IC_{50} values of the six species of *Ocimum* leaves and inflorescence were compared (Table 2). The maximum activity was found in the leaves of *Ocimum tenuiflorum* (Krishna basil/ purple basil) with an IC_{50} value of 3.06 mg/mL ± 0.07 whereas *Ocimum tenuiflorum* (green basil) showed lowest inhibitory activity with an IC_{50} value of 28.26 mg/mL ± 1.71. On the other side, inflorescence of *Ocimum kilimandscharicum* showed highest tyrosinase inhibitory activity with an IC_{50} value of 1.73 mg/mL ± 0.43 subsequently *Ocimum basilicum* with an IC_{50} value of 1.78 mg/mL ± 0.00. IC_{50} value of positive standard (*p*-arbutin) was found 0.025 mg/mL ± 0.00 (Table 3).
Commercially available *Ocimum* drop exhibited very low inhibition with high IC_{50} value (19.59 mg/mL ± 0.15) in comparison to the mixture of different *Ocimum* crude extract (IC_{50} value of 3.55 mg/mL ± 0.2) as shown in Table 2.

3.4. Correlation between some phenolic compounds and tyrosinase inhibition activity

Through metabolomics analysis 20 phenols were detected from leaves and 16 phenols were detected from inflorescences. Among them *p*-arbutin, quinic acid, ferulic acid, 1,2,4-benzenetriol, gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and O-acetylsalicylic acid were detected present both in leaves and florescences of the investigated *Ocimum* species. 4-Hydroxy-3-methoxybenzoic acid was found in inflorescences of all the studied *Ocimum* species. All these phenols showed negative correlation with tyrosinase enzyme inhibition activity. 4-hydroxycinnamic acid present in leaves of different *Ocimum* species, also showed significant negative correlation with this enzyme inhibition. In accordance with the correlation, seven phenolic compounds viz., *p*-arbutin, quinic acid, ferulic acid, 1,2,4-benzenetriol, gallic acid, 4-hydroxy-3-methoxybenzoic acid (vanillic acid) and 4-hydroxycinnamic acid (*p*-coumaric acid) exhibited tyrosinase inhibition activity with significant IC_{50} values (Table 3). However, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and O-acetylsalicylic acid did not show any inhibition against the enzyme.

3.5. Antioxidant activities

All the species of *Ocimum* studied showed DPPH radical scavenging property in a dose dependent mode. In case of leaves, *Ocimum basilicum* and *Ocimum americanum* showed highest DPPH radical scavenging activity with IC_{50} values of 0.04 mg/mL ± 0.00 and 0.04 mg/mL ± 0.00, respectively. Whereas, *Ocimum gratissimum* showed lowest inhibitory activity with an IC_{50} value of 0.24 mg/mL ± 0.00. In case of inflorescences, the 70% methanolic solution of *Ocimum basilicum* showed highest DPPH radical scavenging activity with an IC_{50} value of 0.13 mg/mL ± 0.00, whereas, purple basil (*Ocimum tenuiflorum*) showed lowest DPPH radical scavenging property with an IC_{50} value of 0.13 mg/mL ± 0.00. (Table 2). The commercially available *Ocimum* drop exhibited no DPPH activity, whereas, moderate DPPH scavenging activity was found in mixed crude extracts of *Ocimum* with an IC_{50} value of 0.10 mg/mL ± 0.00.
In super-oxide free radical scavenging activity, *Ocimum basilicum* (for both leaves and inflorescence) showed highest activity (with an IC_{50} value of 0.08 mg/mL ± 0.09 and 0.09 mg/mL ± 0.00, respectively), whereas leaves of *Ocimum gratissimum* and inflorescences of purple basil *(Ocimum tenuiflorum)* showed lowest superoxide radical scavenging activity. In this assay, commercially

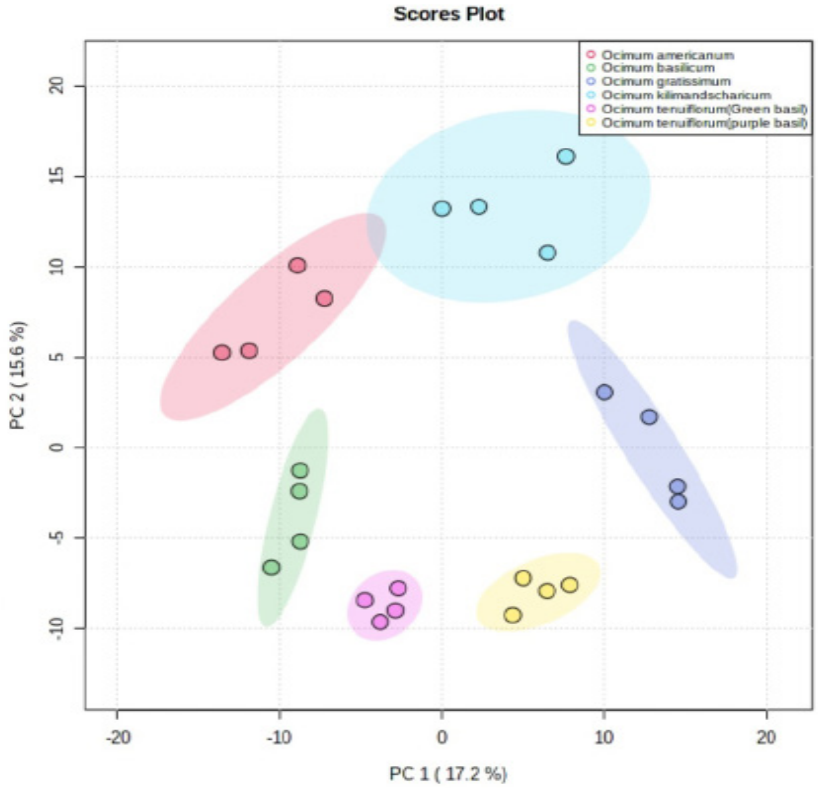


Fig. 2. PCA scores plot among leaves of *Ocimum tenuiflorum* (green and purple basil), *Ocimum basilicum*, *Ocimum americanum*, *Ocimum kilimandscharicum* and *Ocimum gratissimum* based on polar metabolite profile.

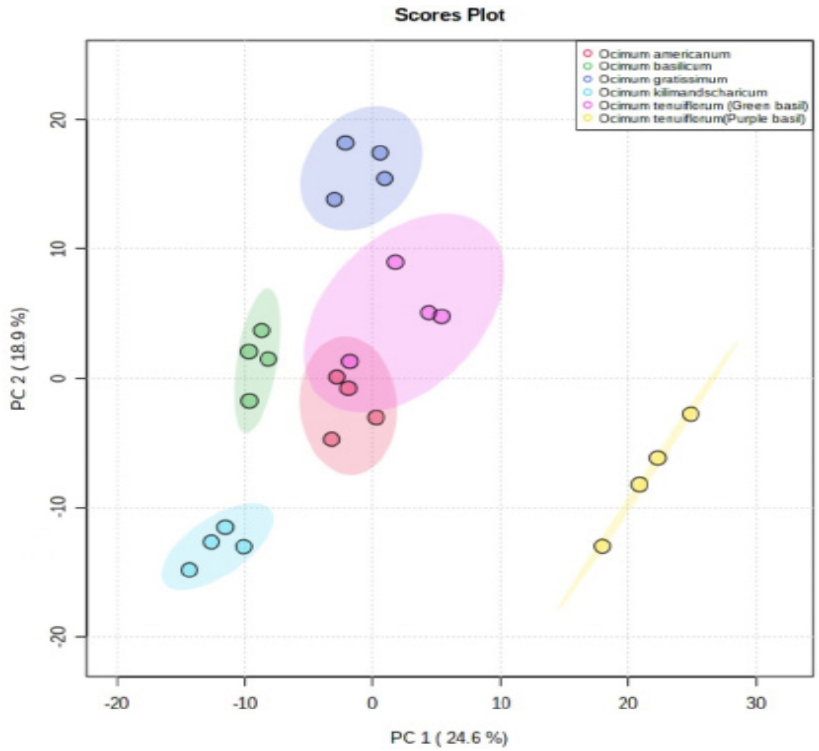


Fig. 3. PCA scores plot among inflorescences of *Ocimum tenuiflorum* (green and purple basil), *Ocimum basilicum*, *Ocimum americanum*, *Ocimum kilimandscharicum* and *Ocimum gratissimum* based on polar metabolite profile.

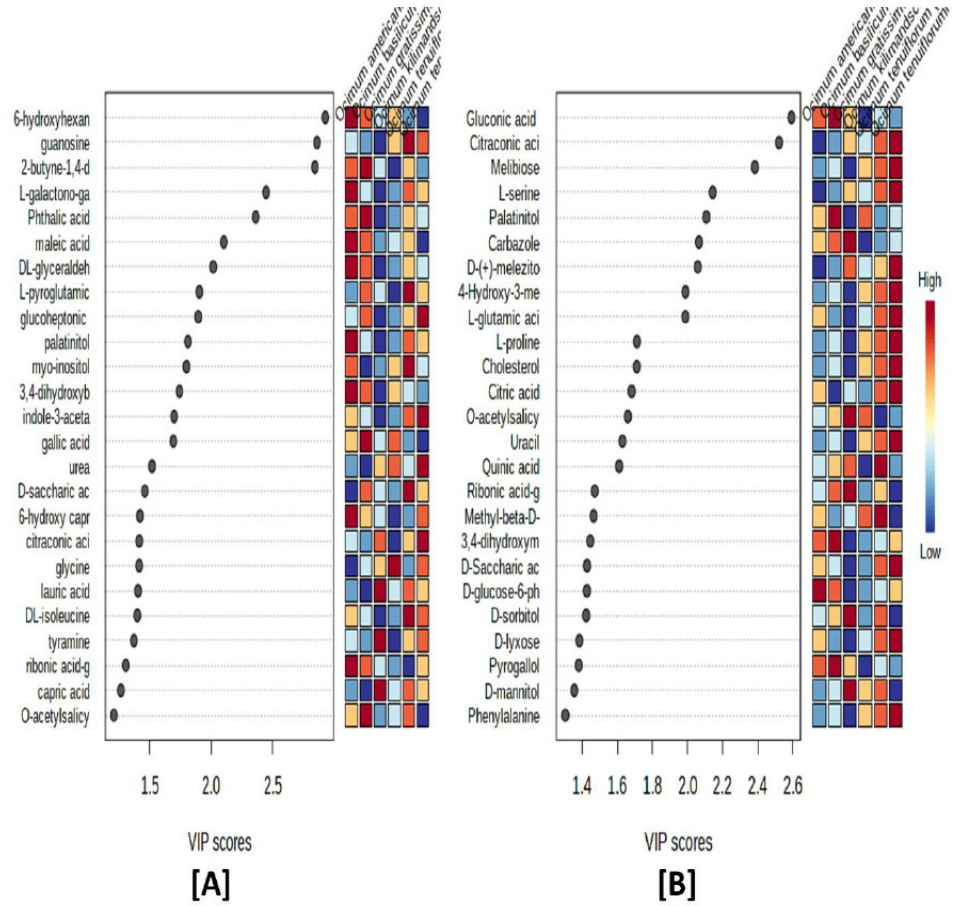
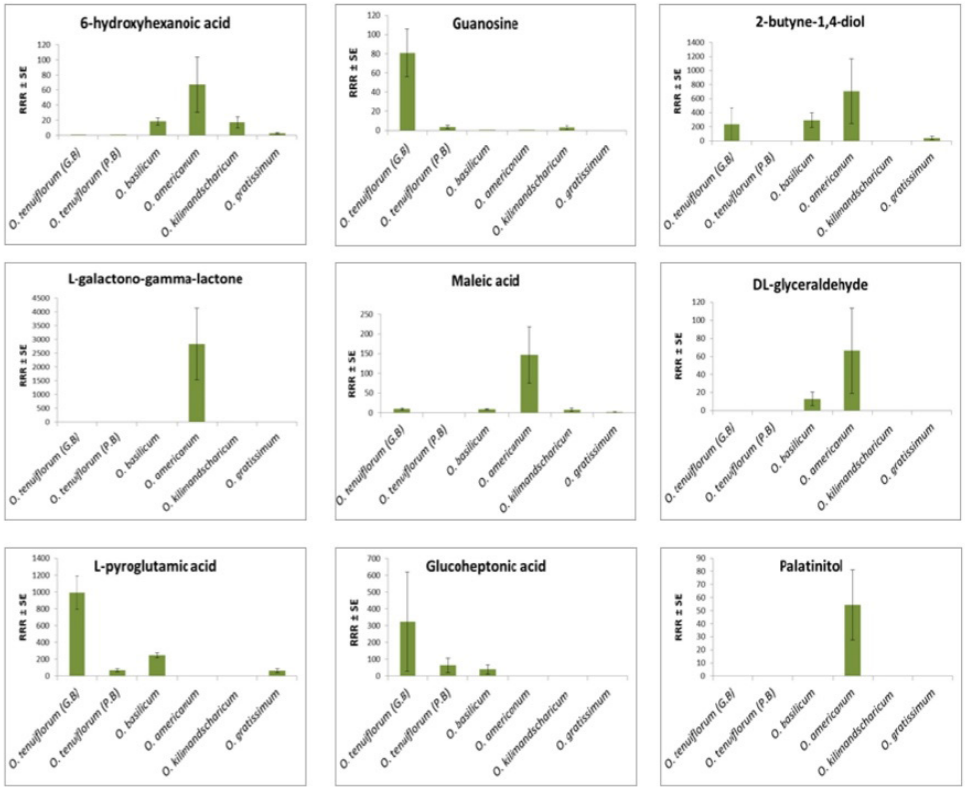
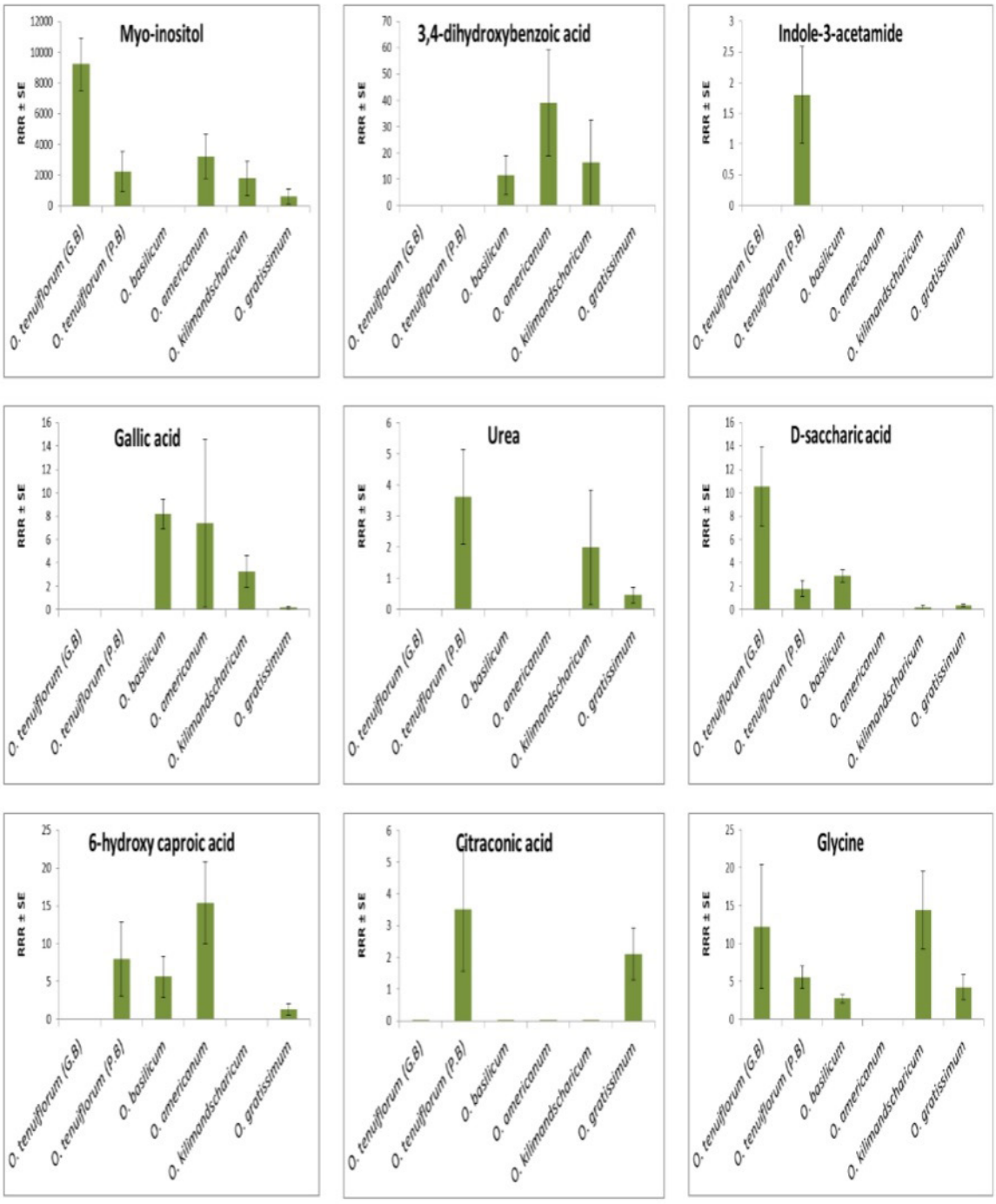
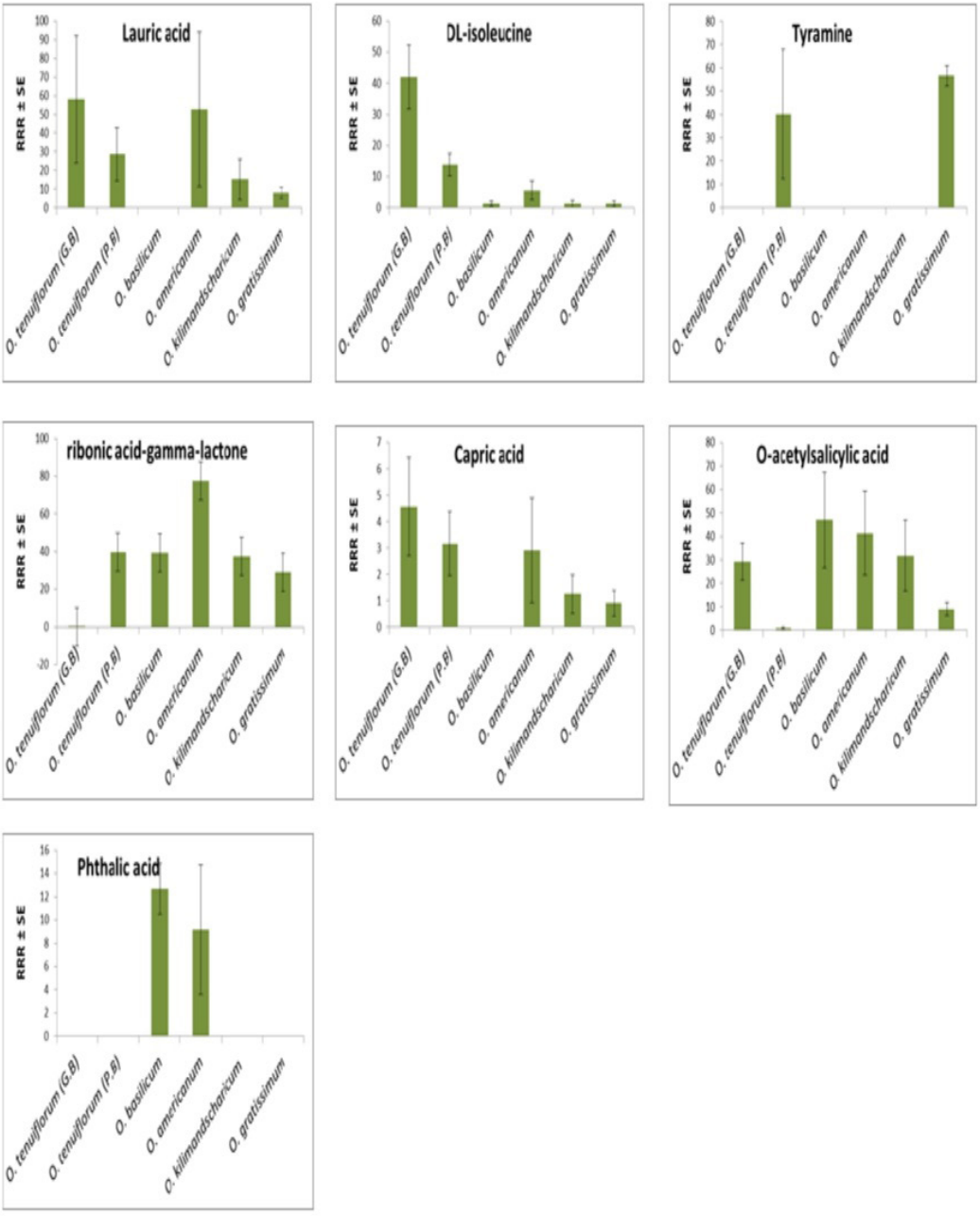


Fig. 4. Loading Plot in PLS-DA showing the variations in concentration among top 25 important metabolites in leaves (A) and inflorescences (B) of six *Ocimum* species, respectively.

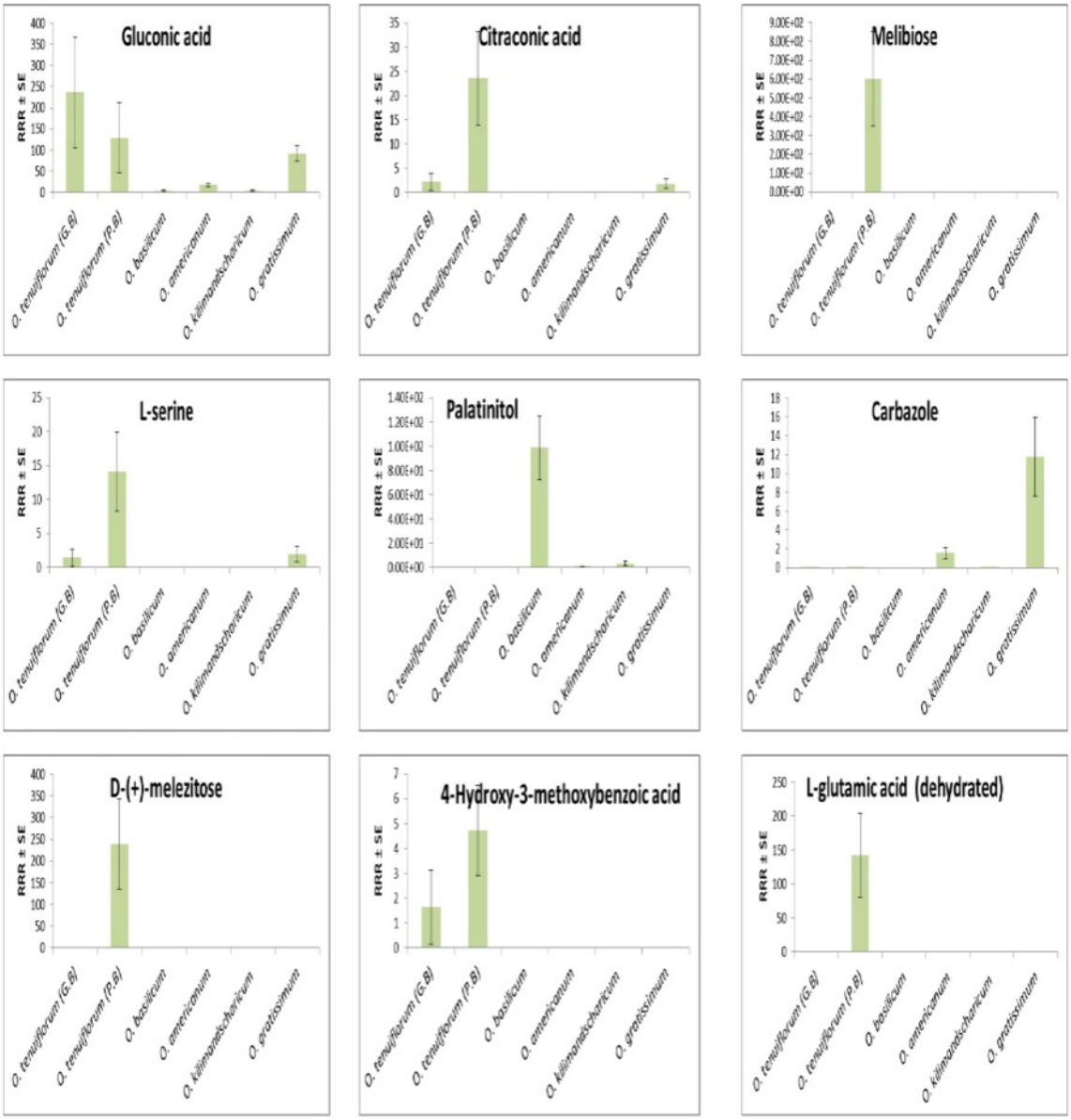


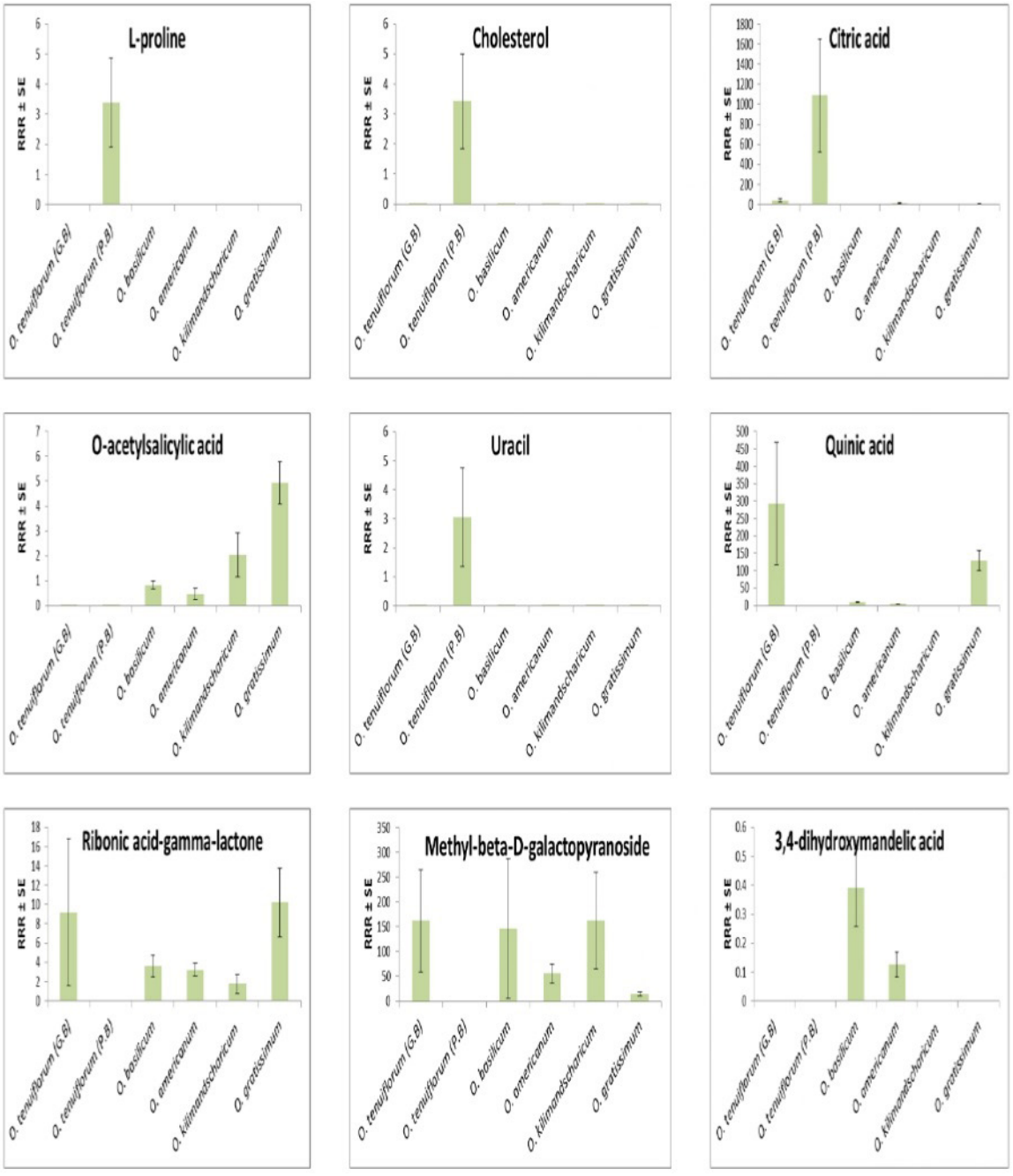

Myo-inositol
3,4-dihydroxybenzoic acid
Indole-3-acetamide
Gallic acid
Urea
D-saccharic acid
6-hydroxy caproic acid
Citraconic acid
Glycine
RRR ± SE
O. tenuiflorum (G.B)
O. tenuiflorum (P.B)
O. basilicum
O. americanum
O. kilimandscharicum
O. gratissimum

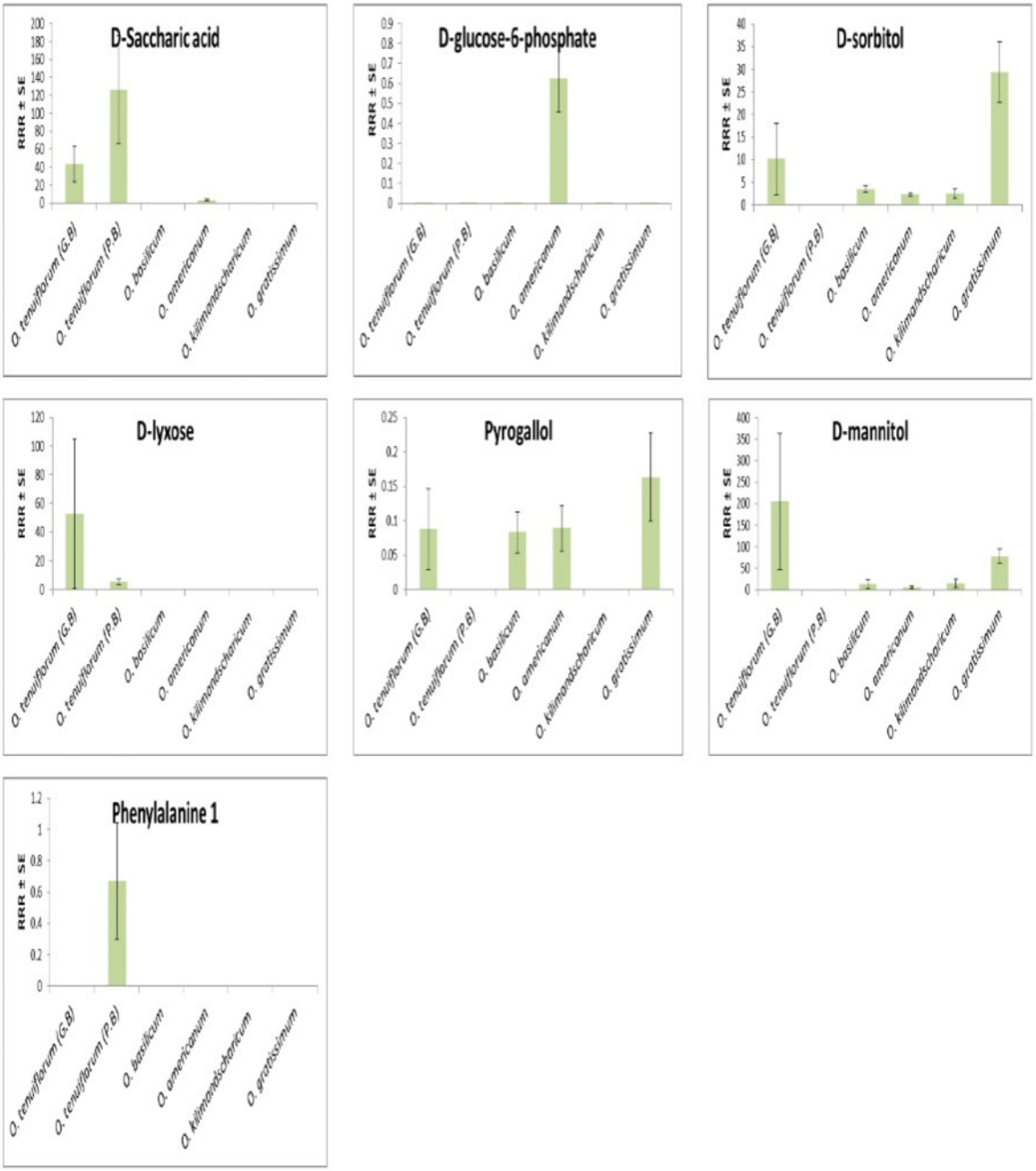


[A]

Fig. 5A. Bar graphs of top 25 metabolites based on their concentration differences among leaves. The error bars are the standard error from the four biological replicates of each species. (X-axis represented to different species of *Ocimum* and Y-axis represented to relative response ratio ± SD).


Gluconic acid
Citraconic acid
Melibiose
L-serine
Palatinitol
Carbazole
D-(+)-melezitose
4-Hydroxy-3-methoxybenzoic acid
L-glutamic acid (dehydrated)
RRR ± SE
O. tenuiflorum (G.B)
O. tenuiflorum (P.B)
O. basilicum
O. americanum
O. kilimandscharicum
O. gratissimum


L-proline
Cholesterol
Citric acid
O-acetylsalicylic acid
Uracil
Quinic acid
Ribonic acid-gamma-lactone
Methyl-beta-D-galactopyranoside
3,4-dihydroxymandelic acid
RRR ± SE
O. tenuiflorum (G.B)
O. tenuiflorum (P.B)
O. basilicum
O. americanum
O. kilimandscharicum
O. gratissimum



[B]

Fig. 5B. Bar graphs of top 25 metabolites based on their concentration differences among inflorescence of different species of *Ocimum* as identified from PLS-DA. The error bars are the standard error from the four biological replicates of each species. (X-axis represented to different species of *Ocimum* and Y-axis represented to relative response ratio ± SD).

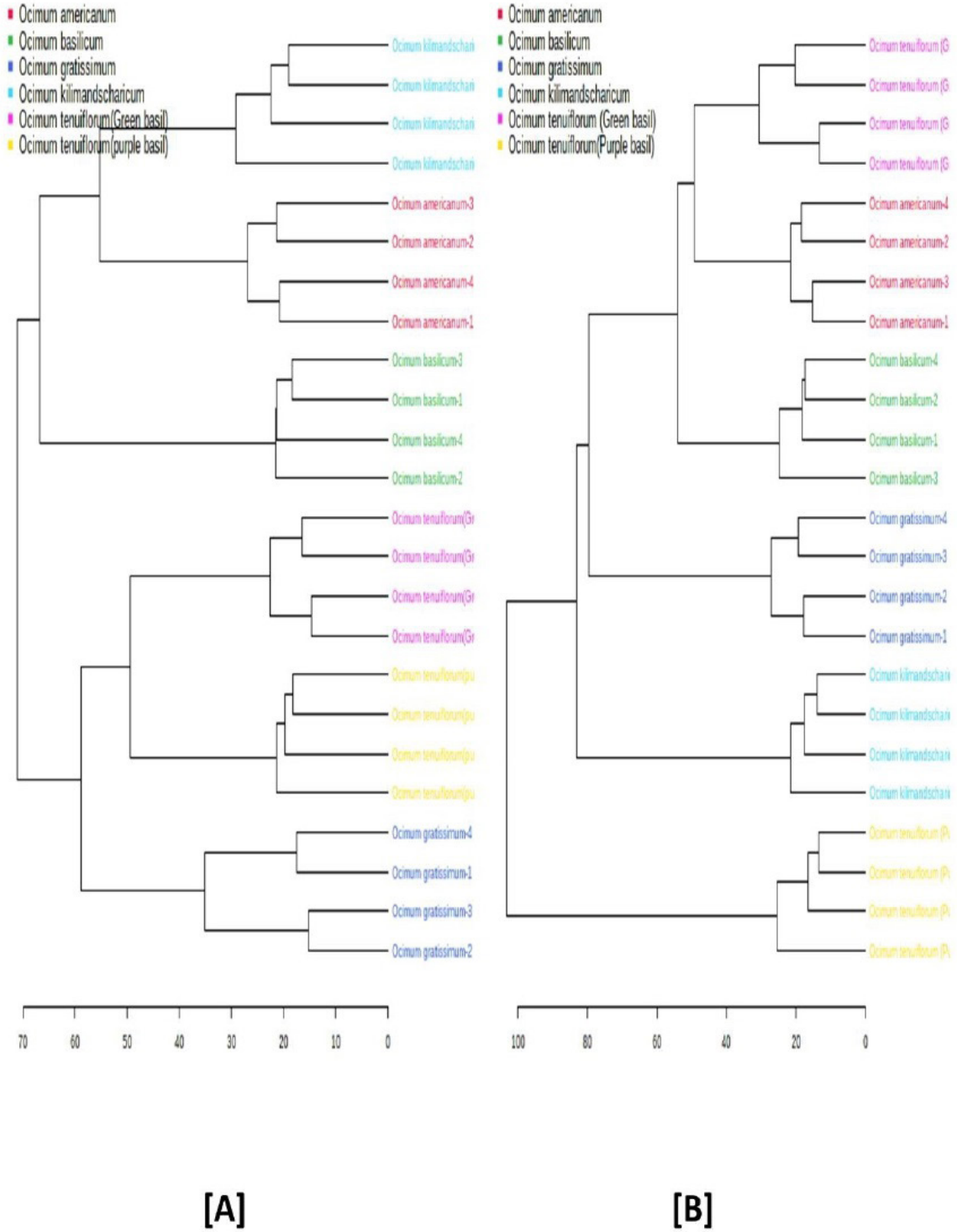


Fig. 6. Dendogram based on Euclidean distance measures and complete clustering algorithms showing separation in leaves (A) and inflorescences (B) of different *Ocimum* species, respectively.

Table 3
Anti-tyrosinase activities showing IC_{50} values of the phenolic compounds reported in the *Ocimum* species.

Standard phenolic compounds	Tyrosinase inhibition IC_{50} value ±SD (mg/ mL)
p-Arbutin	0.025 ± 0.006
Quinic acid	0.15 ± 0.006
Ferulic acid	1.14 ± 0.026
1,2,4-Benzenetriol	3.59 ± 0.104
Gallic acid	6.88 ± 0.0208
Vanillic acid	11.16 ± 0.170
p-Coumaric acid	15.34 ± 0.026

available *Ocimum* drop exhibited very low superoxide radical scavenging activity in comparison to the crude mixture of different *Ocimum* species (Table 2).
With respect to TAC, leaves of both *Ocimum basilicum* (0.07 mg ascorbic acid/ mg crude extract ± 0.03) and *Ocimum americanum* (0.07 mg ascorbic acid/ mg crude extract ± 0.03) showed highest total antioxidant capacity. Whereas, leaves of *Ocimum tenuiflorum* (green basil) showed lowest (0.02 mg ascorbic acid/ mg crude extract ± 0.00) total antioxidant capacity. In case of inflorescences, *Ocimum basilicum* showed highest (0.08 mg ascorbic acid/ mg crude extract ± 0.05) TAC. But surprisingly inflorescence of *Ocimum americanum* showed lowest (0.04 mg ascorbic acid/ mg crude extract ± 0.01) capacity among all the species. So it can be concluded that *Ocimum basilicum* have the highest TAC in both leaves and inflorescences. Commercially available *Ocimum* drop exhibited very low TAC in comparison to the crude mixtures of *Ocimum* species (Table 2). All *Ocimum* species exhibited nitric oxide radical scavenging activity in a dose dependent pattern. Among leaves, *Ocimum basilicum* showed highest nitric oxide anti-radical activity with an IC_{50} value of 1.66 mg/ mL ± 0.01, whereas, *Ocimum tenuiflorum* (purple basil) showed lowest inhibitory activity with an IC_{50} value of 12.67 mg/mL ± 0.14. In case of inflorescences *Ocimum tenuiflorum* (green basil) showed highest nitric oxide radical scavenging activity with an IC_{50} value of 7.64 mg/mL ± 0.09. Whereas, *Ocimum gratissimum* showed lowest nitric oxide radical scavenging activity with an IC_{50} value of 21.94 mg/mL ± 0.03 (Table 2).
Leaves of *Ocimum tenuiflorum* (purple basil) showed highest TPC, with 267. 1 mg gallic acid/ mg crude extract ± 48.04 whereas, leaves of *Ocimum tenuiflorum* (green basil) showed lowest (123.80 mg gallic acid/ mg crude extract ± 11.03) total phenol content. In case of inflorescences, *Ocimum basilicum* showed highest (284.04 mg gallic acid/ mg crude extract ± 35.24) TPC and *Ocimum tenuiflorum* (green basil) showed lowest (134.37 mg gallic acid/ mg crude extract ± 21.65) TPC (Table 2).
Leaves of *Ocimum basilicum* showed highest TFC (55.07 mg catechin/ mg crude extract ± 8.07), whereas, leaves of *Ocimum gratissimum* displayed lowest (10.6 mg catechin/ mg crude extract ± 2.80) total flavonoid content. In case of inflorescences, *Ocimum basilicum* exhibited highest (43.86 mg catechin/ mg crude extract ± 15.31) TFC and inflorescence of *Ocimum tenuiflorum* (green basil) showed lowest (14.31 mg catechin/ mg crude extract ± 3.64) TFC (Table 2).

3.6. Docking analysis

Molecular docking studies of the identified phenolic metabolites against the active-form of tyrosinase enzyme (4OUA) were performed by AutoDockVina. The binding energies of all the ligand-protein interactions were observed to be having significant negative free energies implying positive interaction. However, strength of their interactions varied significantly amongst each set of ligand and protein (Table 4). This is due to variable interactions of ligands with surrounding amino acids with diverse bonds including hydrogen bonding, Vander Waal interaction, hydrophobic interaction, Pi-Pi, Pi-anion, and Pi-cation interactions (Fig. 7).
As shown in Table 4 and Fig. 7, the calculated binding affinity for compounds viz., *p*-arbutin, ferulic acid, quinic acid, 1,2,4-benezenetriol, gallic acid, *p*-coumaric acid and vanillic acid were -7.2 kcal/mol, -6.4 kcal/mol, -5.9 kcal/mol, -5.6 kcal/mol, -6.1 kcal/mol, -6.1 kcal/mol and -6.0 kcal/mol respectively. The calculated binding affinity for 1,2,4-benezenetriol showed that they were not as proficient as other phenolic compounds studies in inhibiting tyrosinase enzyme.
According to Oyewole et al. (2020), higher the binding affinity (in terms of negativity) is anticipated to result in highest inhibitory property of such metabolites, thus, *p*-arbutin (Pubchem ID 440936) with -7.2 kcal/mol manifested to be more controlling than other studied phenolic compounds. The residues involved in the interaction for each ligand was Asp344, Thr345, Gly361, Gly360 for *p*-arbutin (Pubchem ID 440936), Gly360 for ferulic acid (Pubchem ID 445858), Gln294, Ser351, Ser291, Thr345, Asp344 for quinic acid (Pubchem ID 6508), His57 for 1,2,4-benzenetriol (Pubchem ID 10787), Asp252 for gallic acid (Pubchem ID 370), Ser351, Gly360, Val358 for *p*-coumaric acid (Pubchem ID 637542). The residues involved in the interaction could not be detected in vanillic acid (Pubchem ID 8468). The sequence of interaction was noticed in the docking analysis (Table 4 and Fig. 7). The simple phenols such as hydroquinone (Hashimoto et al., 1984; Sakuma et al., 1999) and its derivatives (Chen et a., 2009; Sasaki et al., 2018), deoxyarbutin (Boissy et al., 2005; Chawla et al., 2008) and its derivatives (Chawla et al., 2012), 4-(6-hydroxy-2-naphthyl)-1,3-benzenetriol, resorcinol (resorsin) (Tasaka et al., 1998) and 4-*n*-butylresorcinol (Kolbe et al., 2013), vanillin (Nguyen et al., 2012) and its derivatives (Shirota et al., 1994; Ashraf et al., 2015) have been cited in the scientific published work as possible phenolic inhibitors of tyrosinase. So, in this study, we evaluated anti-tyrosinase activity of *p*-arbutin, ferulic acid, quinic acid, 1,2,4-benzenetriol, gallic acid, *p*-coumaric acid and vanillic acid as these were detected both in leaves and inflorescences of the studied *Ocimum* sp. by GC/MS. These phenolic compounds may also have synergistic effect against tyrosinase enzyme. So, the above evaluated phenolic compounds of *Ocimum* leaves and inflorescences could be effective sources of

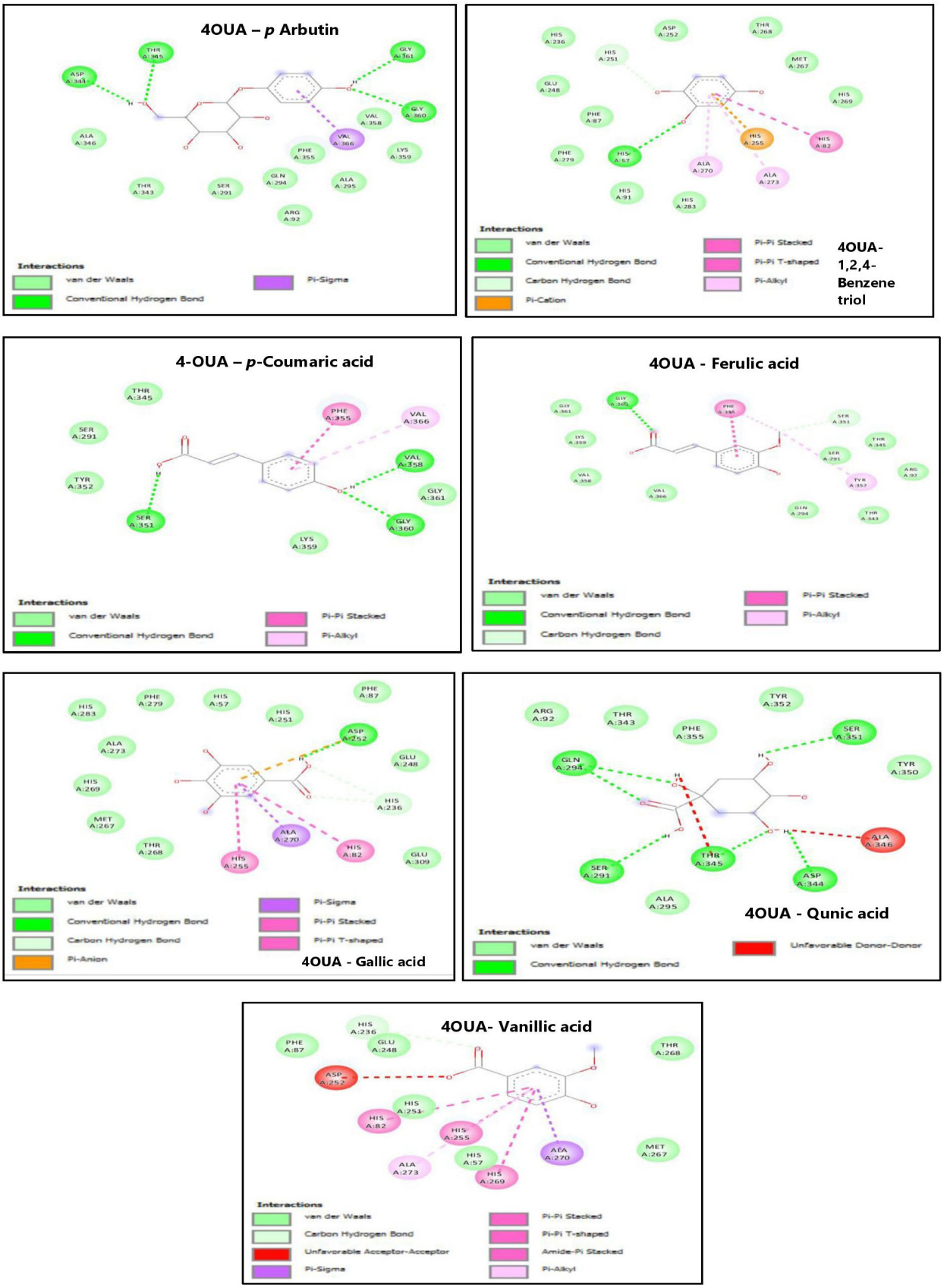


Fig. 7. 2-D structures of interaction between identified phenolic compounds and tyrosinase (PDB ID: 4OUA).

Table 4
Molecular docking studies of mushroom tyrosinase (PDB ID: 4OUA).

S.No.	Protein	Ligand (Pubchem ID)	Free energy ΔG (kcal/mol)	Number of hydrogen bonds
1	Tyrosinase (PDB ID: 4OUA)	p-Arbutin (440936)	-7.2	Asp344, Thr345, Gly361, Gly360
2		Ferulic acid (445858)	-6.4	Gly360
3		Quinic acid (6508)	-5.9	Gln294, Ser351, Ser291, Thr345, Asp344
4		1,2,4-Benzenetriol (10787)	-5.6	His57
5		Gallic acid (370)	-6.1	Asp252,
6		p-Coumaric acid (637542)	-6.1	Ser351, Gly360, Val358
7		Vanillic acid (8468)	-6	--

AutoDockVina parameter: (i) Grid dimension (x, y, z) = 21.303, 50.422, 39.872; (ii) Grid Size (x, y, z) = 54, 54, 56.

drugs/compounds as tyrosinase inhibitors, reported via this study for the first time.

4. Concluding remarks

In conclusion, this study indicated that hydro-methanolic leaf and inflorescence extracts of *Ocimum* species have strong anti-melanogenic and antioxidant potentialities. Among the leafy parts and inflorescences of 6 *Ocimum* species, leaves of *Ocimum tenuiflorum* (purple basil) and inflorescences of *O. kilimandscharicum* showed highest IC_{50} value with 3.06 mg/mL ± 0.076 and 1.73 mg/mL± 0.435 respectively to show their anti-tyrosinase activity. Amongst the GC/MS identified metabolites, correlated phenolic compounds, quinic acid and *p*-arbutin showed very high activity against tyrosinase. Moreover, the species showed high anti-radical properties, possibly because of their high levels of total phenol and flavonoid contents.

In this study, green tulsi and Krishna tulsi showed noticeably different activities against tyrosinase enzyme, antioxidant activities, TPC and TFC and even in their chemical fingerprinting, although they were placed in same species as suggested by taxonomist. On the basis of chemometric analysis and metabolome classification, these two *Ocimum* species should be entitled as different species. These findings propose that leaf and inflorescence extracts of *Ocimum* species and / or the isolated *p*-arbutin, ferulic acid, quinic acid, 1,2,4-benzenetriol, gallic acid, *p*-coumaric acid (4-hydroxycinnamic acid), vanillic acid (4-hydroxybenzoic acid) may serve as a great reservoir of functionally active molecules for controlling hyper-pigmentation and as a skin-whitening agent and also as anti-browning preparations of fruits, fungi and vegetable substances. Further study is needed to authenticate the property of these bioactive components in *in vivo* models.

Conflict of interest

The authors confirm that they there is no conflict of interest.

Acknowledgements

Dr. S. Das is grateful to Science & Technology and Biotechnology DEPARTMENT, Govt. of West Bengal (Vide Memo No. 170(Sanc.)/ST/P/S&T/5G-6/2018 dated 14/02/2019 and Vide Memo No. 660(Sanc.)/ STBT-11012(15)/16/2019 -ST SEC dated 04/10/2021) for providing financial assistance to carry out this research project**.** Mrs. S. Sarkar acknowledges Swami Vivekananda Merit cum Means Fellowship Scheme (fellowship Id WBP191574941302). Gas Chromatography Mass Spectroscopy based analytical study supported by DST-FIST Plan, Govt. of India [Grant Number: SR/FST/LS1-459/2010] is also recognized. The authors are grateful to Narendrapur Ramakrishna Mission, Kolkata and Agri Horticultural Society of India for providing the fresh samples in ample amounts.

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