Diversity of antioxidant ingredients among *Echinacea* species

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ABSTRACT

Echinacea species are important medicinal plants with significant therapeutic potential and are widely used in the pharmaceutical industry. Different *Echinacea* species exhibit various chemical compositions and bioactivities. In the present study, the chemical differences, antioxidant ingredients, and diversity mechanisms of *Echinacea* species were studied. The aerial parts and roots of six different *Echinacea* species (*E. purpurea* (L.) Moench; *E. pallida* (Nutt.) Nutt.; *E. angustifolia* DC.; *E. atrorubens* (Nutt.) Nutt.; *E. paradoxa* (Norton) Britton var. *paradoxa*; *E. sanguinea* Nutt.) were collected for investigation. Through non-target metabolomics following targeted quantitative analysis, chicoric acid, caftaric acid, and echinacoside were found to be the main different components of *Echinacea* species. Coincidentally, these three chemicals were also the dominant antioxidant ingredients of *Echinacea* extracts, as determined by correlation analysis between chemical contents and *in vitro* antioxidant activities. Based on the cloning, sequencing, and measurement of the identified chicoric acid biosynthetic genes, the diversity of chicoric acid and caftaric acid among *Echinacea* species was determined by the precursor content, as well as by the expression levels of key biosynthetic genes. Overall, these results clarified the chemical differences and the possible mechanisms, as well as the bioactive ingredients, in *Echinacea* species and can guide the selection of *Echinacea* species for different industrial applications.

1. Introduction

The genus *Echinacea*, belonging to the Asteraceae family, is a group of nine species native to mid-latitude North America (Tang et al., 2017). The distribution of *Echinacea* covers a wide range of moisture and temperature regimes in North America, from the relative warmth of central Texas, Georgia, and Alabama to the cooler weather of Montana, North Dakota, Minnesota, and Canada (Kindscher, 2016). In the past, *Echinacea* was used by Native Americans for various ailments, including mouth sores, colds, and cough (Borchers et al., 2000). *Echinacea*-derived products are widely used as daily supplements worldwide and are marketed and used as immunostimulants to treat and prevent the common cold, influenza, and upper respiratory tract infections (Cao and Kindscher, 2016). Owing to the health benefits, products from *Echinacea* have drawn increasing attention globally and have become a massive industry. In 2019, in the US market alone, the sales of products from three

commonly used species, including *E. angustifolia* (Ean), *E. pallida* (Epa), and *E. purpurea* (Epu) reached 120 million US dollars, an increase of 4.9% compared with the previous year. In addition, in the first half of 2020, *Echinacea* sales grew sharply by 90.9%, which may be due to the COVID-19 pandemic (Smith et al., 2020).

Modern pharmacology studies on *Echinacea* have identified many bioactivities, such as antioxidant, immunomodulatory, anti-inflammatory, antifungal, and antiviral activities (Barrett, 2003; Binns et al., 2002; Melchart et al., 1995). Caffeic acid derivatives, alkamides, polysaccharides, polyacetylenes, polyenes, flavonoids, and terpenoids have been linked to these bioactivities (Cao and Kindscher, 2016). In addition, synergistic effects of alkamides, caffeic acid derivatives, and polysaccharides have been reported in *E. purpurea* (Dalby-Brown et al., 2005). Two important caffeic acid derivatives from *Echinacea*, chicoric acid and echinacoside, have been widely studied for the remarkable bioactivity and are assumed to be the active ingredients of *Echinacea* (Aiello et al., 2015; Naveed et al., 2018; Silva et al., 2014). The production, stabilization, and changes during storage have been extensively studied (Bergeron et al., 2002; Dalby-Brown et al., 2005; Lin et al., 2011). The chemical compositions of the three commonly used *Echinacea* species (Ean, Epa, and Epu) were different, resulting in various bioactivities (Barnes et al., 2010; Erenler et al., 2015; Perry et al., 2001; Sloley et al., 2001; Thomsen et al., 2012). On a broader level, the genome size and chloroplast genome of the *Echinacea* genus have been compared (Jedrzejczyk, 2020; Zhang et al., 2017).

Recently, the complete biosynthesis pathway of chicoric acid in *Echinacea* has been successfully elucidated (Fu et al., 2021). Chicoric acid and its two substrates, caftaric acid and chlorogenic acid, originate from phenylpropanoid metabolism. Hydroxycinnamoyl-CoA: quinate/shikimate hydroxycinnamoyl transferase (HCT) catalyzes the synthesis of caffeoyl CoA, the most important precursor; hydroxycinnamoyl-CoA: tartaric acid hydroxycinnamoyl transferase (HTT) catalyzes caffeoyl CoA and tartaric acid to synthesize caftaric acid; hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (HQT) combines caffeoyl CoA and quinic acid into chlorogenic acid; and caftaric acid and chlorogenic acid are used as acyl acceptor and acyl donor, respectively, by chicoric acid synthase (CAS) to generate chicoric acid. These four biosynthetic enzymes complete the specific chicoric acid biosynthesis and allow the study of the intrinsic determinants of chicoric acid diversity in *Echinacea* (Fu et al., 2021).

In the present study, the chemical differences among six *Echinacea* species, namely, Epu, Epa, Ean, *E. atrorubens* (Eat), *E. paradoxa var. paradoxa* (Epp), and *E. sanguinea* (Esa), were compared using non-target metabolomics. The active antioxidant ingredients were determined based on the positive relationship between the observed antioxidant activity and the sum of each active ingredient's antioxidant contribution. Finally, the potential mechanisms of chicoric acid and caftaric acid diversity among *Echinacea* species were investigated. All these results will significantly promote the research into *Echinacea* species, such as the selection of materials for special applications.

2. Material and methods

2.1. Reagents

MS-grade methanol, acetonitrile, and formic acid were purchased from Thermo Fisher Scientific Inc. (MA). Chlorogenic acid (98%, CAS: 327-97-9) was obtained from Chengdu Herbpurify Co., Ltd. (Chengdu, China). Caftaric acid (98%, CAS: 67879-58-7), chicoric acid (98%, CAS: 6537-80-0), echinacoside (98%, CAS: 82854-37-3), fluorescein sodium salt, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and inorganic salts for phosphate buffer were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). The RNeasy Plant Mini Kit was purchased from Qiagen (Hilden, Germany). iTaq Universal SYBR Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Plant materials

Seeds of the six *Echinacea* species were obtained from the U.S. National Plant Germplasm System (Epu PI656830, Epa PI631293, Ean PI649026, Eat PI631262, Epp PI633664, and Esa PI649044). Ten seeds were pretreated in 2 mL of water at 40°C for 6 h and then grown in a growth room (23 ± 2°C, 16 h light/8 h dark) for two months. The aerial parts and roots of the three seedlings were collected and frozen immediately in liquid nitrogen. The samples were ground into powder. One part was lyophilized for metabolite extraction. The other part was used for RNA extraction.

2.3. Preparation of the extracts

Extracts were prepared according to a previous study, with some modifications (Thomsen et al., 2012). The dry sample powder was weighed (10 mg) and extracted with 1 mL 70% methanol (v/v) in an ultrasonic bath (Qsonica 700, USA) at 4°C, for 20 s on/40 s off; for a total of 15 min and 50% AMPL. The extract was centrifuged at 5000 × g for 10 min at 4°C, and the supernatant was collected. The residue was extracted under the same conditions. The two supernatants were combined and diluted to a concentration of 2.5 mg/mL (dry material *vs.* solvent). The extracts were further centrifuged at 20,000 × g for 10 min at 4°C, and the supernatant was collected for chemical composition analysis and antioxidant tests.

2.4. Non-target metabolomic analysis

Non-target metabolomics was performed using a liquid chromatography-high-resolution mass spectrum (LC-HRMS) system (Nexera UHPLC LC-30A and AB SCIEX qTOF X500R) coupled with a Turbol V™ source and SCIEX OS software (version 1.7) (Fu et al., 2021). Sample separation was performed using a Hypersil Gold C18 column (100 × 2.1 mm, 1.9 μm; Thermo Fisher Scientific, USA) at 40 °C with a flow rate of 0.4 mL/min. 0.1 % formic acid (A) and acetonitrile (B) were adopted as mobile phases, and the gradient was as follows: 0-0.5 min 2% B; 0.5-6 min 2%-20% B; 6-10 min 20%-95% B; 10-12 min 95% B; 12-12.1 min 95%-2% B; and 12.1-–15 min 2% B. The sample (1.0 μL) stored at 15 °C was injected. An information-dependent acquisition (IDA) model was used for data acquisition with an electrospray ionization (ESI) source in negative polarity. The MS parameters were set as follows: ion source gas 1: 50 psi; ion source gas 2: 50 psi; curtain gas: 35 psi; CAD gas: 7; temperature: 450 °C; spray voltage: −4500 V; for TOF-MS, the mass range: 100–1000 Da; declustering potential (DP): −80 V; DP spread: 0; collision energy (CE): −10 V; CE spread: 0; accumulation time: 0.1 s; IDA criteria: small mole-

cule; for TOF-MS/MS, the mass range: 50–1000 Da; DP: −80 V; DP spread: 0; CE: −40 V; CE spread: 20 V; accumulation time: 0.05 s. Non-targeted screening workflow of SCIEX OS software (version 1.7) was adopted with a medium peak detection sensitivity to export peak area for statistical analysis.

2.5. Quantitative analysis of main caffeic acid derivatives

Quantification of major components was performed on a DIONEX UltiMate 3000 UHPLC system with Chromeleon software (Thermo Fisher Scientific, USA) (Fu et al., 2021). Sample separation was performed using the same column used for the LC-HRMS at 40 °C. The mobile phases were 0.1 % formic acid water (A) and acetonitrile (B) and the gradient was as follows: 0-0.5 min 2% B; 0.5-1.5 min 2%-14% B; 1.5-7 min 14%-18% B; 7-9 min 18%-80% B; 9-10 min 80% B; 10-10.1 min 80%-2% B; and 10.1-−13 min 2% B. The flow rate was 0.5 mL/min, and the injection volume was 2.0 μL. The sampler temperature was set at 10 °C. UV–vis absorption spectra was recorded on-line from 190 to 800 nm during UPLC analysis. Photodiode array detection was performed at 330 nm for quantitative purposes. Quantification was carried out using the external standard method with calibration curves (1, 2, 4, 8, 16, 32, 64, and 128 μg/mL). The calibration curve coefficients for caftaric acid (y = 0.1728x-0.0018), chicoric acid (y = 0.2199x-0.0286), chlorogenic acid (y = 0.1407x-0.0014), and echinacoside (y = 0.0618x-0.0005) were 0.9999, 0.9999, 0.99996, and 0.99993, respectively. The results are expressed as mg of each caffeic acid derivative per 1 g of dry material.

2.6. Antioxidant activity tests

2.6.1. Oxygen radical antioxidant capacity (ORAC) assay
The ORAC assay was performed as previously reported with slight modifications (Fu et al., 2016). Briefly, 40 μL of extracts (100 μg/mL, obtained by dilution of 25 times from *Echinacea* extracts), Trolox (50, 100, 200, 300, 400, and 500 μM dissolved in 70% methanol), or 70% methanol were mixed with 40 μL fluorescein solution (50 μM in PBS, pH = 7.4) and incubated for 20 min at 37 °C. Afterward, 170 μL of freshly prepared AAPH (100 mM in PBS, pH = 7.4) was added, and the fluorescence was recorded immediately at an excitation wavelength of 485 nm and an emission wavelength of 538 nm every 2 min for 150 min using a BioTek Synergy H1 microplate reader.

Calculations were based on the area under the fluorescence decay curve (AUC). The ORAC values were calculated using a regression equation for linear regression using Trolox standards. The net area under the curve was obtained by subtracting the area under the curve for the blank values from the curves of samples and standards. The ORAC values were expressed in μmol Trolox equivalents per gram dw (μmol TE/g dw).

The ORAC values of single compounds, including caftaric acid, chicoric acid, and echinacoside were determined using the above method with a 10 μg/mL sample concentration.

2.6.2. ABTS radical scavenging assay
The ABTS radical scavenging activity of *Echinacea* extracts was determined using a previously published method (Fu et al., 2014). The ABTS radical solution was prepared by reacting 7 mM ABTS with 2.45 mM potassium persulfate (final concentration, in PBS (pH = 7.4)) at 25 °C in the dark for 12 h. The solution was then diluted with PBS to acquire an absorbance of 0.7 ± 0.02 at 734 nm. Fifty microliters of samples

(6.25–100 μg/mL) or Trolox (6.25–100 μM) were mixed with 50 μL of the diluted ABTS solution. The mixtures were incubated at 25 °C for 3 min, and A_{734} was measured using a microplate reader. The radical scavenging activity was calculated as follows:

Radical scavenging activity (%) = (1-(A_{sample}-A_{blank})/($A_{control}$-A_{blank})) × 100

where A_{sample} represents the A_{734} of the sample extracts, A_{blank} represents the A_{734} of the blank, and A_{blank} represents the A_{734} of the control.

The IC_{50} values were determined using the GraphPad software (La Jolla, CA, USA). The ABTS radical scavenging activity was also expressed as TEAC (μmol TE/g dw) by comparing the IC_{50} values of the samples and Trolox.

The ABTS radical scavenging activities of single compounds were also determined using the method described above, with concentrations ranging from 0.625 to 10 μg/mL.

2.7. Gene cloning and sequencing

The RNeasy Plant Mini Kit (Qiagen) was used to extract the total RNA. First-strand cDNA was synthesized from 1 μg RNA using a Prime-Script™ RT reagent Kit with gDNA Eraser (Takara). Coding sequences of *HCT*, *HQT*, *HCT*, and *CAS* genes from different species were amplified *via* PCR using primers used for Epu in a previously study (Fu et al., 2021). The PCR products were cloned into the pEASY-T1 vector (TranGen Biotech, China) and used for sequencing. The sequences were aligned using DNAMAN (version 6).

2.8. qRT-PCR

qRT-PCR was conducted according to a previously published method using the same primers (Fu et al., 2021). qRT-PCR was performed using a Bio-Rad CFX384 and iTaq Universal One-Step RT-qPCR Kit (Bio-Rad) according to the manufacturer's instructions. The data were analyzed and calculated using Bio-Rad CFX manager software. Tubulin was used as an internal control. Relative gene expression was calculated using the ΔCt method.

2.9. Statistical analysis

All experiments were conducted at least three times, and the results are expressed as the mean ± SD. GraphPad Prism 8.01 (La Jolla, CA, USA) was used for the statistical analysis of different significance and correlation ships. Principal component analysis and cluster analysis were performed using SIMCA 13.0 (Umea, Sweden) and MEV (version 4.9.0), respectively.

3. Results and discussion

3.1. The chemical diversity of Echinacea species

Six of the nine known *Echinacea* species, namely, Epu, Epa, Ean, Eat, Epp, and Esa were germinated and cultivated in a greenhouse under the same conditions for 2 months. The phenotypes of the *Echinacea* species are shown in Fig. 1a. Among them, Epu showed a distinct phenotype with more fibrous roots and wide blades. The aerial parts and roots were separately extracted and analyzed.

First, a liquid chromatography-high resolution mass spectrometry (LC-HRMS)-based non-target metabolomic analysis
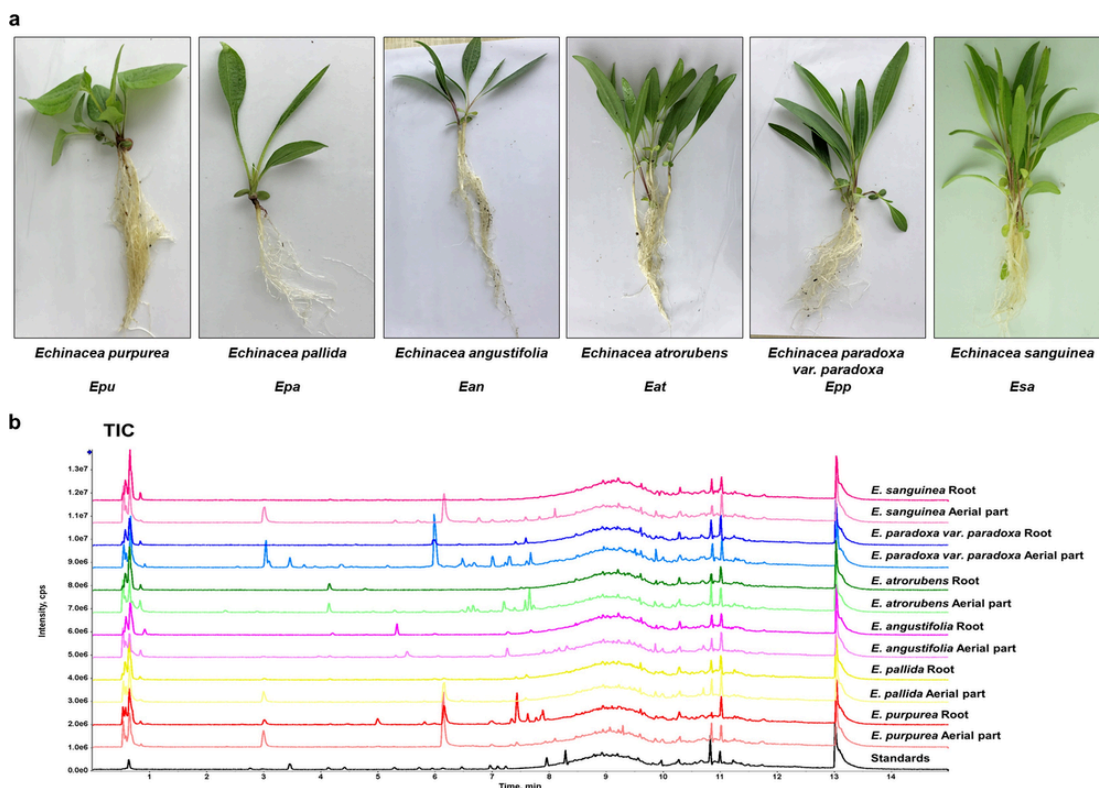
Fig. 1. Non-target metabolomic analysis of extracts from six *Echinacea* species. (a) Phenotypes of *Echinacea* species. Six species including *E. purpurea*, *E. pallida*, *E. angustifolia*, *E. atrorubens*, *E. parapdoxa var. papradoxa* and *E. sanguinea* were successfully germinated and cultivated in a greenhouse under the same conditions. Two-month-old seedings' aerial parts and roots were separately collected and used for metabolomic analysis. (b) Total ion chromatograms (TICs) of extracts from aerial parts and roots of different *Echinacea* species detected by LC-HRMS.

was adopted to investigate the differences in constitution. Typical total ion chromatograms (TICs) of the aerial parts and roots of *Echinacea* species acquired from LC-HRMS are shown in Fig. 1b. Extracts of aerial parts and roots from the same species exhibited similar chemical compositions. However, there was a clear difference between the species (Fig. 1b). All peaks were aligned and exported for principal component analysis (PCA). Two principal components (pc1 and pc 2) were included in the illustration. As shown in the score plots (Fig. 2a), the roots of different species appeared to be grouped together. Among the aerial parts, those of Epu and Epp showed great differences compared with the aerial parts of other species. From the loading plots (Fig. 2b), the major different ions were identified as caftaric acid, chicoric acid, and echinacoside. Chicoric acid and echinacoside were also identified as the major bioactive compounds in *Echinacea* (Perry et al., 2001).

Using a UPLC method, the contents of echinacoside and chicoric acid and the substrates caftaric acid and chlorogenic acid were quantitatively measured. As shown in the heatmap (Fig. 3), the whole plant of Epu contained high levels of chicoric acid and caftaric acid. The chicoric acid content reached up to 4.2% and 2.8% in the aerial parts and roots, respectively. The aerial parts of Epa and Esa also contained relatively high amounts of chicoric acid and caftaric acid. The whole plant of Epp contained echinacoside, especially aerial parts with up to 80 mg/g dry weight. This content is far higher than that reported previously for Epa and Ean. Chlorogenic acid, on the other hand, was widely distributed among the *Echinacea* species, even at low concentrations. In summary, the differences of caffeic acid derivatives in the present study were

similar to those reported previously (Pellati et al., 2004; Pellati et al., 2005; Perry et al., 2001; Sloley et al., 2001).

Through non-target metabolomic and target quantitative analysis, the main difference among *Echinacea* species is owing to the distinct composition of caffeic acid derivatives, especially chicoric acid and echinacoside. As all species were grown under the same conditions to exclude environmental effects on the chemical content, the composition differences were supposed to be caused by genetic variation. The variety of types and contents among *Echinacea* species provides guidance for selecting the material for different applications. *Echinacea sanguinea* can be a new choice for producing chicoric acid in addition to the widely utilized Epu and Epa. *Echinacea paradoxa var. paradoxa* showed higher echinacoside content than the currently known resources, such as *Cistanche*, *Syringa*, and *Penstemon* (Wang et al., 2020; Wang et al., 2019; Xie et al., 2010), making it suitable for the echinacoside industry.

3.2. Antioxidant ingredients of Echinacea species

The diversity of chemical composition among *Echinacea* species provides an opportunity to explore the bioactive ingredients. Antioxidant tests have been widely used to screen for potential biological activity (Fu et al., 2015; Fu et al., 2013; Giuffrè et al., 2018; Giuffrè, 2019). The *in vitro* antioxidant activities of *Echinacea* species were determined using the ORAC and ABTS methods which have been widely used to determine the antioxidant activity of plant methanol extracts (Celano et al., 2021; Muscolo et al., 2020). The ORAC method is considered preferable because of its biological relevance to the *in vivo* antioxidant efficacy (Bisby et al., 2008). The ORAC values of
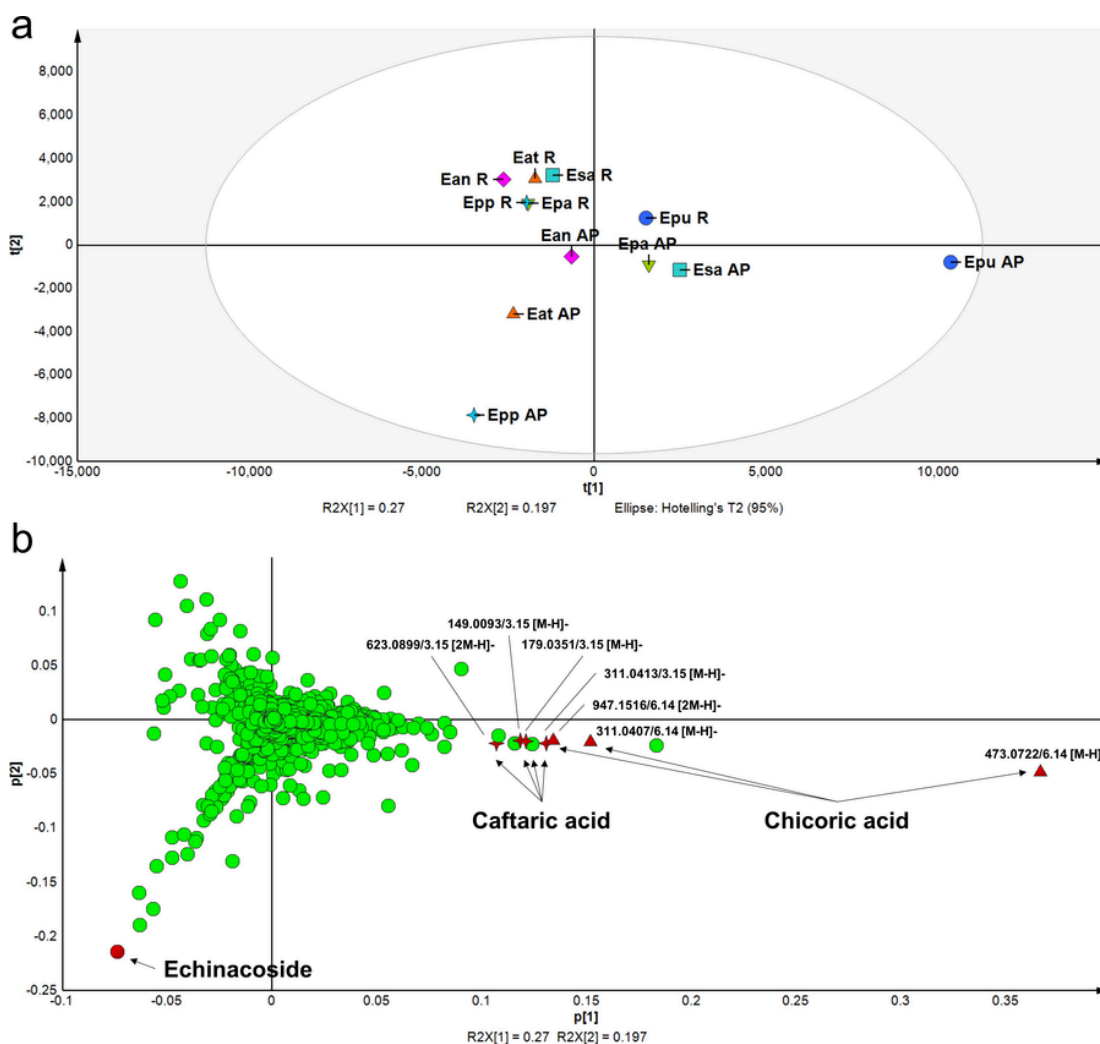
Fig. 2. Main different metabolites among different *Echinacea* species. (a) Score plot. (b) Loading plot. Each point represents the mean of three extracts duplications.

different parts and species, which to our knowledge were first determined in *Echinacea* species, are presented in Fig. 4a. The highest ORAC value was found in the extract of the aerial parts of Epp (2633 µmol Trolox/g), while the lowest was found in the root extract of Ean (245.3 µmol Trolox/g) (Fig. 4a). The ABTS radical scavenging assay has also been widely used to evaluate antioxidant activity in food and biological systems. Trolox equivalent antioxidant capacity (TEAC, µmol Trolox/g) was used to evaluate ABTS radical scavenging capacity. The ABTS radical scavenging activity was similar to the ORAC results. The antioxidant activities of different *Echinacea* species root extracts tested using ORAC and ABTS methods were similar to the previously published 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities (Pellati et al., 2005). The antioxidant activity of three commonly used species in the order of Epu > Epa > Ean was in line with previously published report (Sloley et al., 2001). The antioxidant properties of all *Echinacea* species were compared using the two widely used antioxidant determination methods. In summary, the aerial parts of Epp showed the highest antioxidant capacity. *Echinacea purpurea* showed high antioxidant capacities in both the aerial parts and roots extracts.

It is still uncertain whether the distinct antioxidant activities are related to the different chemical contents. The correlations between each quantitative chemical content and the antioxidant activity, expressed as ORAC and TEAC values were calculated (Fig. 5). Caftaric acid, chicoric acid, and echinacoside showed potential positive relationships, especially echinacoside with a significant positive correlation ($p < 0.05$), but not chlorogenic acid. Caftaric acid, chicoric acid, and echinacoside were responsible for the antioxidant activity of different extracts. The antioxidant activities of these compounds were determined for further studies.

The ORAC values of these three compounds are shown in Fig. 6a. Chicoric acid exhibited significantly stronger antioxidant activity than echinacoside and caftaric acid ($p < 0.05$). The ABTS radical scavenging capacity was also found in the order of chicoric acid > echinacoside > caftaric acid. These results are in line with a previous report that chicoric acid showed higher antioxidant activity than echinacoside in $CuSO_4$-induced oxidation of low-density lipoprotein (Dalby-Brown et al., 2005).

The contribution of a single compound to the ORAC value of the extract was calculated by multiplying the content and ORAC value of every single compound. The ORAC values of each compound were added together (assumed as Predicted), then compared with the actual ORAC values of extracts, and a highly and significantly positive correlation ($r = 0.8988$) was

	Caffaric acid	Chicoric acid	Chlorogenic acid	Echinacoside
Epu AP	17.79±1.89[a]	42.21±4.78[a]	0.18±0.01[b]	0[c]
Epu R	7.43±0.42[b]	28.06±1.33[b]	0.95±0.07[b]	0[c]
Epa AP	10.26±1.39[b]	16.85±2.98[c]	0.27±0.04[b]	0[c]
Epa R	2.56±0.40[c]	2.48±0.67[d]	0.88±0.04[b]	9.83±4.05[b]
Ean AP	1.14±0.14[c]	0.23±0.02[d]	0.91±0.22[b]	0.93±0.24[c]
Ean R	0[c]	0.10±0.02[d]	1.22±0.05[b]	2.63±0.99[c]
Eat AP	0[c]	0[d]	5.00±2.51[a]	0[c]
Eat R	0[c]	0[d]	4.05±0.40[a]	0[c]
Epp AP	0[c]	0[d]	0[b]	80.11±4.61[a]
Epp R	0[c]	0[d]	0.43±0.03[b]	13.17±4.69[c]
Esa AP	19.96±4.15[a]	23.40±5.02[bc]	0.27±0.04[b]	0[c]
Esa R	0.84±0.32[c]	1.05±0.55[d]	0.59±0.18[b]	0[c]

Fig. 3. Contents heatmap of main different caffeic acid derivatives. The contents were determined using UPLC method and expressed as mean ± SD (n = 3). Statistical significance was calculated by One-way ANOVA. Different lowercases indicate significant different at the $p < 0.05$ levels.

found (Fig. 6c). Similar results were obtained in the ABTS radical scavenging assay (Fig. 6d). The predicted values were lower than the actual values, indicating that other compounds, such as alkamides and polysaccharide, also contributed to the antioxidant capacity of *Echinacea* (Dalby-Brown et al., 2005). However, the highly positive correlation also demonstrated that caffaric acid, chicoric acid, and echinacoside were the active ingredients of *Echinacea* species and were responsible for the antioxidant activities.

Chicoric acid and echinacoside are considered the primary active compounds in *Echinacea* species (Barrett, 2003). Currently, chicoric acid and echinacoside are used as quality indicators, and most commercial *Echinacea* products are labeled with the chicoric acid and echinacoside contents. The results of the present study support this finding. In addition, caffaric acid is an active ingredient. As chicoric acid is metabolized to caffaric acid *in vivo* (Liu et al., 2015), more attention should be paid to the activity of caffaric acid. With respect to the combined application of *Echinacea* species for commercial use, Epp leaf and Epu whole plant could be a better combination than the widely used Epu and Ean root combination if only caffeic acid derivatives are considered. However, this hypothesis needs to be verified through further investigations. In summary, chicoric acid, caffaric acid, and echinacoside were found to be the main antioxidant active ingredients in *Echinacea*.

3.3. The biosynthesis of chicoric acid among Echinacea species

Recently, the conserved biosynthetic route of chicoric acid in *Echinacea* has been elucidated (Fu et al., 2021). The coding sequences (CDS) of these biosynthetic genes from different *Echinacea* species were cloned and sequenced. When comparing the amino sequences, HCT and HQT were highly conserved
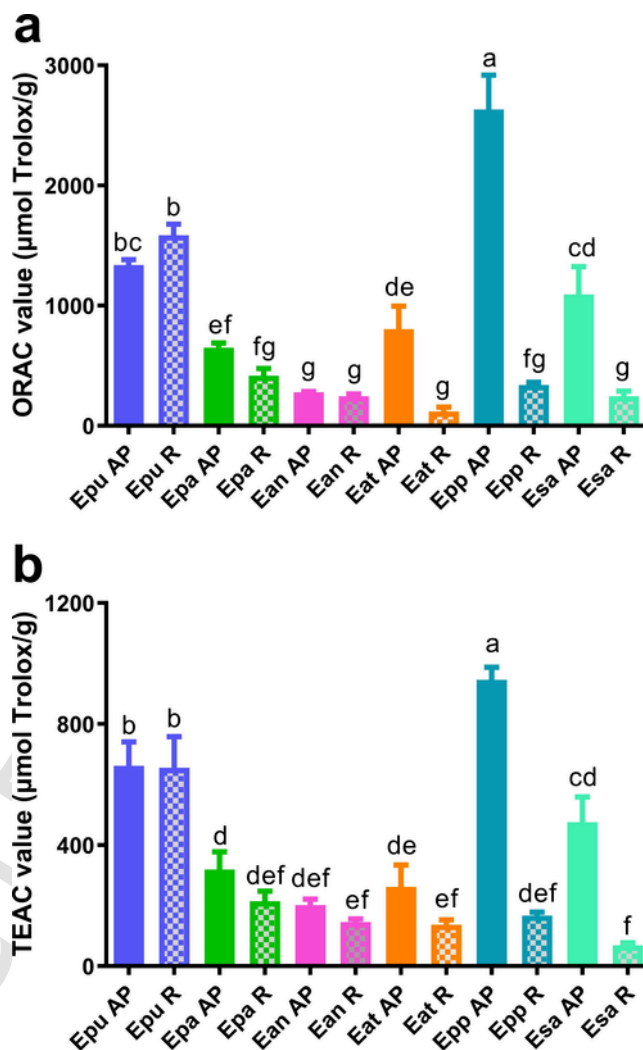
Fig. 4. *In vitro* antioxidant activities of different *Echinacea* species. (a) Antioxidant capacities expressed as ORAC values. (b) ABTS radical scavenging activities expressed as TEAC values. Data are mean ± SD (n = 3). Statistical significance was calculated by One-way ANOVA. Different lowercases indicate significant different at the $p < 0.05$ levels.

among all six *Echinacea* species with conserved HHAAD and HTLSD motifs, respectively (Fig. S1−2) (Bontpart et al., 2015; D'Auria, 2006). Identical CAS protein sequences were also found in all *Echinacea* species, except Ean, which exhibited a few mutations (Fig. S3). However, highly homologous *HTTs* of *EpuHTT* were only present in Ean and Esa. In Epa, a relatively low identical sequence was found with the conserved HHLVD motif. Although no *HTTs* were found in Eat and Epp (Fig. S4). Based on the sequence analysis and preliminary experiment, qRT-PCR primers used in previous study could also be used for the quantification of these biosynthetic genes among *Echinacea* species (Fig. S5−8) (Fu et al., 2021). Subsequently, the expression levels of these four genes in different parts and species were determined: *HCTs* were constitutively highly expressed since they are important biosynthetic genes of phenylpropanoid metabolism and are conserved across all land plants (Fig. 7a) (Weng and Chapple, 2010); and *HQTs* were expressed at lower levels than *HCTs*, but with a similar expression pattern, except for the extraordinarily low expression in Epp (Fig. 7b). However, the other two specific genes, *HTT* and *CAS,*
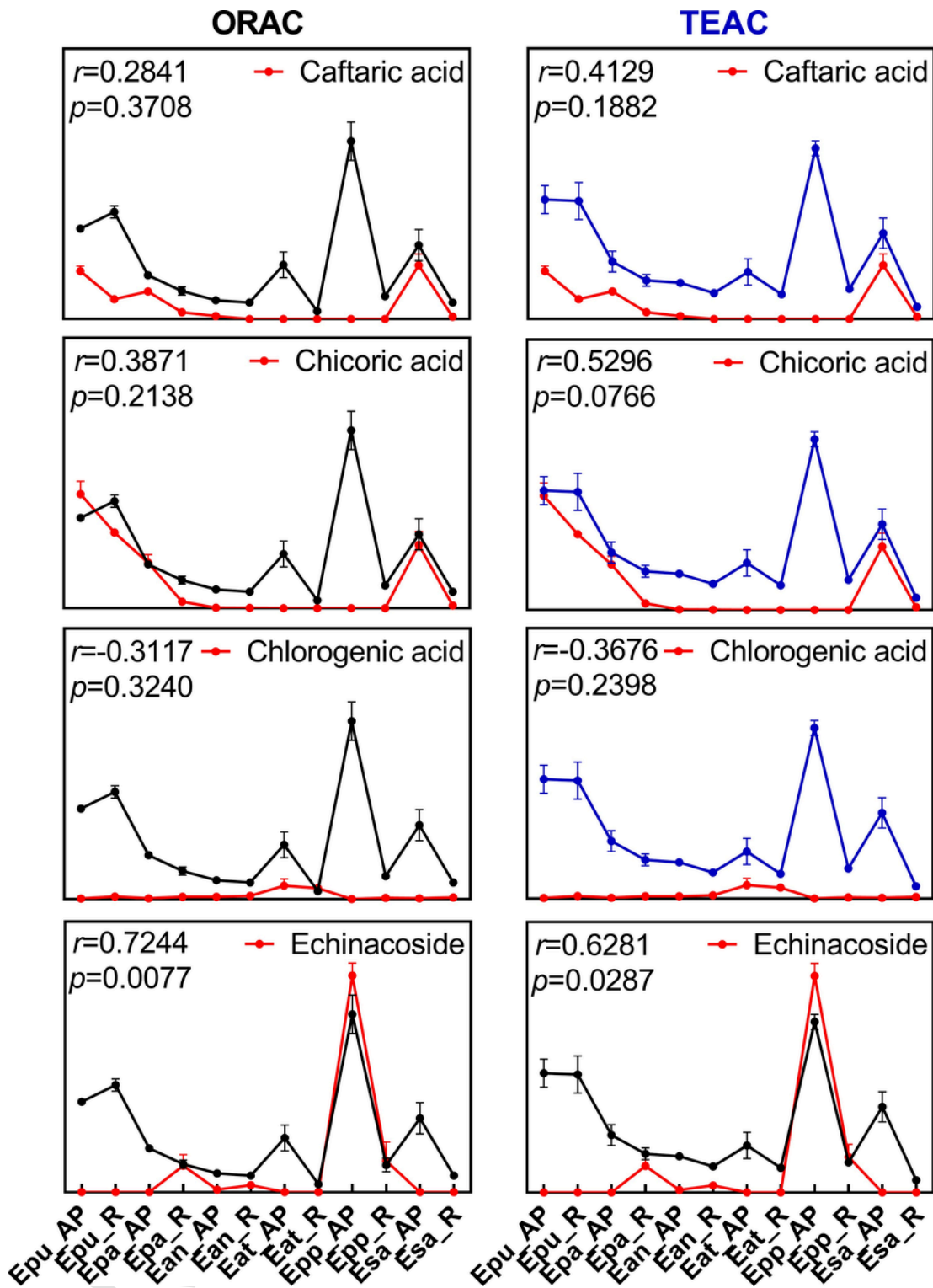
Fig. 5. Co-relationships between of main caffeic acid derivatives and antioxidant activities. The linear relationships between single component content and the ORAC or TEAC values were determined as Pearson correlation coefficients (r) and two-tailed *p* values. Data are mean ± SD (n = 3).

showed distinct expression patterns: *HTT* and *CAS* expression levels in whole Eat and Epp were quite low (Fig. 7c-d). The low expression levels of *HTT* in Eat and Epp were in line with the clone results that no *HTTs* were amplified from the mRNA.

When the contents of metabolites and the expression levels of biosynthetic genes were combined, through *Z*-score stan-

dardization and Pearson correlation clustering, the chemical contents were positively correlated with the biosynthetic gene' expression levels (Fig. 8). In particular, *HTT* expression levels were highly positively correlated with caftaric acid content; *CAS* expression and chicoric acid content were grouped into the same cluster. In addition, *HQT* expression and chlorogenic
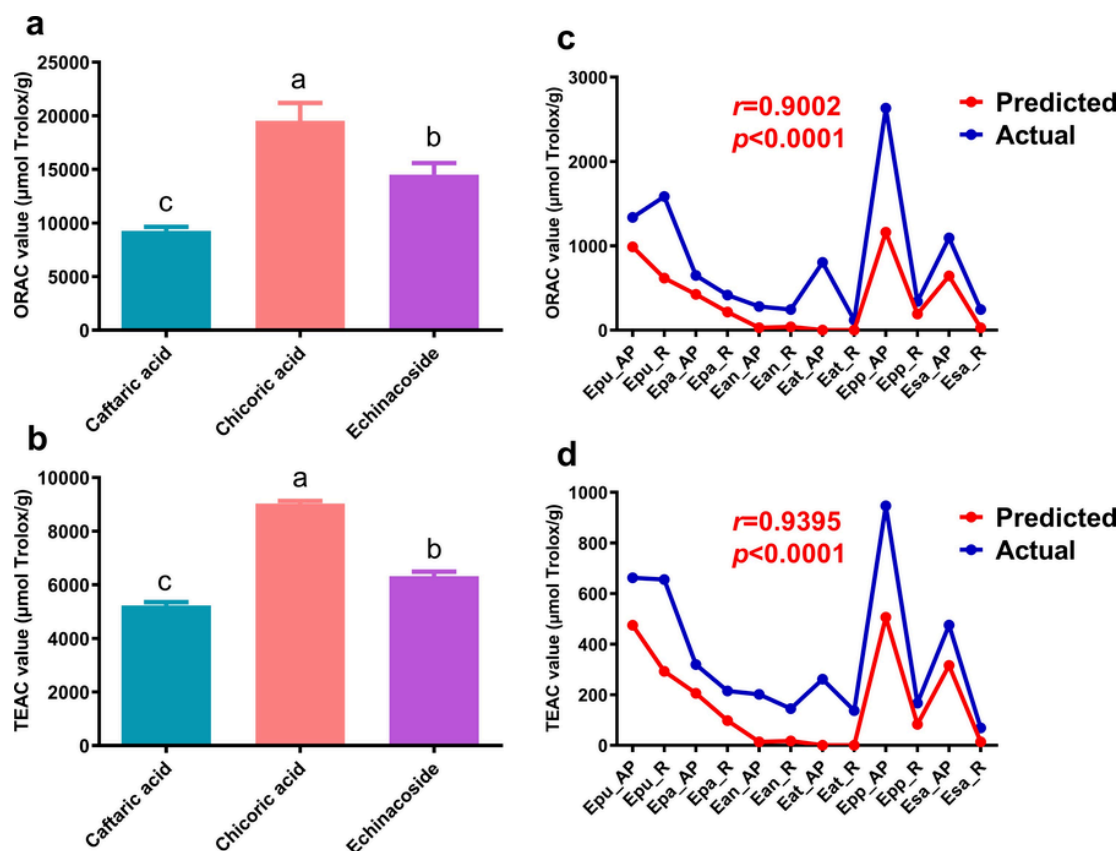
Fig. 6. The major antioxidant bioactivates of *Echinacea* species. Antioxidant activities of three main caffeic acid derivatives determined as (a) ORAC and (b) TEAC values. Data are mean ± SD (n = 3). Statistical significance was calculated by One-way ANOVA. Different lowercases indicate significant different at the *p* < 0.05 levels. The predicted and actual (c) ORAC and (d) TEAC values. The predicted values were calculated by multiplication of content and ORAC value of each single compound and then addition of that of the main three compounds.

acid content were gathered into a branch; *HCT* was observed to show a similar tendency as that of an upstream biosynthetic gene. In contrast, echinacoside showed a distinct pattern compared with that of the other components (Fig. 8).

It seems that the accumulation of chicoric acid and caftaric acid is determined by the expression levels of the biosynthetic pathway genes. However, when re-checking the metabolomic analysis results, the area of tartaric acid (the necessary precursor for caftaric acid and chicoric acid biosynthesis) was also in line with the contents of caftaric acid and chicoric acid. In Eat and Epp, there was almost no tartaric acid (Fig. 9). These results demonstrated that the diversity of chicoric acid and caftaric acid among *Echinacea* species was determined not only by the expression levels of biosynthetic genes, but also by the precursor content.

In summary, based on the RT-qPCR analysis of the recently identified biosynthetic genes and chemical contents among *Echinacea* species, the intrinsic determinants for the diversity of chicoric acid and caftaric acid were found. The diversity was determined by both gene expression and precursor content. However, due to the lack of an identified biosynthetic pathway, it is impossible to clarify the diversity among *Echinacea* species for the other active ingredient compound, echinacoside.

4. Conclusion

Echinacea species have received increasing attention owing to the health benefits and great potential for industrial applica-

tions. Diversity in chemical composition exists within the genus. In the present study, *Echinacea* species were cultivated under the same conditions to exclude environmental effects on the chemical contents. The chemical diversity was comprehensively studied and analyzed. *Echinacea sanguinea* has considerable potential for the production of chicoric acid, in addition to the widely used Epu and Epa, while Epp is a good echinacoside resource. The main chemical differences among *Echinacea* species were caftaric acid, chicoric acid, and echinacoside, which are also suggested to be the antioxidant ingredients of *Echinacea* species. The diversity of chicoric acid among *Echinacea* species was determined by the content of the precursor and the expression levels of biosynthetic genes. The variation in echinacoside needs further explanation because of the lack of an identified biosynthesis pathway. In summary, these results provide information about the chemical differences, bioactive ingredients, and intrinsic determinants of chicoric acid in *Echinacea* species, and provide guidance for the selection of *Echinacea* species for different applications and are fundamental to further research on *Echinacea*.

CRediT authorship contribution statement

Rao Fu: Conceptualization, Methodology, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Pingyu Zhang:** Methodology, Investigation, Resources. **Zongbi Deng:** Methodology, Investigation, Resources. **Ge Jin:** Methodology, Investigation, Resources. **Yiran Guo:** Conceptualization, Writing - review &
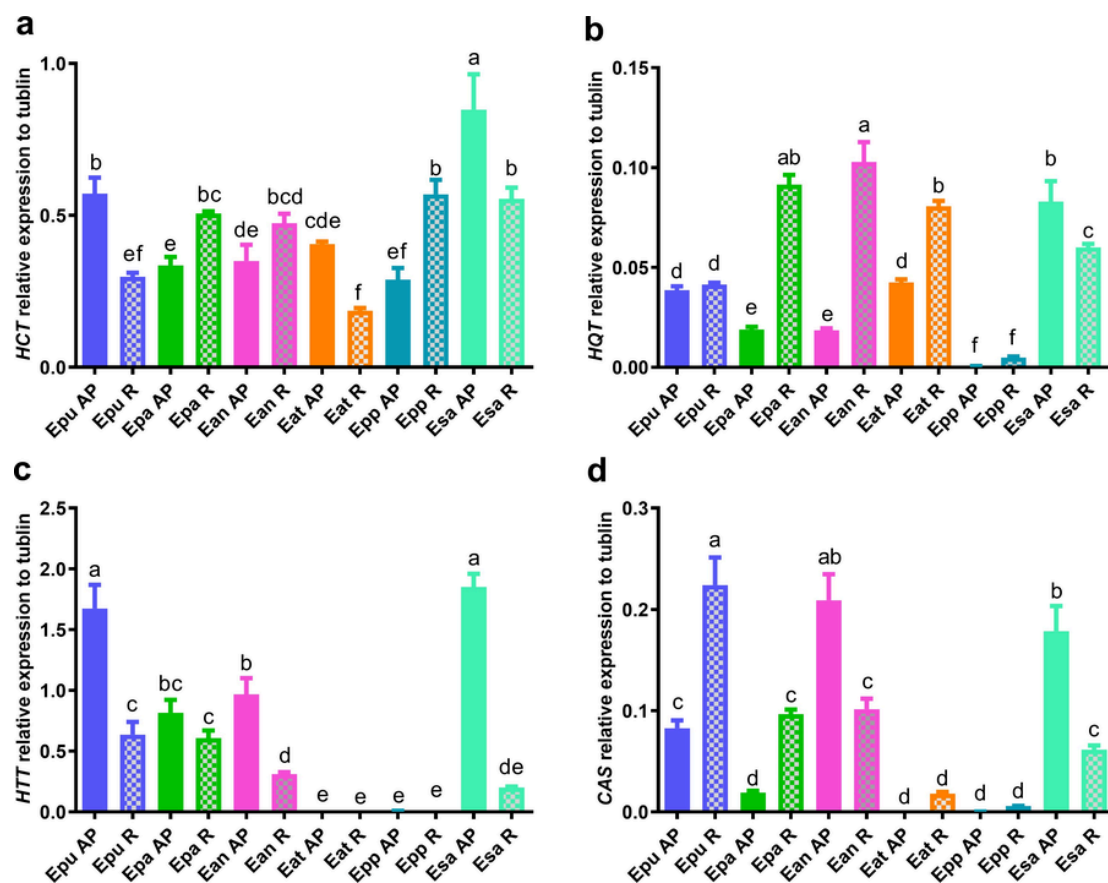
Fig. 7. Chicoric acid biosynthetic genes expression levels among different *Echinacea* species. (a) *HCT*. (b) *HTT*. (c) *HQT*. (d) *CAS*. Data are mean ± SD (n = 3). Statistical significance was calculated by One-way ANOVA. Different lowercases indicate significant different at the *p* < 0.05 levels.
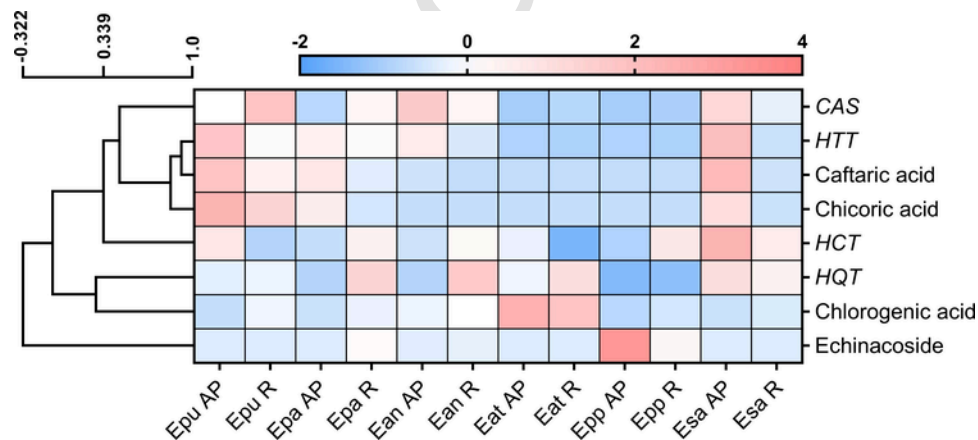

Fig. 8. Hierarchical clustering of biosynthetic gene expression levels and chemical contents. The data per row is *Z*-score standardized to -2 to 4. The distance metric for hierarchical clustering is Pearson correlation.

editing, Project administration, Funding acquisition. **Yang Zhang:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

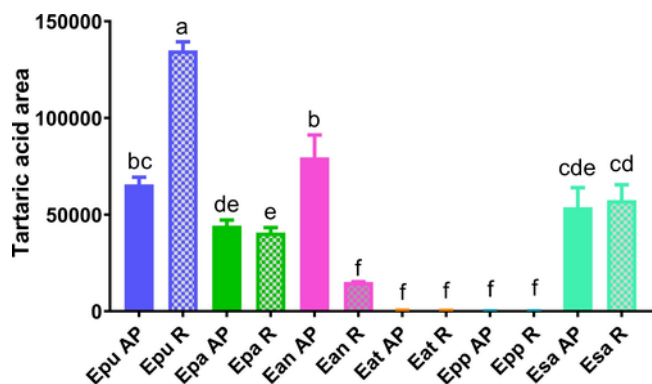The authors report no declarations of interest.

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Fig. 9. Tartaric acid area among *Echinacea* species. The area was retrieved from LC-HRMS analysis. Data are mean ± SD (n = 3). Statistical significance was calculated by One-way ANOVA. Different lowercases indicate significant different at the $p < 0.05$ levels.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113699.

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