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Identification of chemical components in *Diantthus* determined by widely targeted metabolomics

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Abstract: The chemical composition of the secondary metabolites is of great significance to the quality control of agricultural products. The genus *Dianthus* is famous for its beautiful flowers in the cut flower trade and also used in the traditional Chinese medicinal system and food market. However, the chemical composition in *Dianthus* is still unknown. The current study examined the levels of different metabolites of the flowers in *Dianthus caryophyllus, Dianthus chinensis* and *Dianthus superbus* via the use of the widely targeted metabolomic strategy. We obtained the structure and content of 423 metabolites in *Dianthus*, which included the primary and secondary metabolites. The principal component analysis was able to clearly seperate *Dianthus caryophyllus*, *Dianthus chinensis* and *Dianthus superbus* based on the flower metabolites. The differential metabolites can be categorised into 11 different classes, the majority were flavonoids, amino acids and their derivatives, phenolic acids and lipids. The results of this study provide important information for the effective use of *Dianthus* flowers in edible, medicinal and therapeutic aspects.

Keywords: bioactive compounds; edible flowers; *Dianthus*; LC-MS; phytochemistry

The genus *Dianthus* is famous for its beautiful flowers in the cut flower trade and also used in the traditional Chinese medicinal system and food market. The genus Dianthus is an abundant natural resource and most of the plants (flowers) contain phytochemicals with recognised health benefits. The food industry and the recognised health benefits on edible flowers are attracting more and more attention. are attracting more and more attention (Lu et al. 2015). Flowers are an important part of expanding the food market because of their suitable sensory and nutritional properties. Edible flowers retain proteins, fatty acids, vitamins, mineral elements and antioxidants especially when they are consumed in their natural or minimally processed form (Rop et al. 2012; Fernandes et al. 2019). Recently, some biological characteristics have also been associated with edible flowers, such as improvement of ulcerative colitis (Meurer et al. 2019), having a neuroprotective activity against oxidative effects (Yang et al. 2019), an antihyperglycaemic and anticholinergic activity (Nowicka, Wojdyło 2019) and even an anticancer activity (Nguyen et al. 2019).

Dianthus L. is an annual or perennial herb that belongs to the Angiosperm's family Caryophyllaceae, subclass *Caryophyllidae* and tribe *Caryophyllidae*, which is a cultivated plant and grown worldwide (Chandra et al. 2016). Some species of *Dianthus* are considered important in medicine as they have

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been widely used to treat various infections and diseases in China, Iran, the South Korea and Mongolia for thousands of years (Mutlu et al. 2016; Weon, Ma 2016). Pharmacological studies have shown that the *Dianthus caryophyllus* has anticancer, antibacterial, antifungal, antiviral, insecticidal, repellent, antioxidant, renoprotective, anaesthetic and analgesic and other effects (Al-Snafi 2017). Kaempferide triglycoside isolated from *D. caryophyllus* inhibited the proliferation of colon cancer cells with both natural and oestrogen receptor β overexpression (Martineti et al. 2010). The antiviral activity of *D. caryophyllus* against *herpes simplex virus-1* (HSV-1), hepatitis A virus-27 (HAV-27) and human immunodeficiency virus (HIV) has been determined (Lee-huang et al. 1991; Barakat et al. 2009; Al-Snafi 2017). *Dianthus superbus* is a more potent natural herb with antimicrobial, anticancer, antioxidant and anti-inflammatory properties, as well as being immunosuppressive, having osteoblastic proliferation and preventing peanut allergies (Gou et al. 2011; López-Expósito et al. 2011; Tong et al. 2012; Ding et al. 2013). Researchers revealed that *D. superbus* extracts showed cytotoxicity against liver, breast and lung cancer cell lines (Yu et al. 2007, 2012). *Dianthus chinensis* flowers, commonly known as "Chinese pink", are widely used in cooking preparations, being also recognised for their bioactive ingredients and antioxidant properties (Koike 2019). The petals of *D. chinensis* have a spicy flavour and are applied in salads and in the aromatisation of vinegar and wine (Koike 2019). *D. chinensis* has also been used in the treatment of cough, menostasis, and gonorrhoea and as an emmenagogue and diuretic (Chandra et al. 2016).

Based on the qualitative and quantitative analysis of metabolites, metabonomics can be used to analyse the metabolic pathways or networks, and to evaluate the safety of food and drugs. An extensive targeted metabolomic analysis is a novel method combining the advantages of non-targeted metabonomics and targeted metabonomics (Wang et al. 2018). The development of a high-throughput synthesis technology and the idea of metabonomics provides new strategies for the analysis of active components (Zhang et al. 2010). In *Dianthus*, most of the medicinal and edible constituents are unknown. The purpose of this study was to conduct a broad targeted metabolic analysis of *Dianthus* flowers, and to link the molecular breeding of high-quality *D. caryophyllus*, *D. chinensis* and *D. superbus* by determining the important metabolites of *Dianthus* associated with specific medicinal and edible properties.

MATERIAL AND METHODS

Sample preparation and extraction of metabolites. Flowers of the *D. caryophyllus* cultivar 'Master' (Master), *D. chinensis* (DC) and *D. superbus* (DS) were collected from six plants and pooled to create one biological sample, respectively (Figure 1). One biological replicate was analysed per sample. All of the samples were taken from the same locality in Kunming city, Yunnan province, China, in 2020. The *Dianthus* flowers were obtained and macerated with liquid nitrogen and stored at –80 °C until the metabolite extraction. The freeze-dried samples were crushed in a mixing mill (MM 400; Retsch, Germany) containing zirconia beads at 30 Hz for 1.5 minutes. 100 mg powder of crushed flowers was weighed and extracted overnight with a 0.6 mL 70% methanol solution at 4 °C. After centrifugation at 10 000 g for 10 min, a CNWBOND Carbon-GCB SPE cartridge (250 mg,

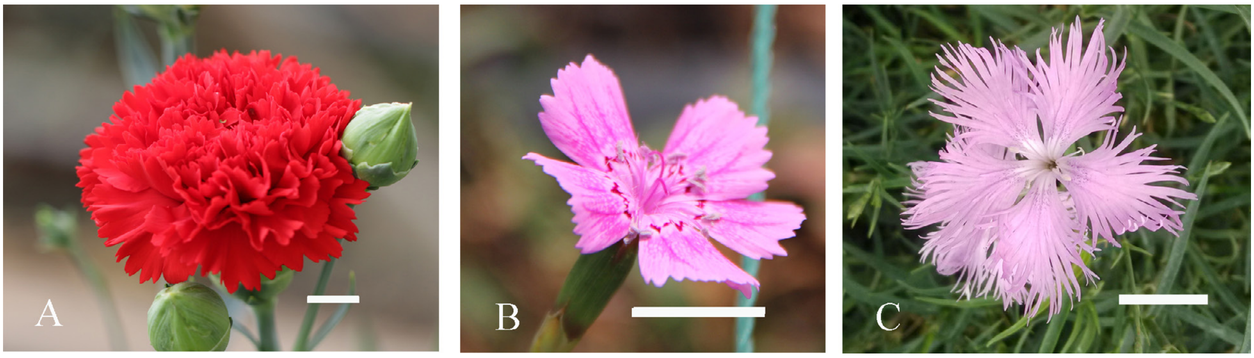

Figure 1. Flowers from *Dianthus caryophyllus* 'Master' cultivar (A), *Dianthus chinensis* (B) and *Dianthus superbus* (C)

Scale bar = 1 cm

3 mL; ANPEL, Shanghai) was used for adsorption. After filtration (SCAA-104, 0.22 μm; ANPEL, Shanghai), an ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis was performed.

UPLC Conditions. The sample extracts were analysed by an ultraperformance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) system (HPLC, Shim-pack UFLC SHIMADZU CBM30A system; Shimadzu, Japan and MS, Applied Biosystems 4500 Q TRAP; Applied Biosystems, USA). Furthermore, 4 μL was pplied to a Waters ACQUITY UPLC HSS T3 C18 (1.8 μm, 2.1 mm × 100 mm). The mobile phases were pure water with 0.04% acetic acid (solvent A) and acetonitrile with 0.04% acetic acid (solvent B). The sample was determined by a gradient program and the initial condition was 5% B. Within 10 min, the program used a linear gradient to obtain 95% B, and 95% B was maintained for one minute. Then, the composition of 5.0% B was adjusted to within 0.10 min and kept for 2.9 min. The flow rate was 0.35 mL/min at 40 °C.

ESI-Q TRAP-MS/MS analysis. Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (Q TRAP), API 4500 Q TRAP UPLC/MS/MS System, equipped with an electrospray ionisation (ESI) Turbo Ion-Spray interface, which operates in both positive and negative Ion modes and with Control Analyst 1.6.3 software (Sciex Inc., Framingham, USA). The operating parameters of the ESI source are as follows: ion source, turbine spray; source temperature of 550 °C; ion spray voltage (IS) 5 500 V (positive ion mode)/–4 500 V (negative ion mode); ion source gas I, gas II and curtain gas are set at 50, 60, and 30.0 psi, respectively; collision gas high. The instrument was tuned and calibrated using 10 and 100 μmol/L of a polypropylene glycol solution in the QQQ and LIT modes, respectively. The QQQ scans were acquired as multiple reaction monitoring (MRM) experiments with the collision gas (nitrogen) set to five psi. The declustering potential and collision energy of the single MRM transition were further optimised. A specific set of MRM transitions was monitored based on the eluted metabolites during each time period.

MS data and statistical analysis. The MS data acquisition and processing was according to the methods described earlier (Zou et al. 2020). Metabolites were annotated using the Metware in-house MS2 spectral tag library (Wuhan Metware Biotechnology Co., Ltd.; http://www.metware.cn, Wuhan, China). The unsupervised principal component analysis (PCA) used the "prcomp" function in R version 3.5.0 (www.r-project.org).

RESULTS AND DISCUSSION

Widely targeted metabolome analysis in *Dianthus*. In order to better understand the chemical constituents of *Dianthus*, we carried out an extensive targeted LC-MS/MS-based metabolite profiling of the *D. caryophyllus* cultivar 'Master', *D. chinensis* and *D. superbus* A total of 423 metabolites were identified, including the primary and secondary metabolites [Table S1 in Electronic Supplementary Material (ESM)]. A total of 379, 391 and 387 metabolites were detected in the *D. caryophyllus* cultivar 'Master', *D. chinensis* and *D. superbus*, respectively. The majority of the biochemical categories were flavonoids, lipids, amino acids and their derivatives and phenolic acids (Figure 2).

Multivariate analysis of the identified metabolites. A hierarchical clustering heat map was constructed using the metabolite ion intensity data, and a PCA was performed on the samples. The PCA revealed the overall metabolic differences among the groups and the variability between the intra-group samples by using several principal components. In Figure 3A, the PCA result shows that the mixed samples used for the quality control were all close to the origin (0 : 0), indicating that there was very little technical variability among the runs. Two principal components, PC1 and PC2, accounted for 51.91% and 41.11% of the variability, respectively. Moreover, the cumulative contribution rate reached 93.02%. The samples from the three species and the quality control (QC) samples were clearly separated from one another, which implies that there is a differential accumulation of a large number of metabolites among these samples (Figure 3A).

In order to eliminate the influence of the quantity on the pattern recognition, the peak area of each metabolite was transformed by $\log_{10}$, followed by a hierarchical clustering analysis. In the heat map, the metabolite contents of the Master, DC and DS were significantly different, while the metabolite contents of DC and DS were basically the same. Although DC and DS are grouped in the same category, the contents of the metabolites are also greatly different

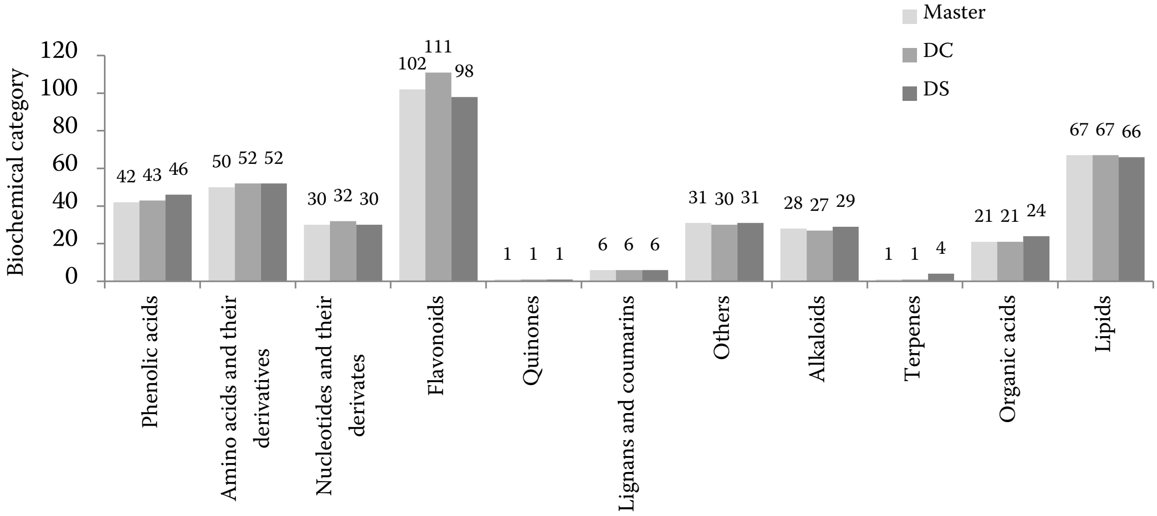

Figure 2. The number of biochemical categories in *Dianthus*
Master – *Dianthus caryophyllus*; DC – *Dianthus chinensis;* DS – *Dianthus superbus*, respectively

in the heat map. This analysis revealed three distinct groups associated with the *D. caryophyllus* cultivar 'Master', *D. chinensis* and *D. superbus*, respectively. Therefore, together, the PCA and cluster analysis showed that the three plants had distinct metabolic characteristics (Figure 3B).

Differentially accumulated metabolites in *Dianthus*. To detect metabolites that were different among the three species of *Dianthus*, we compared the ionic strength of the various metabolites among the different samples and the compounds with a fold change ≥ 2 or fold chang; *e* ≤ 0.5 (Wang et al. 2020) were retained as differentially accumulated metabolites (DAMs). The result was 228 DAMs for DC vs DS, 283 DAMs for Master vs DC and 267 DAMs for Master vs DS (Table S2 in ESM).

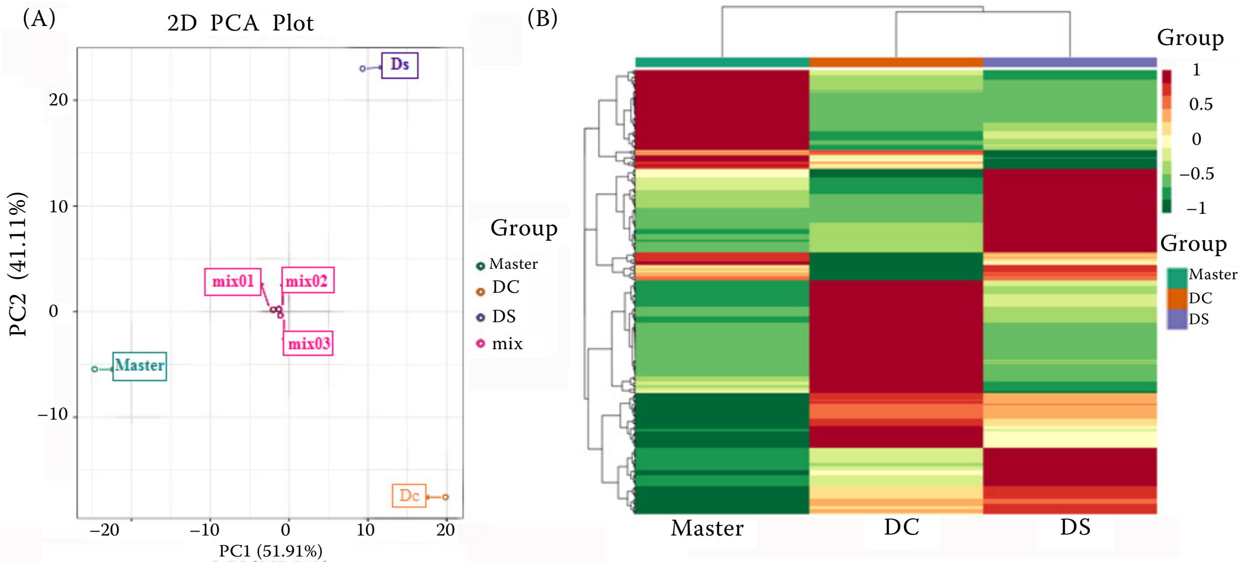

Figure 3. Differential chemical composition in *Dianthus*. (A) PCA of the metabolites identified from the *D. caryophyllus* cultivar 'Master', *D. chinensis* and *D. superbus*. Equal volumes of the *D. caryophyllus* cultivar 'Master', *D. chinensis* and *D. superbus* samples were mixed for use as a quality control (QC); (B) Clustering analysis of the metabolites from the *D. caryophyllus* cultivar 'Master', *D. chinensis* and *D. superbus*
The colours indicate the accumulation level of each metabolite, ranging from low (green) to high (red). The Z-scores are measured in units of a standard deviation from the mean. Master – *Dianthus caryophyllus*; DC – *Dianthus chinensis* and DS – *Dianthus superbus*, respectively

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Overall, about two-thirds of the global metabolome (350 metabolites) experienced significant changes (Table S3 in ESM), indicating differences in the metabolite levels among the different species.

By cross-comparing the different DAMs detected in the control group, 125 metabolite composition changes were found in all the samples, and each test group had its own unique differences in the metabolites. Therefore, the different metabolites can clearly distinguish the *Dianthus* species (Figure 4).

The genus *Dianthus* is well known as a horticultural herb, but the medicinal importance of its members is little known. In recent years, different medicinal plants of the genus *Dianthus* and their biomedical characteristics have been reviewed. However, only a few reviews focused on the medicinal and edible aspects of *Dianthus* (Wetzel et al. 2010; Chandra et al. 2016; Al-Snafi 2017). To the best of our knowledge, this is first time that an LC-MS/MS based metabolomics approach has been widely used to analyse the major and secondary metabolites of the flowers in *D. caryophyllus*, *Dianthus chinensis* and *D. superbus* (Table S1 in ESM). We obtained the structure and content of 379, 391 and 387 metabolites in the flowers of *D. caryophyllus*, *D. chinensis* and *D. superbus* respectively. The majority of the metabolites were flavonoids, phenolic acids, lipids, amino acids and their derivatives.

Flavonoids belong to a class of secondary plant metabolites that have a polyphenolic structure. The regular consumption of them can reduce the risk of many chronic diseases, including cardiovascular disease, cancer and neurodegenerative disorders (Kozłowska, Szostak-Wegierek 2014). Flavonoids also contribute to the quality of food as preservatives, pigments and strong antioxidants (D'Amelia et al. 2018). Amino acids, which are components of peptides and proteins, play important roles in nutrition, and sensory and biological regulation (Kamei et al. 2020). Free amino acids are also involved in the complex taste of many foods which exhibit one or more of the five flavours (sweet, sour, salty, bitter, umami) (Ninomiya 2016). Amino acids and their derivatives also have biological regulatory functions, for example, creatine and taurine improve exercise performance and body strength (Luckose et al. 2015), arginine has the function of relaxing blood vessels and enhancing immunity (Brittenden et al. 1994; Li et al. 2007), and gamma aminobutyric acid regulates blood pressure (Hayakawa et al. 2002; Kazuhito et al. 2004). Phenolic acids are phenolic compounds containing a carboxyl group (Kumar, Goel 2019). Phenolic acids are widely available, and there is ample evidence that they have other health protective effects, such as being antimicrobial, anticancer, anti-cardiovascular, anti-inflammatory, anti-diabetes and many more (Saibabu et al. 2015; Rosa et al. 2016). The antioxidant activities of phenolic acids are extensively implemented in food-based systems as preservatives and maintain their sensory characteristics (Waraho et al. 2011; Phonsatta et al. 2017). Lipids are small, hydrophobic molecules that perform many important functions. Lipids act as structural elements in biofilms and signal molecules in cellular response pathways (Finkelstein et al. 2014). Functional lipids are healthy dietary components that have the potential to affect human health, reduce the risk of disease and improve the quality of life. Health benefits include the reduced susceptibility to atopic dermatitis, heart disease, depression, obesity, Alzheimer's disease and Parkinson's disease (Alabdulkarim et al. 2012).

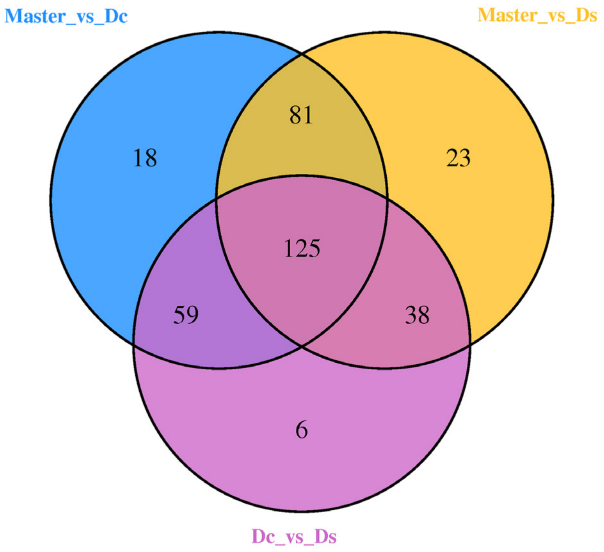

Figure 4. Venn diagram describing the shared and unique differences in the accumulation of the metabolites among the comparison groups

This study explains the strong antioxidant activity of *Dianthus* plants well because they contain good amounts of flavonoids and phenolics. Their flowers are also a good food source for containing high amounts of metabolites, such as flavonoi, amino acids and lipids. However, *D. caryophyllus* has some contraindications and side effects. Occupational exposure to *D. caryophyllus* can cause allergy and asthma symptoms (Sánchez-Guerrero et al. 1999).

CONCLUSION

In recent years, although people use flowers in their food to add flavour and colour and enhance the visual appeal, the potential of flowers as a food is still undervalued. The development of edible ornamental flower products is the solution to the sustainable development of the cut flower industry because cut flowers lack ornamental value and tonnes of fresh flowers are discarded every day regardless of their nutritional and functional value. In this study, we evaluated the medicinal and edible aspects of flowers from three *Dianthus* species, the *D. caryophyllus* 'Master' cultivar, *D. chinensis* and *D. superbus* The aim is to provide reference for the future development of innovative food and folk medicine. The plants of the *Dianthus* genus contain good amounts of flavonoids, amino acids and their derivatives, phenolic acids and lipids. These results point to the potential of *Dianthus* flowers as an innovative health food and disease treatment drug.

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