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Research Paper

Diversity of flavonoids profile in China aster [*Callistephus chinensis* (L.) Nees.] genotypes

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ABSTRACT

Flavonoids in fifteen genotypes of China aster, grouped by their flower color, namely red, deep pink, light pink, white, light purple, deep purple and yellow, were analysed using liquid chromatography–mass spectrometry. Flower petals were a particularly rich source and contained eleven flavonoid compounds: apigenin, catechin, epicatechin, epigallocatechin, hesperetin, leutolin, myricetin, naringenin, quercetin, rutin, and umbelliferone. The genotype Matsumoto Pink was the richest source (26.74×10^3 µg/100 g) and Matsumoto Yellow, the poorest (1.17×10^3 µg/100 g). Apigenin was the major compound irrespective of flower color. Based on hierarchical clusters of the flavonoid compounds, the genotypes were grouped into two. Results of principal component analysis were also in conformity with the results of cluster analysis.

1. Introduction

China aster, *Callistephus chinensis* (L.) Nees., belongs to the family Asteraceae and is a native of China (Navalinskien et al., 2005). The genus *Callistephus* derives its name from two Greek words, *kalistos*, which means 'most beautiful', and *stephos*, which means 'a crown'. *Callistephus* is a monotypic genus, containing the single species *C. chinensis* (Khangjarakpam et al., 2014). The species is a somewhat hardy annual, commercially grown for cut flowers and loose flowers, which are used in floral decoration, in bouquets and garlands. The flowers come in many colors, the main coloring compounds in flower petals being flavonoids (including anthocyanins) a group of secondary metabolites (Tanaka et al., 2008).

Flavonoids are a class of secondary metabolites found in plants and fungi. In general, all flavonoids are derivatives of 2-phenylchromone, synthesized from phenylalanine, with a characteristic $C_6{-}C_3{-}C_6$ carbon skeleton. More than 5000 flavonoid compounds have been isolated from various plants, broadly classified as flavanols, flavanones, flavones, isoflavones, flavonols, and anthocyanins (Beecher, 2003). The pigments that color most flowers, fruits, and seeds are flavonoids and contain glycosides, which help in co-pigmentation. Flavonoids have received considerable attention because of their anti-allergic, anti-inflammatory, antiviral, anti-proliferative, and anticarcinogenic activity (Ren et al., 2003). The present study is an attempt to identify the genetic variability in China aster in terms of flavonoids and to determine their profiles by using liquid chromatography and mass spectrophotometry (LC–MS/MS). It gives information about the genotype which can be exploited commercially for flavonoid i.e. apigenin extraction from the flowers of China aster for nutraceutical industry.

2. Materials and methods

2.1. Plant material

Fifteen genotypes of China aster were selected from the experimental fields of ICAR-IIHR (Indian Council of Agricultural Research – Indian Institute of Horticultural Research), Bengaluru (Karnataka, India). The experimental site was geographically located at 13° 58′ N Latitude, 78°E Longitude and at an elevation of 890 m above mean sea level with an average rainfall of 890 mm. The type of soil is red loamy with pH 7.35 and E.C. of 0.26 d Sm^{-1}. The genotypes were divided into seven groups based on their flower color at the harvesting stage, as follows: red (Matsumoto Scarlet), deep pink (Arka Aadya, Arka Kamini, and Matsumoto Red), light pink (IIHRE10, IIHRCC31A and Matsumoto Pink), white (Arka Archana, Local White and IIHRJ22), light purple (IIHRI69-2, and IIHRCC5-1A) deep purple (Arka Violet Cushion, and

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IIHRI69), and yellow (Matsumoto Yellow) Fig. 5. Flowers were collected for analysis in the morning hours (9.00 AM-10AM) in the month of January 2016. Petals were removed from the flowers with scissors and stored in 80% methanol at −40 °C.

2.2. Chemicals and reagents

Flavanoid standards for apigenin, catechin, epicatechin, epigallocatechin, hesperetin, leutolin, myricetin, naringenin, quercetin, rutin, and umbelliferone, were procured from Sigma-Aldrich, St. Louis, Missouri, USA. Chromatographic or MS-grade organic solvents were used for the analysis; all the other reagents were of analytical grade. The mobile phases were prepared using ultra-purified water and filtered through a fine membrane (pore size 0.45 µm).

2.3. Calibration curve

The calibration curves for flavonoids were developed by the multiple reaction monitoring (MRM) method of LC–MS or MS using the parent mass (m/z) and most abundant fragmented daughters.

2.4. Sample preparation and extraction

Flavonoids for the LC–MS/MS analysis were extracted in 80% methanol, using, with slight modifications, the method described by Chen et al. (2001). Each extract was evaporated to dryness under vacuum at 45 °C, and the residue was dissolved in water and extracted three times into 30 mL ethyl acetate. The ethyl acetate extract was also evaporated to dryness under vacuum. To the dried residue was added 4 mL of 2 M NaOH, and the mix was set aside for alkaline hydrolysis for 6 h. After acidification to pH 2 using 4 mL of 2 M HCl, the mix was again set aside for acid hydrolysis for 1 h in a boiling water bath. Flavonoids released from soluble glycosides were extracted from the hydrolyzate using ethyl acetate. Discarding the aqueous layer, the ethyl acetate layer, which carried the flavonoids, was evaporated to complete dryness under vacuum at room temperature; the residue dissolved in 2 mL of the mobile phase; and filtered through a 0.2 µm nylon filter prior to injection into LC–MS or MS for estimating the flavonoids.

2.5. Equipments

An acquity UPLC H-class Bio System coupled with a triple quadruple mass spectrometer (TQMS) (Waters Corporation, Milford, Massachusetts, USA) with an electrospray ionization (ESI) source was used for the determination of flavonoids. The system was equipped with a degasser, a quaternary pump, and an automatic injection system (0–10 µL) with a diode array detector and a temperature control compartment for the analytical column. The overall system was controlled by Mass Lynx, a software package. The mass spectra were obtained using the negative ionization mode (ESI^-) for the most abundant forms of de-protonated $[M\text{-}H]^-$ molecules of the flavonoids, which were then fragmented by their respective collision energy (CE) to develop the MRM methods (Table 1).

2.6. LC and MS/MS conditions

The mobile phase comprised an aqueous phase, which was 0.1% formic acid in water (A), and an organic phase, which was 0.2% formic acid in methanol (B). The initial gradient consisted of 90% A and 10% B, held for 2.5 min. At 4.0 min, the gradient was changed to 70% A and 30% B and held for 1.0 min. At 5.0 min, a linear gradient was followed to reach 60% A and 40% B and held for 5.0 min. At 10.0 min, the gradient was changed to 80% A and 20% B and held for 2.0 min. The final step consisted of 90% A and 10% B and held for 2.0 min. The system was then returned to the initial conditions at 14 min and this condition was held for 1 min for equilibrating before the next injection. The flow rate was 0.3 mL/min. The analytical column was a 2.1 × 50 mm UPLC BEH C18 column (Waters) with 1.7 µm particles, protected by a VanGuard BEH C18 1.7 µm guard column (Waters), and the column temperature was maintained at 25 °C. The sample injection volume for flavonoids was 5 µL throughout. The metabolites were pumped directly without any split into the TQD-MS/MS system (Waters), which was optimized for flavonoids analysis with a source temperature of 135 °C, disolvation gas flow of 650 L/h, and a temperature of 350 °C.

2.7. Statistical analysis

The data on flavonoid content were analysed using MS Excel 2007; analysis of variance (ANOVA) at $p \leq 0.05$ was used to ascertain whether the differences between means were significant. Cluster analysis and principal component analysis (PCA) of the LC–MS/MS data were performed using XLSTAT (Addinsoft, 2017).

3. Results and discussion

3.1. Flavonoid composition

A total of eleven flavonoid compounds were identified and categorized into three groups, namely flavones, flavanones, and flavonols. The compounds identified were apigenin, catechin, epicatechin, epigallocatechin, hesperetin, leutolin, myricetin, naringenin, quercetin, rutin, and umbelliferone. The amounts (µg/100 g) of the detected compounds in petals of different colors are given in Table 2.

The amount of flavonoids varied significantly with color and also within genotypes that produced flowers of the same color. The highest amount of flavonoids was reported in the light pink genotype Matsumoto Pink (26.74×10^3 µg/100 g), followed by IIHRE10 (8.80×10^3 µg/100 g), whereas Matsumoto Yellow recorded the lowest (1.17×10^3 µg/100 g).

Apigenin was present in maximum amounts, followed by naringenin, whereas quercetin content was the lowest; the remaining eight flavonoids did not show much variation across the genotypes. The highest amount of apigenin was recorded in Matsumoto Pink

Table 1
Multiple reaction monitoring (MRM) details for LC–MS/MS estimation of flavonoids.

Flavonoids	Formula/Mass	t_R	Parent m/z $[M + H]^-$	Cone Voltage (V)	Daughters	Collision Energy (CE)	Ion Mode
Apigenin	270.00	10.20	268.97	46	107.04	30	ES-
Catechin	290.00	4.26	289.03	38	245.15	12	ES-
Hesperetin	302.00	7.31	300.97	42	286.15	16	ES-
Naringenin	272.00	10.55	271.03	34	151.00	16	ES-
Leutoline	286.00	6.21	284.90	54	150.99	26	ES-
Myricetin	318.00	7.49	317.03	42	151.06	28	ES-
Quercetin	302.00	9.48	301.03	36	151.12	20	ES-
Rutin	610.00	6.91	609.10	60	300.20	42	ES-
Umbelliferone	162.14	5.82	161.04	42	133.07	18	ES-