JJBS

Volume 15, Number 4,December 2022
ISSN 1995-6673
Pages 579 – 592
https://doi.org/10.54319/jjbs/150406

Jordan Journal of Biological Sciences

In Vitro Antioxidant, Antihyperglycemic and Antiglycation Properties of Bulbs, Flower Buds and Flowers Extracts of *Lilium* Species and their Chemometric Profiling

The Su Moe [1, 2], Mingfang Zhang [1], Jing Xue [1], Mya Thida [3], Yixin Liu [1], Mohammad Sayyar Khan [1, 4], Yunpeng Du [1*] and Xiuhai Zhang [1,*]

[1]Beijing Agro-Biotechnology Research Center（Beijing Key Laboratory of Agricultural Genetic Resources and Biotechnology, Beijing Engineering Technology Research Center of Functional Floriculture）, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, P.R. China; [2] Pharmaceutical Research Laboratory, Biotechnology Research Department, Ministry of Education, Mandalay Division, Kyaukse 05151, Myanmar;[3]Cell Culture Laboratory, Biotechnology Research Department, Ministry of Education, Mandalay Division, Kyaukse 05151, Myanmar;[4]Genomics and Bioinformatics Division, Institute of Biotechnology and Genetic Engineering (IBGE), the University of Agriculture, Peshawar 25000 Khyber Pakhtunkhwa, Pakistan

Received: June 28, 2021; Revised: January 11, 2022; Accepted: January 31, 2022

Abstract

This objective of this study was to explore the antioxidant, antihyperglycemic, and antiglycation properties of 13 methanolic extracts from 8 different *Lilium* species by various *in vitro* assays and their chemometric profiles to provide the evidence-based information for further exploration. Chemometric profiling was performed using the MetaboAnalyst and GNPS: Global Natural Products Social Molecular Networking platforms to explore and identify the metabolites from *Lilium* metabolomics profiles. *In vitro* bioactivity studies showed that flower bud extract of *L. lancifolium* was the most active extract with the strongest radical scavenging activity against DPPH and SO radicals, reducing activity of ferric ions and highest total phenolic contents among tested extracts. Univariate and multivariate statistical analysis revealed that the chemical variation between the sample classes: parts used (bulb vs flower); bioactivity (active vs inactive) and studied species. 13 bioactive metabolites were putatively identified and some of them were confirmed by comparing the MS/MS spectrums with the standards. Results suggest that metabolite profiling along with the bioactivity study could be useful to explore the chemical compositions, functional and developmental potentials of valuable plant species, and lay the foundation for identification, separation and assessment of their potential medicinal applications and species classification.

Keywords: *Lilium*; antioxidant; antihyperglycemic; antiglycation; chemometric profiling

1. Introduction

The genus *Lilium* from the family Liliaceae, consisting of about 280 genera and 4,000 species(Carr), was comprised of 110-115 species. It is a rich source of chemical diversity and substantial natural resources that have great ornamental, medicinal, and edible value. Among them, 55 species and 18 varieties originated from China, which had a noticeable history for the richness of *Lilium* resources(Tang *et al.*, 2010). Lily flowers and bulbs are regularly consumed as food and medicine all over the world, particularly in Asia(Munafo JrGianfagna, 2015). In China, the flowers and bulbs of some *Lilium* species are used for culinary and/or medicinal purposes. Local people believed that dried bulbs are potent for the lung diseases, have tonic properties, and are commonly used to flavor and thicken the soup(Royal Botanic Gardens, 1889). Moreover, specialized metabolites from some *Lilium* species showed the potential biological activities such as anti-inflammatory (Francis *et al.*, 2004), hepatoprotective (Tang *et al.*, 2015), antidiabetic (Tang, *et al.*, 2015), antioxidant (Guo *et al.*, 2014), antifungal (Uhlig *et al.*, 2014), antitumor (Shimomura *et al.*, 1987), antibacterial activities (Li, 2007) and cardiac disease(Zhou *et al.*, 2012) by various *in vitro* and *in vivo* assays. Due to these great potentials and traditional knowledge of *Lilium*, it has drawn an interest for commercial purposes.

On the other hand, taxonomic classification is critical for the plant species to recognize and effectively exploit their effectiveness for different purposes. Morphological (ViscosiCardini, 2011), physiological (Dunlop *et al.*, 1999), genetics(Du *et al.*, 2015), and chemical characterization(Rivière *et al.*, 2012) has been used to classify the plant species by comparing the difference characteristics between them. Among these taxonomic classification methods, chemical characterization or chemotaxonomy became a useful tool for the plant taxonomy. It is a method based on the dissimilarity of the chemical constituents of the plant species to classify according to their phylogeny(Kim *et al.*, 2012). Since the *Lilium* species are extremely related, several researches

* Corresponding author. e-mail: duyunpeng@baafs.net.cn ; zhangxiuhai@baafs.net.cn.

have been carried out to classify the *Lilium* species with different approaches such as floral morphology(Gao *et al*., 2015), SSR molecular markers(Du, *et al*., 2015), RAPD analysis(Ikinci, 2010) and DNA barcoding(Zheng *et al*., 2014). However, chemical characterization has not been utilized to understand the differences of chemometric profiles of *Lilium* species so far.

In recent years, metabolomics approaches has been broadly used to discover the chemical diversity of bioactive natural products, especially for the targeted isolation of structurally or biologically noble bioactive compounds(Raterink *et al*., 2014). Metabolomics is an approach that can characterize and quantify the primary and secondary metabolites or low molecular weight molecules present in the complex biological samples. Characterization of primary and secondary metabolites by metabolomics approach can be used to explore the relationship between the chemometric profiles of the studied species and identify the differences associated with the underlying study question and generate the new hypotheses(BoufridiQuinn, 2016).

In this research, bioactivities such as antioxidant activity, antihyperglycemic and antiglycation properties of bulbs, flower buds and flowers extracts of *Lilium* species were investigated by various *in vitro* assays to determine the potential application of different varieties of lily and provide the evidence-based information for further development of research and innovation. MetaboAnalyst and Global Natural Products Social Molecular Networking (GNPS) platform were used to explore the *Lilium* chemometric profiles mainly based on their bioactivity and studied species, and to facilitate the identification of MS spectra in the complex metabolomics datasets of selected *Lilium* species.

2. Materials and Methods

2.1. Chemicals and reagents

Analytical grade chemicals such as 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl (DPPH), ascorbic acid, gallic acid, sulphanilic acid, glacial acetic acid, sodium nitroprusside, N-(1-naphthayl) ethylenediamine dihydrochloride, ethylenediamine tetra-acetic acid (EDTA), Folin-Ciocalteu's reagent, sodium carbonate, riboflavin, nitro blue tetrazolium (NBT), potassium ferricyanide, trichloroacetic acid , ferric chloride, acarbose, α-Glucosidase, p-Nitrophenyl- α-D-Glucopyranoside (PNPG), fructose, bovine serum albumin (BSA) and sodium azide were purchased from companies such as Sigma Chemicals Co. (St. Louis, USA), and TCI Development Co. Ltd (Shanghai, China). Acetonitrile, dimethyl sulfoxide (DMSO), methanol and formic acid were purchased from Thermo Fisher Scientific, USA. Standards used for the compound identification were purchased from VEGSCI, Inc., China.

2.2. Plant materials

Lilium bulbs, flower buds and flowers used in this study were grown and collected form the greenhouses at the germplasm conservation center at the Beijing Academy of Agriculture and Forestry Sciences. The selected *Lilium* species used in this study are shown in Table 1.

Table 1. Selected *Lilium* species

Sample code	Botanical name	Family name	Parts used	Yield %
ZDM1	*L. lechtlinii var. maximowiczii*	Liliaceae	Bulb	16.96
ZDM2	*L.A.hybrids* 'Tresor'	Liliaceae	Bulb	11.99
ZDM3	*L. lancifolium*	Liliaceae	Bulb	10.49
ZDM4	*L. regale*	Liliaceae	Bulb	17.99
ZDM5	*L. pumilum*	Liliaceae	Bulb	10.46
ZDM6	*L. davidii*	Liliaceae	Bulb	14.03
ZDM7	*L. brownie*	Liliaceae	Bulb	12.65
ZDMF1	*L. davidii*	Liliaceae	Flower	35.65
ZDMF2	*L. henryi*	Liliaceae	Flower	31.64
ZDMF3	*L. lancifolium*	Liliaceae	Flower bud	26.32
ZDMF4	*L. lancifolium*	Liliaceae	Flower	36.18
ZDMF5	*L.A.hybrids* 'Tresor'	Liliaceae	Flower bud	40.98
ZDMF6	*L.A.hybrids* 'Tresor'	Liliaceae	Flower	53.76

2.3. Extraction

A grinding machine (BJ-800A, China) was used to crush freeze-dried plant samples into a fine powder. Samples were soaked for one week in 95 percent methanol then filtered, and solvents were removed using a rotary vacuum evaporator (RE 52AA, China). This step was repeated three times to get the maximum yield. Yield % was calculated using the formula: Yield % =Weight of the dry extract x 100/ Weight of the dry plant (Table 1). After removing the solvents, crude extracts were freeze-dried and stored at -80˚C for further experimentation.

2.4. Biological activity assays

2.4.1. In vitro antioxidant activity assays

Free radical-scavenging activity of the extracts was explored using the stable DPPH free radicals as mentioned by Lee et al.(Lee *et al*., 1998). The detailed experimental protocol could be seen on this previous publication of Moe et al. (2018). The optical density (OD_{515}) was measured using a Thermo Scientific Multiskan FC microplate reader. DMSO was used as the blank, and ascorbic acid was used as a positive control. Inhibition rate (%) was calculated through comparison to the blank.

Nitric oxide (NO) radical scavenging assay was performed as mentioned by Hertog et al.(Hertog *et al*., 1993). The detailed experimental protocol could be seen on this previous publication of Moe et al. (2018). The optical density (OD_{540}) was measured using a Thermo Scientific Multiskan FC microplate reader.

The extracts' free radical scavenging activity was also determined using a superoxide (SO) radical scavenging assay modified from Patel Rajesh's methodology.(Patel RajeshPatel Natvar, 2011). The detailed experimental protocol could be seen on this previous publication of Moe et al. (2018). The optical density (OD_{560}) was measured using a Thermo Scientific Multiskan FC microplate reader. In both NO and SO assays, ascorbic acid was useed as a positive control.

To assess the reducing activity of each extract, ferric reducing antioxidant power (FRAP) assay described by

Takashi Kuda et al. was used with slight modifications (Kuda *et al.*, 2005). 200 µL of extracts (10 mg/mL in DMSO), 200 µL of 0.2M Sodium Phosphate Buffer (pH 6.6) and 200 µL of 1% Potassium ferricyanide [K3Fe (CN)6] were mixed in a 2 ml micro-capped tubes and incubated at 50 ˚C for 30 min. Then, the mixture was cooled down, and 200 µL of 10% trichloroacetic acid was added to the reaction mixture. After that, 125 µL of this mixture were transferred to a 96-well microplate, and 20 µL of 0.1% Ferric chloride (FeCl3-6H2O) was added to each well. The optical density (OD_{620}) was measured using a Thermo Scientific Multiskan FC microplate reader. Gallic acid was used as a positive control.

2.4.2. In vitro antihyperglycemic activity assay

α-glucosidase inhibitory assay protocol described by Peyman Salehi et al. was used to detect the antihyperglycemic potentials of *Lilium* extracts (Salehi *et al.*, 2013). 10 µL of the extracts (0.4 mg/mL in DMSO), 120 µL of 0.1 M phosphate buffer (pH 6.9) and 20 µl of α-glucosidase (0.5 unit/ml) were mixed in a 96-well microplate and incubated at 37°C for 15 min. Subsequently, 20 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside was added to initiate the enzymatic reaction, and the reaction mixture was incubated at 37°C for 15 min. After that, the reaction was neutralized with 80 µL of 0.2 M sodium carbonate solution, and optical density (OD_{405}) was measured using a Thermo Scientific Multiskan FC microplate reader. The reaction mixture without plant extracts was used as control, and that of without enzyme was used as blank for correcting the background absorbance. Acarbose was used as a positive control.

All the assays mentioned above were carried out in at least triplicate for each sample and positive controls, and inhibition rates for all the above tested assays were calculated using the following formula:

Inhibition rate (%) = [1 – (OD tested / OD control)] × 100

2.4.3. In vitro antiglycation activity assay

The ability of the extracts to inhibit the formation of advanced glycation end products (AGE) was measured to explore the i*n vitro* antiglycation activity of the extracts as described by Choudhary (Choudhary *et al.*, 2011). The detailed experimental protocol could be seen on this previous publication of Moe et al. 2018. AGEs formation was measured at the excitation of 340 nm and emission of 440 nm fluorescence's intensity by using an Agilent Cary Eclipse Fluorescence spectrophotometer (G9800, US). Rutin (1mM) was used as a positive control. The reaction mixture without fructose was used as the negative reaction control and that of without extracts as positive reaction or glycated control. Inhibition rate (%) was calculated by using the following formula:

Inhibition rate (%) = [1 - (fluorescence of tested sample / fluorescence of positive control)] × 100

2.4.4. Total phenolic content measurements

The measurement of total phenolic content present in each plant extract was done using the method described by Andrew L. Waterhouse with a few modifications(Waterhouse, 2002). Briefly, 2 µL of the extracts (1mg/mL in 70% methanol) or Gallic acid in different concentrations (0, 0.5, 1, 1.5, 2, 2.5, 5 µg/ml in 70% methanol) were put into the 96 well microplate. 148 µL of the distilled water and 20 µL of 1N Folin-Ciocalteu's reagent were added. The reaction mixtures were mixed thoroughly and incubated at room temperature for 5 min. Then, the reaction was neutralized by adding 30 µL of 0.2 g/ml sodium carbonate. After that, the samples were incubated at 40°C for 30 min, and the OD_{765} was measured with Thermo Scientific Multiskan FC microplate reader. For each sample, at least three replicate assays were performed. The total phenolic content (TPC) was represented as Gallic acid equivalent (GAE) in mg/g of extract using the equation generated from the Gallic acid standard curve ($y = 0.0007x$, $R^2 = 0.9995$).

2.5. Sample preparation for UHPLC-LTQ-XL-IT-MS/MS analysis

20 mg of each extract was dissolved in 1 mL of 70 % methanol and filtered through a 0.2 µm PTFE (polytetrafluoroethylene) filter, prior to ultrahigh performance liquid chromatography LTQ XL linear ion trap mass spectrometry/mass spectrometry (UHPLC-LTQ-XL-IT-MS/MS) analysis. Pierce™ LTQ ESI positive ion calibration solution was used to calibrate the LCMS system to check the system performance. 70% Methanol was employed in the LC-MS analysis as a blank sample for background subtraction.

2.6. UHPLC-LTQ-XL-IT-MS/MS analysis

UHPLC-LTQ-IT-MS/MS analysis was performed using the method adapted from Lee et al. with a few modification (Lee *et al.*, 2015b). Thermo Fischer Scientific LTQ XL linear ion trap mass spectrometry equipped with electrospray interface (Thermo Fischer Scientific, San José, CA, USA), DIONEX UltiMate 3000 RS Pump, RS Auto sampler and RS Column Compartment (Dionex Corporation, Sunnyvale, CA, USA) was used for metabolite profiling of the *Lilium* extracts. Samples were separated on a Thermo Scientific Hypersil GOLD C18 column with 1.9 µm particle size. The mobile phase consisted of A (0.1% (v/v) formic acid in water) and B (0.1% (v/v) formic acid in acetonitrile) and the gradient conditions were increased from 10% to 100% of solvent B. Scanning was set to start after 1 min to source. Solvent gradient time was set over 19 min, and re-equilibrated to the initial condition for 4 min by setting the divert valve to waste. The flow rate was set at 0.3 mL/min and the injection volume was 10 µL. Temperature of the column during measurement was maintained at 35 ˚C. Ion trap was performed in positive and full-scan ion modes within a range of 150–1000 m/z. The operating parameters were as follows: source voltage; ±5 kV, capillary voltage; 39 V, capillary temperature; 275 ˚C, auxiliary gas flow rate; 10−20 arbitrary units, sheath gas flow rate; 40−50 arbitrary units, spray voltage; 4.5 kV. Tandem MS (MS/MS) analysis was performed by scan-type turbo data-dependent scanning (DDS) under the same conditions used for MS scanning for the six most intense ions. MS data was acquired by Xcalibar software, Thermo Fischer Scientific(Khan *et al.*, 2020).

2.7. Data pre-processing

The UHPLC-LTQ-IT-MS/MS data were acquired with Xcalibar software (version 2.00, Thermo Fischer Scientific). The raw data files were converted from the Xcalibar standard data-format to .mzXML format using

the MSConvert software, part of the ProteoWizard package version 3.0.19140 (Chambers *et al*., 2012). All .mzXML were pre-processed using MZmine 2.51 for mass detection, chromatogram building and deconvolution, deisotoping and peak alignment (Pluskal *et al*., 2010). The aligned peak list files were exported using the built-in options "Export to MetaboAnalyst file".

2.8. Statistical analysis and putative identification of metabolites

For the LC-MS data analysis, .csv file created by MZmine 2.51 with feature lists (m/z, RT), sample name, group and the area of each peak was uploaded to MetaboAnalyst and statistical analysis was performed using unsupervised (principal components analysis - PCA and hierarchical cluster analysis - HCA) and supervised (Partial Least Squares - Discriminant Analysis - PLS-DA) methods. Samples were classified into three different groups: parts used (bulb vs flower), species names and bioactivity (inactive vs active). Analysis was performed based on the sample groups. Then the univariate and multivariate statistical analysis was processed using MetaboAnalyst 4.0 (Chong *et al*., 2019). The data integrity check was done by default, data filtering was performed by mean intensity value and auto scaling was used for the normalization. Principal component analysis (PCA) and partial least-square discriminant analysis (PLS-DA) were performed to explore the metabolite differences between samples. The most significant metabolites were selected based on variable importance in the projection (VIP) score.

After statistical analysis, metabolites were identified by using the standard compounds by comparing both mass spectra and retention time. If the standard compounds could not purchase, putative identification was done by the GNPS public spectral database which comprised of the library containing the natural product compounds from user contributions and third party databases such as Massbank, ReSpect, HMDB, CASMI and Sumner Spectral Libraries (http://gnps.ucsd.edu) (Wang *et al*., 2016b). mzXML files converted from the raw LC-MS files were uploaded to GNPS and molecular network was created with the MSCluster algorithm enabled(Frank *et al*., 2008) using the default parameters. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.6 and at least 4 matched peaks. Blank Spectra were also uploaded and filtered before networking. MASST Search (Mass Search Tool) was also used to search single MS/MS spectrum of interest against all public spectral libraries on GNPS (Wang *et al*., 2020).

For the bioactivity assays, all data were expressed as mean ± standard error mean of at least triplicate experiments. One-way analysis of variance (ANOVA) and Dunnett's or Tukey's multiple comparison tests were performed to compare the difference between the plant extracts and standard control or between the tested plants extracts. In each analysis, $P < 0.05$ was considered to be statistically significant. Correlation analysis was also performed to evaluate the correlations between DPPH radical scavenging assay and the rest of tested bioactivity assays using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results

3.1. In vitro antioxidant, antihyperglycemic and antiglycation activities of Lilium extracts

In this research work, potential antioxidant, antihyperglycemic and antiglycation activity of 13 methanolic extracts of bulbs, flower buds and flowers from 8 different *Lilium* species were explored by using the various *in vitro* assays for evidence-based validation. Comparison between the bioactivities of each plant extract and the standard or comparison between the samples was carried out to distinguish the potentially bioactive plant extract for further study and samples that were significantly different ($P< 0.05$) from the standard or from each other were also stated.

ZDMF3 (flower bud extract of *L. lancifolium*) emerged as the most active plant extract in four of the seven bioassays that were evaluated. It demonstrated the highest ferric ion reducing activity with the maximum absorbance value of 2.02±0.08 and the highest radical scavenging activity against DPPH and SO radicals with inhibition rates of 91.54±0.38 %, 82.35 ± 1.25%, respectively (Table 2) and highest total phenolic contents of 112.74 mg GAE/g of extract (Figure 1). While many other extracts had significantly lower activity than the standard for these assays, ZDMF3 was comparable to the standard and could be a potential source of antioxidants and valuable for reducing oxidative stress.

Table 2. *In vitro* antioxidant activities of selected *Lilium* extracts

Sample code	Inhibition rate (%)			
	DPPH free radical scavenging activity	Nitric oxide radical scavenging activity	Superoxide radical scavenging activity	FRAP activity (Absorbance)
ZDM1	0.00*	28.02±3.72*	12.93±1.92*	0.18±0.002*
ZDM2	0.00*	35.92±2.66*	15.61±3.49*	0.20±0.002*
ZDM3	44.02 ± 4.49*	54.54±0.24*	69.21±3.38*	0.85±0.025*
ZDM4	57.96 ± 0.80*	60.19±1.44*	80.26±2.12	0.89±0.020*
ZDM5	3.80 ± 3.52*	36.60±2.52*	2.77±6.67*	0.32±0.003*
ZDM6	0.85 ± 0.33*	37.46±3.75*	11.28±5.21*	0.21±0.001*
ZDM7	8.66 ± 3.15*	20.38±6.91*	36.66±4.22*	0.16±0.002*
ZDMF1	23.14 ± 3.73*	45.64±2.17*	42.57±6.56*	1.22±0.017*
ZDMF2	17.90 ± 0.47*	36.60±3.90*	51.44±2.42*	0.39±0.015*
ZDMF3	91.54 ± 0.38	56.48±1.95*	82.35±1.25	2.02±0.089*
ZDMF4	68.05 ± 1.29*	52.46±0.32*	68.59±4.68*	1.29±0.028*
ZDMF5	60.56 ± 2.17*	36.51±1.12*	53.07±3.37*	0.94±0.011*
ZDMF6	26.51 ± 0.73*	21.10±5.51*	45.59±2.97*	1.24±0.020*
Ascorbic Acid	97.48 ± 0.16	74.88±0.86	89.56±0.58	-
Gallic Acid	-	-	-	3.82±0.031

Results were shown as the average of at least triplicates of experiments ± standard error of mean.*P < 0.05, extract *vs* standard in each group. DPPH: 1, 1-diphenyl-2-picrylhydrazyl; FRAP: Ferric Reducing Antioxidant Power; GAE: gallic acid equivalent.

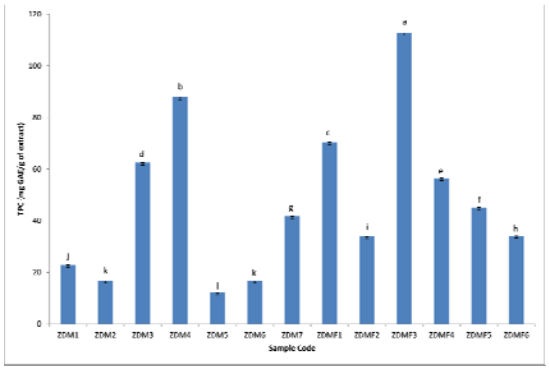


Figure 1. TPC of *Lilium* extracts determined by the Folin-Ciocalteu's assay and calculated as GAE in mg/g extract based on dry weight. Results were the average of triplicates ± standard error of the mean. Different letters (a–l) indicated significant difference (P < 0.05) from each group. GAE: gallic acid equivalent; TPC: total phenolic content

For the inhibition of NO radicals, ZDM4 (bulb extract of *L. regale*) exhibited the best activity against NO radicals among the tested samples. The inhibition rate was 60.19±1.44%, while the standard ascorbic acid showed the inhibition rate of 74.88±0.86% (Table 2). ZDM4 also has the moderate activity for antihyperglycemic activity through α-glucosidase inhibition assay with the rate of 61.51±9.66% (Table 3). It also possesses the second highest total phenolic contents of 87.86 mg GAE/g of extract (Figure 1). The highest activity of α-glucosidase inhibition among the tested extracts was to be possessed by ZDM7 (bulb extract of *L. brownii*) with the inhibition rate of 63.38±2.33%.

Table 3. *In vitro* antihyperglycemic and antiglycation activities of selected *Lilium* extracts

Sample code	Inhibition rate (%)	
	Antihyperglycemic activity	Antiglycation activity
ZDM1	29.16±2.36*	27.92±12.52*
ZDM2	15.35±2.96*	12.84±2.68*
ZDM3	38.38±9.99*	84.26±4.62
ZDM4	61.51±9.66*	22.06±9.88*
ZDM5	34.72±5.18*	29.33±6.06
ZDM6	0.00*	20.17±1.92*
ZDM7	63.38±2.33*	22.88±6.02*
ZDMF1	28.88±1.24*	9.22±7.44*
ZDMF2	38.13±2.91*	0.00*
ZDMF3	19.56±2.54*	0.00*
ZDMF4	10.45±5.67*	5.30±12.36*
ZDMF5	41.60±1.80*	0.00*
ZDMF6	43.95±6.92*	27.98±4.28*
Acarbose	98.67±0.33	-
Rutin	-	56.73±3.35

Results were shown as the average of at least triplicates of experiments ± standard error of mean.*P < 0.05, extract *vs* standard in each group.

Then the inhibition of AGE formation was evaluated by a non-enzymatic glycation reaction. Among all the tested samples, ZDM3 (bulb extract of *L. lancifolium*) revealed the highest antiglycation activity with inhibition rate of 84.26±4.62% (Table 3). Its activity was remarkably higher than the standard, rutin, with that of 56.73±3.35% at the tested concentration.

3.2. Metabolite Profiling of Lilium Extracts by MetaboAnalyst workflow

We analyzed the *Lilium* extract metabolomics data by classifying the samples based on their plant parts (bulb vs flower) to determine whether the samples are correctly classified using this statistical model. As shown in Figure 2, PCA score plot clearly distinguished the metabolites from the bulb and flower extracts. In the PCA plot, 2

components account for 34 % of the variance. With the exception of one bulb extract, ZDM4 (*L. regale*), which clustered into the flower extract, hierarchical clustering analysis (Figure 3) likewise clearly distinguished between the two groups: one group comprising the flower extracts and the other group including the bulb extracts. *L. regale* bulb: purple in color, was different from all the other tested bulb extracts, which were all white. These color giving metabolites from ZDM4 extracts might be quite similar to that of the flower extracts, and this could be the reason that ZDM4 was clustered into the flower extracts. As per the result obtained, MetaboAnalyst platform was considered to be suitable for the analysis of *Lilium* metabolomic data sets.

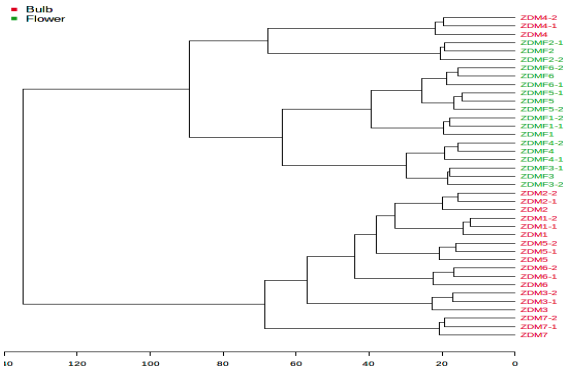


Figure 3. Dendrogram showing the hierarchical clustering pattern of *Lilium* metabolomics data based on the class defined by parts used (Bulb vs Flower).

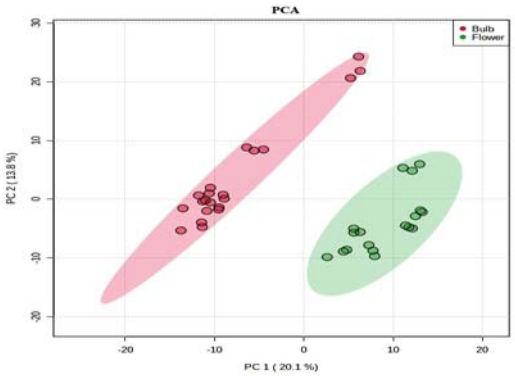
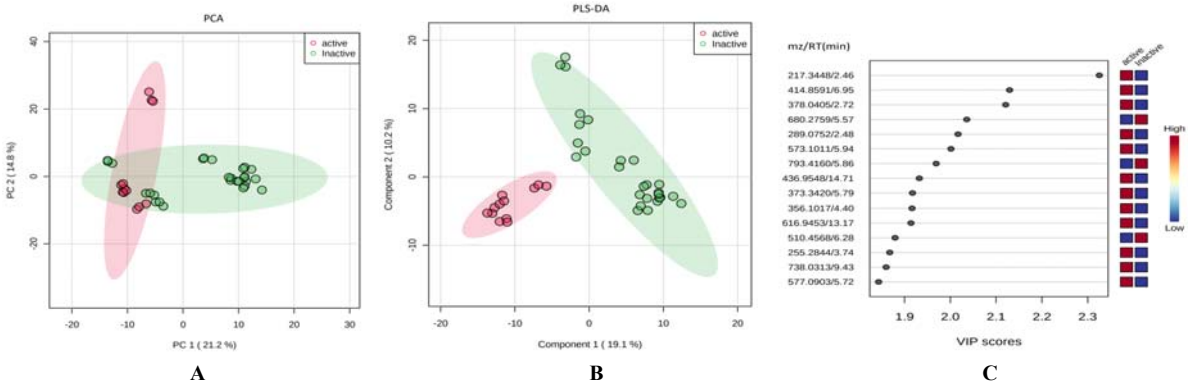


Figure 2. PCA scores plot for the unsupervised analysis of *Lilium* metabolomics data based on the class defined by part used (Bulb vs Flower).

We then investigated the *Lilium* metabolomics data sets by classifying the sample class information based on their bioactivities: active vs inactive extracts to understand the metabolites separation between them. Bioactivity was classified based on the DPPH radical scavenging activity as this activity was positively correlated with the other antioxidant activities, such as NO radical scavenging activity(r= 0.45), SO radical scavenging activity(r= 0.79), FRAP activity(r= 0.74) and total phenolic contents(r= 0.71). Extracts with ≥50 % of the inhibition rate of DPPH radicals were considered as active extracts, and those with < 50% were regarded as inactive extracts. For this analysis, 2 components account for 36% of the variance in PCA score plot (Figure 4A), and active extracts were clearly separated from the inactive extracts with 2 components account for 29% of the variance in PLS-DA plot (Figure 4B). The metabolites that separated the active and inactive extracts can be seen in Figure 4C. As we can see in the dendrogram, almost all the active extracts are from the flower parts. As we previously mentioned, one bulb extract ZDM4 that revealed as bioactive appeared clustering into the flower extract (Figure 5A). PLS-DA analyses allow us for selecting the most important features or metabolites that differentiate between groups through its VIP score in the sPLS plot [23]. Of the 15 most important features of group separation, four metabolites were putatively identified as solasodine, chlorogenic acid, hydroferuloylglucose and 15-Oxospirosolan-3-yl 2-O-(6-deoxy- alpha-L-mannopyranosyl) -beta- D-Glucopyranoside (Steroidal saponins) by the GNPS public spectral database (Table 4).



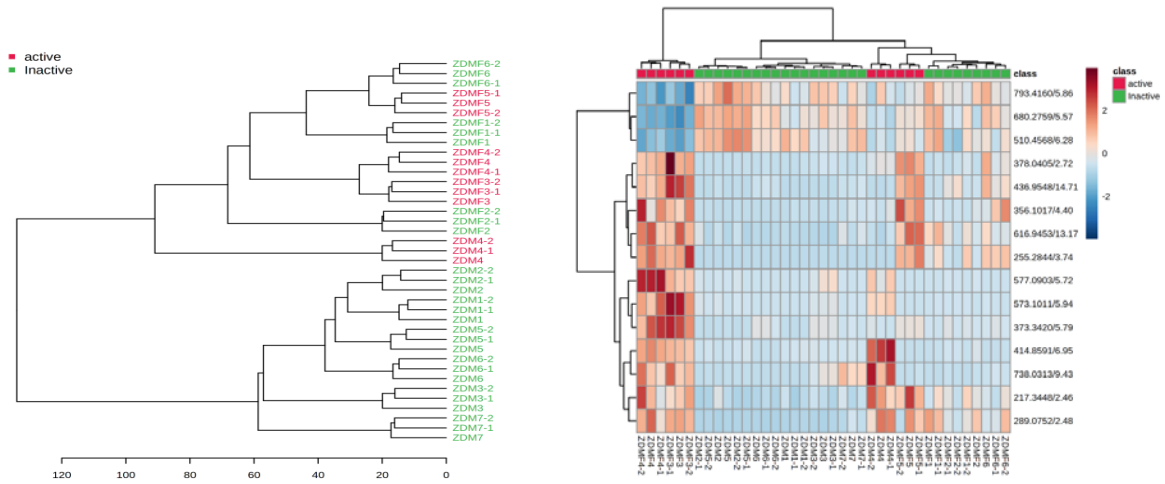
A **B** **C**

Figure 4. Unsupervised and supervised analysis of *Lilium* metabolomics data based on their bioactivity: (A) PCA scores plot; (B) PLS-DA scores plot and (C) PLS-DA loadings for the 15 most important variables (metabolites) that could discriminate between the active and inactive extracts.

Table 4. Discriminant metabolites from bioactivity-based grouping

No.	Parent ion (mz)	Retention Time (min)	Proposed Identity	Molecular Formula	Identifier	GNPS library ID
1	217.3448	2.46	NI	-	GNPS	-
2	414.8591	6.95	Solasodine	C27H43NO2	GNPS/Standard	CCMSLIB00004690970
3	378.0405	2.72	Chlorogenic acid	C16H18O9	GNPS	CCMSLIB00003139010
4	680.2759	5.57	NI	-	GNPS	-
5	289.0752	2.48	NI	-	GNPS	-
6	573.1011	5.94	NI	-	GNPS	-
7	793.4160	5.86	NI	-	GNPS	-
8	436.9548	14.71	NI	-	GNPS	-
9	373.3420	5.79	Hydroferuloylglucose	C16H20O10	GNPS	CCMSLIB00000209861
10	356.1017	4.40	NI	-	GNPS	-
11	616.9453	13.17	NI	-	GNPS	-
12	510.4568	6.28	NI	-	GNPS	-
13	255.2844	3.74	NI	-	GNPS	-
14	738.0313	9.43	15-Oxospirosolan-3-yl 2-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-Glucopyranoside	C39H63NO12	GNPS	CCMSLIB00000852029
15	577.0903	5.72	NI	-	GNPS	-

NI: Not Identified



A B

Figure 5. Clustering pattern of *Lilium* metabolomics data based on the class defined by bioactivity: (A) Dendrogram; (B) Heatmap (distance measure using euclidean, and clustering algorithm using ward.D).

We also studied metabolomic data of the *Lilium* bulb samples by classifying the samples based on their species to discover if the chemical characterization could differentiate the bulbs of these closely related *Lilium* species. For the unsupervised analysis, 2 components account for 45% of the variance in PCA score plot. As shown in the Figure 6A, *Lilium* species were closely related and three species such as *L.brownii*, *L. lancifolium* and *L. regale* were separated from the area in which the rest species are grouping into close manner. However, as shown in the Figure 6B, supervised analysis could discriminate the species with 2 components account for 41% of the variance. The top 15 significant metabolites that discriminate these species and their relative concentration can be seen in the PLS-DA loading plot (Figure 6C). Among these 15 most important metabolites, relative concentration of 3 metabolites such as 534.5637mz/14.08min, 517.0777mz /14.08min, and 532.5118mz /13.13min were found to be highest in *L.brownii* than the rest of the species. The concentration of 7 metabolites such as 485.4668mz/13.29min, 696.6569mz/12.85 min, 694.7240mz/11.73min, 589.8393mz /12.52min, 522.9131mz /14.26min (PC(0:0/18:0)), 613.9326mz /11.75 min, and 502.0174mz /13.29min were highest in *L.A.hybrids* 'Tresor'. The rest five most important metabolites appeared as 332.0139 mz /4.76 min, 234.1394mz /12.28 min, 397.3256mz /1.58min, 453.9508mz /4.60min, and 579.2074mz /5.88min (2-(hydroxymethyl)-6-(2,20,21-trihydroxydocosan-3-yloxy)oxane-3,4,5-triol) were relatively highest in concentration in *L.regale*.

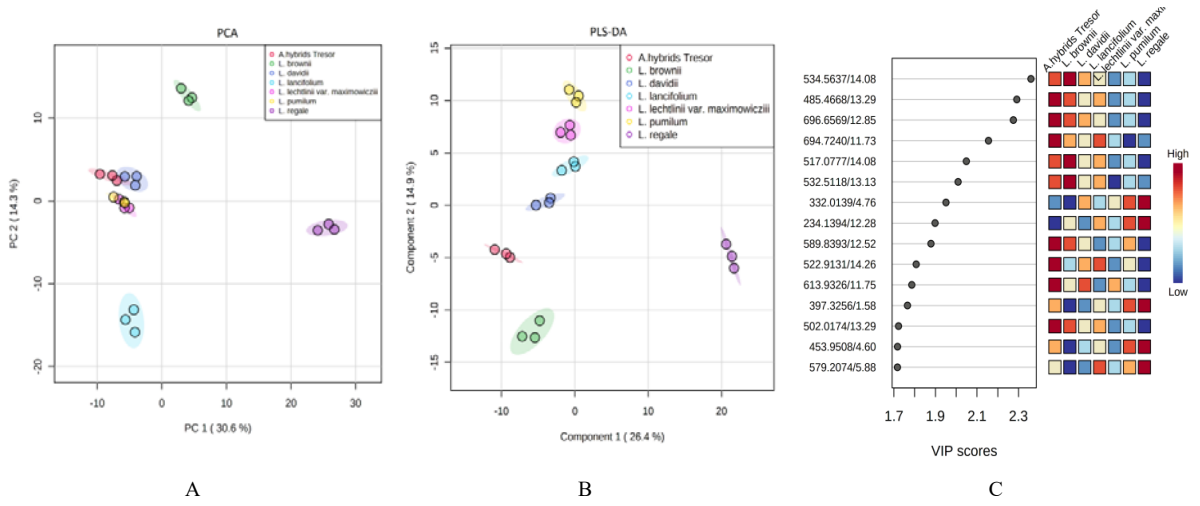


Figure 6. Unsupervised and supervised analysis of metabolomics data of *Lilium* bulb samples based on species: (A) PCA scores plot; (B) PLS-DA scores plot and (C) PLS-DA loadings for the 15 most important variables (metabolites) that could discriminate between the closely related *Lilium* species.

3.3. Chemical Interpretation of Lilium Extracts by GNPS workflow

After the identification of discriminant metabolite, the bioactive compounds were further explored to get the more comprehensive chemical information into the metabolomics profiles of the methanol extracts of *Lilium*. As the classic untargeted metabolomics study with the statistical analysis was hindered by metabolite annotation due to its big and complexity data type and the availability of the chemical standards for the structural identification, classic molecular networking from GNPS was applied to identify the bioactive compounds present in the *Lilium* metabolomics profiles. Molecular network of the methanol extracts of *Lilium* was created on GNPS platform which organizes the metabolomics data by the relatedness of the structures through the similarity between fragmentation patterns (MS/MS) of precursor ions. The created molecular network can be found at the GNPS website via the following links:

https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=42a7896ae4094dbf8d745f525b0e8b90.

The molecular network was generated for the spectra with a minimum of four shared fragment ions, and by merging all identical spectra into individual consensus nodes. Blank spectra from the solvent control were filtered before the networking. In the created *Lilium* molecular network, 7597 nodes were produced from the 91681 MS/MS spectra establishing the 788 molecular families, which were formed based on the structural relatedness. MS/MS spectra which do not have any similarity with others formed as the self-loop single node. 47.3 % of the molecular families composed of 2824 nodes were putatively identified by GNPS spectral libraries with the unique compound names of 966 in total and the lists can be browsed at the link provided above. All the structural annotations were level 2 annotations according to the 2007 Metabolomics Standards Initiative (MSI) (Sumner *et al.*, 2007). Among them, 13 metabolites which were previously found in the Liliaceae genus could be detected in this *Lilium* network by the GNPS spectral library matching (Table 5). In order to determine the presence of some compounds in the Lilium extracts, several reference compounds were purchased, their MS/MS spectra were generated using the same method as the crude extracts, and they were submitted to the GNPS website for data analysis. Ten bioactive compounds were confirmed to be present in *Lilium* species by comparing the MS/MS spectral data with standards. Retention time and spectral data of these compounds can be seen in Table 5.

Table 5. LC-MS characteristics of metabolites to be present in selected *Lilium* extracts

No	Proposed Identity	Retention Time (min)	Parent ion (mz)	MS2 ions	Molecular formula	Identifier	GNPS library ID
1	Regaloside C	2.16	417.08	255,163,399,325	C18H24O11	Standard	CCMSLIB00005720288
2	Gallic acid	2.71	172.47	125,144,150,121	C7H6O5	Standard/ GNPS	CCMSLIB00005720302
3	Chlorogenic acid	2.75	355.48	163	C16H18O9	GNPS	CCMSLIB00000221163
4	Rutin	4.69	611.49	303,465	C27H30O16	GNPS	CCMSLIB00005435850
5	Daucosterol	5.89	579.11	399,416	C35H60O6	Standard	CCMSLIB00005720278
6	Stigmasterol glucoside	6.48	574.28	530,681,737,482, 624,511,455	C35H58O6	Standard	CCMSLIB00005720298
7	Solasodine	9.52	414.89	397,253,271,158	C27H43NO2	Standard/ GNPS	CCMSLIB00005720295
8	Quercetin	10.72	301.44	240	C15H10O7	Standard	CCMSLIB00004705497
9	Quercetrin	11.77	448.49	304,416,286	C21H20O11	Standard/ GNPS	CCMSLIB00005720287
10	Diosgenin/Spirost-5-en-3-ol, (3beta,25R)-	12.70	415.65	272,253,283	C27H42O3	GNPS	CCMSLIB00000855694
11	Gibberellic acid GA3	13.08	346.43	329,311,285	C19H22O6	Standard/ GNPS	CCMSLIB00005720279
12	Riboflavin	13.50	413.70	396,354	C17H20N4O6	Standard	CCMSLIB00004689658
13	Isopimaric acid	17.92	303.47	257,285,179,247, 123	C20H30O2	Standard/ GNPS	CCMSLIB00005720283

4. Discussion

In China, *Lilium* species are considered as commercially important and widely used as edible and medicinal purposes. Many researches demonstrated that *Lilium* species have a wide range of biological activities including antioxidant and antidiabetic activities. Plant extracts or compounds which have the antioxidant, antihyperglycemic and anti-glycation activities might have great therapeutic potential for treating diabetic complications (Chinchansure *et al.*, 2015). Zhang et al. studied that polysaccharides extracted from one of the *Lilium* species, *Lilium davidii var. unicolor* Salisb has certain antiglycation activity at different stages of non-enzymatic glycosylation process(Zhang *et al.*, 2018). However, very few researches have been carried out for the evidence-based validation, especially for antidiabetic potentials of *Lilium* species through glycation inhibition activity. The advanced glycation end-products (AGEs) formation is significantly increased in hyperglycemic conditions(Moe *et al.*, 2018). Therefore, inhibition of AGEs is essential to prevent AGEs related biological disorders. Many researches have been carried out to search the natural or synthetic compounds, which can inhibit the AGEs. As anti-glycemic agents like metformin, amino guanidine and irbesartan have some side effects, compounds isolated from natural resources have been considered safer and cheaper options for the inhibition of AGEs formation(Kaewnarin *et al.*, 2014).

Among the studied species, we could have presumed that different parts of *L. lancifolium* could be useful for different purposes, as its flower bud extract was active for antioxidant activity when we tested its activity with four different *in vitro* assays such as DPPH, SO, FRAP and TPC, and its bulb extract was active for antidiabetic activity through glycation inhibition properties. This might be due to the specialized metabolite localization being different between the plant parts, and bioactivities could differ with the choice of the part used. Since the oxidative stress and non-enzymatic protein glycation was linked to various diseases such as Alzheimer's disease, rheumatoid arthritis, diabetes and atherosclerosis(Perera *et al.*, 2013, RahbarFigarola, 2003), *L. lancifolium* could be a promising plant species with therapeutic potential that merits further investigation, or could be used to develop the functional components or nutraceutical products using its different parts.

Following the investigation of bioactivities of selected *Lilium* species, we further explore the chemical information that could give us the background information for different type of sample presenting the bioactivity in different ways. Hence, chemometric profiling of crude extracts of *Lilium* species was performed to explain this purpose. UHPLC-LTQ-IT-MS/MS analysis with data-dependent scanning (DDS) mode was done and MetaboAnalyst platform (https://www.metaboanalyst.ca) was used to explore the metabolite characteristics of selected *Lilium* species.

MetaboAnalyst is an extensive web-based platform for analysis of complex metabolomics datasets: particularly for data pre-processing, statistical analysis, and functional interpretation which requires little statistical or computational background (XiaWishart, 2016). The parent ion mass and retention time information can help the analyst to locate the target compounds in a complicated metabolomic data set even if the MetaboAnalyst platform is unable to annotate the identification of the discriminant features (Demarque et al., 2020). Finding the known metabolites within complex biological samples—a process sometimes referred to as dereplication—is one of the main obstacles to finding the noble bioactive compound. It is crucial to identify the MS spectra of the metabolites of complex metabolomics dataset by spectral database matching or standard compound comparison. Therefore, in order to search for compound identity using the spectral database, the information provided by MetaboAnalyst is crucial. Moreover, by using the grouping or class

information, it could also provide the information to determine whether the samples are correctly classified as it should be or the chemical information of discriminant compounds, which could be used as biomarker based on the studied questions. From this exploratory statistical analysis, we could detect the pattern of the significant metabolites based on the predefined grouping information. For instance, the metabolites that distinguish between the parts used are distinct from those that distinguish between groups based on bioactivity or species. Hence, we could extract the important chemical information using this exploratory statistical analysis. Even though the selected *Lilium* species belong to one genus, the chemical constituents were mainly related to different parts of the species, this may be due to the fact that the metabolites have different functions at different stages of the plant growth or plant parts based on the demand of metabolism and protective system of the plant (Kadhim *et al.*, 2019).

Among the identified important features of bioactivity-based group separation, solasodine detected in the *Lilium* extracts is bioactive alkaloid, and previous studies indicated that it has antimicrobial, anti-oxidant, neuroprotection, anticonvulsant and central nervous system depressant activities (Chauhan *et al.*, 2011, Kumar *et al.*, 2009, Lecanu *et al.*, 2011, Niño *et al.*, 2009, Sharma *et al.*, 2014). Solasodine, iridoid glycoside, is a typical Solanaceae metabolite. In addition to Solanaceae, other plant families such as Asparagaceae, Compositae, Smilacaceae, Rhamnaceae, and Rosaceae, were examined for the presence of iridoid glycosides(Chi *et al.*, 1981). However, there is no previous report that Liliaceae can biosynthesize the iridoid glycosides. Even though the presence of solasodine was confirmed by comparing the MS2 spectra with the standard in this current research, it is still needed to confirm its presence in this species by the targeted isolation. As shown in the Figure 5B, the relative concentration of this bioactive compound tentatively identified as solasodine was higher in *L. regale* extracts than the rest of the extracts. NO radical scavenging activity and antihyperglycemic activity of *L. regale* extracts might be due to the presence of this compound.

Hydroferuloylglucose, which was putatively identified by the GNPS library matching, was also found to have similar MS2 fragmentation pattern of that reported in the work of Ma et.al (Figure 7) (Ma *et al.*, 2007). Ma et.al reported that Hydroferuloylglucose, one of the plant phenols, phenylpropanoid glycosides type compound could be one of the responsible compounds for the biological activity of ethanolic extract of *Ananas comosus* L. leaves, which exhibited the antidiabetic, antihyperlipidemic and antioxidative effects. The presence of this compound may have influenced the antioxidant and antidiabetic properties of *Lilium* species.

The metabolites which are specifically found to be highest concentration in particular plant species may serve as potential biomarkers for discriminating the closely related species. Therefore, the metabolites, showed up as important features for species based group separation (Table 6), could be used as biomarkers to distinguish *L.brownii*, *L.A.hybrids* 'Tresor' and *L.regale* from the rest of the species, as the relative concentration of these 15 metabolites were quite low in the rest species. However, only two metabolites were putatively identified as PC (0:0/18:0) and 2-(hydroxymethyl)-6-(2, 20, 21-trihydroxydocosan-3-yloxy) oxane-3, 4, 5-triol by the GNPS public spectral database. With the help of GNPS library matching, some of the discriminant metabolites were able to identify putatively. However, many of them were still needed for the identification.

Table 6. Discriminant metabolites from species-based grouping for the Lily bulbs

No.	Parent ion (mz)	Retention Time (min)	Proposed Identity	Molecular Formula	Identifier	GNPS library ID
1	534.5637	14.08	NI	-	GNPS	-
2	485.4668	13.29	NI	-	GNPS	-
3	696.6569	12.85	NI	-	GNPS	-
4	694.7240	11.73	NI	-	GNPS	-
5	517.0777	14.08	NI	-	GNPS	-
6	532.5118	13.13	NI	-	GNPS	-
7	332.0139	4.76	NI	-	GNPS	-
8	234.1394	12.28	NI	-	GNPS	-
9	589.8393	12.52	NI	-	GNPS	-
10	522.9131	14.26	PC(0:0/18:0)	C26H55NO7P	GNPS	CCMSLIB00003121778
11	613.9326	11.75	NI	-	GNPS	-
12	397.3256	1.58	NI	-	GNPS	-
13	502.0174	13.29	NI	-	GNPS	-
14	453.9508	4.60	NI	-	GNPS	-
15	579.2074	5.88	2-(hydroxymethyl)-6-(2,20,21-trihydroxydocosan-3-yloxy)oxane-3,4,5-triol	C28H56O9	GNPS	CCMSLIB00000847180

NI: Not Identified

As the classic untargeted metabolomics study with the statistical analysis was hindered by metabolite annotation due to its big and complexity data type and the availability of the chemical standards for the structural identification,

classic molecular networking from GNPS was applied to identify the bioactive compounds present in the *Lilium* metabolomics profiles. Molecular network of the methanol extracts of *Lilium* was created on the GNPS platform which organizes the metabolomics data by the relatedness of the structures through the similarity between fragmentation patterns (MS/MS) of precursor ions.

Among the identified metabolites which were involved in the *Lilium* molecular network created by GNPS, regaloside C is a glycerol glucoside, isolated from the bulbs of *Lilium* genus and its biological activities involve anti-inflammatory and cardiomyocyte protective activity (Qiu *et al.*, 2020). Some bioactive phenolic acids such as gallic acid and chlorogenic acid were found to be present in the *Lilium* molecular network. Gallic acid is well known bioactive metabolite, which is present in a variety of fruits and a number of plants, and showed several biological activities such as antioxidant, anti-inflammatory, hepatoprotective, antimicrobial, antidepressant, antiparkinson, antidiabetic, antimalarial, diuretic, cardioprotective, antiviral, antifungal, wound healing, anthelmintic, and anxiolytic (Kahkeshani *et al.*, 2019, Nayeem *et al.*, 2016). Chlorogenic acid also plays several biological and pharmacological roles, such as anti-microbial, antioxidant, anti-inflammatory, antipyretic, hepatoprotective, cardioprotective, neuroprotective, anti-obesity, antiviral, anti-hypertension and as a central nervous system (CNS) stimulator and modulator for many metabolic disorders such as diabetes, hepatic steatosis, cardiovascular disease and obesity (Naveed *et al.*, 2018).

Some phytosterol were also detected in the *Lilium* molecular network. A phytosterol, namely daucosterol, was among them and it possesses some biological activities, such antimicrobial, antiproliferative, neuroprotective, anti-cancer, and apoptotic effects by several *in vitro* assay results (Jiang *et al.*, 2015, Lee *et al.*, 2015a, Lee *et al.*, 2009, SultanaAfolayan, 2007, Wang *et al.*, 2016a, Zhao *et al.*, 2015). Another phytosterol, Stigmasterol glucoside, was also present in this network. Khatun et. al discussed the possible biological activity of plant sterols, and they assumed that glucose moiety of the sterol glucoside may prevent the esterification of cholesterol, and this might inhibit the entry of cholesterol into the blood vessel (Khatun *et al.*, 2012). Quercetin, a medicinally important flavonoid, and its derivatives, quercetrin and rutin, were identified in this molecular network. Many studies reported several beneficial health effects on quercetin such as antioxidant, antidiabetic, antiviral, anti-inflammatory, cardioprotective, neuroprotective, hepatoprotective and antimicrobial through various *in vitro* assays (Pejin *et al.*, 2015). Moreover, it's derivative, bioactive rutin, was previously reported to be present in the bulb extract of *L. lancifolium* (Jin *et al.*, 2012), and it had been identified in this *Lilium* molecular network. As rutin has wide range of bioactivities such as anticancer (Alonso-Castro *et al.*, 2013), antidiabetes (Niture *et al.*, 2014), antihypertensive (Olaleye *et al.*, 2014), anti-inflammation (Choi *et al.*, 2014) and antioxidant (Sikder *et al.*, 2014), the presence of this compound could contribute the antioxidant and antiglycation activity of *Lilium* species. Zhang et.al reported that quercetrin inhibited colorectal cancer cell growth and facilitated apoptosis, and might be potential candidate for colorectal cancer chemotherapy (Zhang *et al.*, 2017). Diosgenin or Spirost-5-en-3-ol, (3beta,25R), a well-known plant steroid sapogenin compound found in this species, also has numerous pharmacological potentials such as anti-diabetes, anticancer, immunomodulatory, estrogenic, cardiovascular protective, neuroprotective and skin protective effects (Chen *et al.*, 2015). Gibberellic acid GA3: plant growth hormone and vitamin B2 were also detected. A widely available tricyclic diterpenoid, isopimaric acid, which has interesting biological and pharmaceutical properties, such as antimicrobial, antiviral, antiallergenic, and anti-inflammatory activities, was also present in the *Lilium* network.

5. Conclusions

It could be concluded that *L. lancifolium* showed most promising activities among the studied species as its flower bud extract was active for antioxidant activity in four different *in vitro* assays, and its bulb extract was active for antidiabetic activity through glycation inhibition property. *L. lancifolium* may therefore be a promising plant species with potential therapeutic properties and is deserving of additional in-depth study for targeted isolation of compound of interest. Two MS2-based untargeted metabolomics approaches, MetaboAnalyst and GNPS, assist to explore the specialized metabolite profiles of selected *Lilium* species. To our knowledge, this study was the first to explore the specialized metabolite profile of *Lilium* species by these approaches. Using these methods, it was possible to recover the chemical information of 13 previously identified bioactive chemicals as well as some of the discriminant molecules that may be utilized as biomarkers for *Lilium* species. Many compounds present in the *Lilium* extracts could not confirm their identities through the public database searches. This could be for a number of reasons: either these metabolites were potentially new and noble compounds that required isolation and identification using various chromatographic techniques, or their spectra were already known but not included in this public database for spectral matching. It's also possible that commercially available spectral databases could identify more metabolites than the public database we used. Although the selected *Lilium* species belong to one genus, their chemical composition was mainly related to the choice of the plant parts. Since the metabolites have different functions at different stages of the plant growth, or depend on the demand of metabolism and protective system of the plant, the chemical composition of the specific parts of the plant might be different. It appeared that the major metabolites detected in this study seem to have potential therapeutic uses. However, further studies are still needed for the practical usage, as most studies are based on *in vitro* assays, and their effects on humans still need to go through clinical trials. From the current work of our research, metabolite profiling of selected *Lilium* species along with the bioactivity study will further pave the way for the targeted isolation of structurally or biologically noble bioactive compounds, and will provide evidence-based information of antioxidant and antidiabetic activity for further development and application of these *Lilium* species for therapeutic uses. Moreover, we expect that our study could be an invaluable proof to promote the

exploration and research on this and other edible and medicinal plants.

6. Supplementary Materials

TIC chromatograms of each sample can be available as supplementary materials. (Figure S1. TIC chromatogram of methanolic extract of ZDM1, Figure S2. TIC chromatogram of methanolic extract of ZDM2, Figure S3. TIC chromatogram of methanolic extract of ZDM3, Figure S4. TIC chromatogram of methanolic extract of ZDM4, Figure S5. TIC chromatogram of methanolic extract of ZDM5, Figure S6. TIC chromatogram of methanolic extract of ZDM6, Figure S7. TIC chromatogram of methanolic extract of ZDM7, Figure S8. TIC chromatogram of methanolic extract of ZDMF1, Figure S9. TIC chromatogram of methanolic extract of ZDMF2, Figure S10. TIC chromatogram of methanolic extract of ZDMF3, Figure S11. TIC chromatogram of methanolic extract of ZDMF4, Figure S12. TIC chromatogram of methanolic extract of ZDMF5, Figure S13. TIC chromatogram of methanolic extract of ZDMF6.)

Acknowledgments

This research work has been financially supported by Ministry of Science and Technology of the People's Republic of China through Talented Young Scientist Program (TYSP), which is greatly appreciated by the authors. The authors are also grateful to Beijing Agro-Biotechnology Research Center, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China for supporting this research work.

Conflict of Interests

We declare that we have no conflict of interests.

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