Amelioration of saline-alkali land by cultivating Melia azedarach and characterization of underlying mechanisms via metabolome analysis

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March 17, 2023

Abstract

Soil salinization is a serious problem leading to ecological degradation. *Melia azedarach* is highly salt-tolerant, and its application to saline-alkali land is a promising strategy for restoring degraded lands. In this study, we analyzed the soil properties and metabolome of *M. azedarach* roots grown in low- (< 3 g/kg; L), medium- (5˜8 g/kg; M), and high- (> 10 g/kg; H) salinity soils to explore the amelioration effect and adaptation mechanism of *M. azedarach* to soils with differential salinity. Cultivation of *M. azedarach* was associated with a decrease in the concentration of Na^+ and increases in organic matter content and alkaline phosphatase and urease activities in the rhizosphere soil. The metabolome analysis revealed that a total of 382 (ESI+) and 277 (ESI-) differential metabolites (DEMs) were detected. The number of DEMs in roots rose with increased soil salinity, such as sugars and flavonoids in H vs. L, and amino acids in M vs. L. The most up-regulated DEMs were 13-S-hydroxyoctadecadienoic acid, 2'-Deoxyuridine and 20-hydroxyleukotriene B4. Combined analysis of soil properties and *M. azedarach* DEMs indicated that alkaline phosphatase activity was positively correlated with traumatic acid concentration. Taken together, these results indicate that *M. azedarach* has the potential to reduce soil salinity and enhance soil enzyme activity, and it can adapt to salt stress by regulating metabolites like sugars, amino acids, and flavonoids . This study provided a basis for understanding the mechanism underlying the adaptation of *M. azedarach* to saline-alkali soil and its amelioration.

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ABSTRACT

Soil salinization is a serious problem leading to ecological degradation. *Melia azedarach* is highly salt-tolerant, and its application to saline-alkali land is a promising strategy for restoring degraded lands. In this study, we analyzed the soil properties and metabolome of *M. azedarach* roots grown in low- (< 3 g/kg; L), medium- (5~8 g/kg; M), and high- (> 10 g/kg; H) salinity soils to explore the amelioration effect and adaptation mechanism of *M. azedarach* to soils with differential salinity. Cultivation of *M. azedarach* was associated with a decrease in the concentration of $Na^+$and increases in organic matter content and alkaline phosphatase and urease activities in the rhizosphere soil. The metabolome analysis revealed that a total of 382 (ESI+) and 277 (ESI-) differential metabolites (DEMs) were detected. The number of DEMs in roots rose with increased soil salinity, such as sugars and flavonoids in H vs. L, and amino acids in M vs. L. The most up-regulated DEMs were 13-S-hydroxyoctadecadienoic acid, 2'-Deoxyuridine and 20-hydroxyleukotriene B4. Combined analysis of soil properties and*M. azedarach* DEMs indicated that alkaline phosphatase activity was positively correlated with traumatic acid concentration. Taken together, these results indicate that *M. azedarach* has the potential to reduce soil salinity and enhance soil enzyme activity, and it can adapt to salt stress by regulating metabolites like sugars, amino acids, and flavonoids. This study provided a basis for understanding the mechanism underlying the adaptation of *M. azedarach* to saline-alkali soil and its amelioration.

Key words: Adaptation; In-situ restoration; *Melia azedarach* ; Root metabolome; Salt stress; Soil salinization

1. Introduction

Soil salinization, as one of the major factors compromising crop growth and leading to soil degeneration in natural ecological environment, which have become a global concern (Haj-Amor et al., 2022; Singh, 2016). Currently, 20% of cultivated land and 50% of irrigated land globally are under the threat of salinization (Hopmans et al., 2021; Rozentsvet et al., 2017). With climate change, population increase, intensive food production and irrigation practices, soil salinization is expected to be exacerbated (Singh, 2021). The area of saline-alkali land in China is approximately 36 million ha, distributed mainly in the north-eastern and north-western parts and along the coast (Jiang et al., 2022; Wang et al., 2020).

Excessive salt in saline-alkali soils have always been regarded to be the greatest hazard to crop production (Zörb et al., 2019). Salt stress have profound influences on the regulation of plant morphology, physiology and metabolic process (Yang & Guo, 2018), which can lead to plant dehydration, ion toxicity, and even plant death (McDowell et al., 2022).

Plants possess a wide range of metabolic strategies to promote osmotic adaptation and antioxidant mechanisms under salt stress (de Freitas et al., 2019). Roots are the main organ for sensing salt stress signals and responding to excess salt (Redwan et al., 2017). The analysis of root metabolite differences under different salinity conditions is important in elucidating the salt tolerance mechanism of plants (Barding et al., 2013; Ben Hsouna et al., 2022).

M. azedarach has high adaptability and strong salt tolerance; hence, growing *M. azedarach* on saline-alkali land has been considered as a promising strategy for degraded land restoration (Dias et al., 2014; Husain & Anis, 2009). *M. azedarach* can effectively increase bacterial communities diversity and enhance environmental carrying capacity (Li et al., 2021b; Shahbaz & Ashraf, 2013).

Understanding of how *M. azedarach* metabolome responds to different soil salinities is poor. In this paper, soil properties and*M. azedarach* root metabolome were characterized under high, medium and low salinity to explore the ameliorative effects of *M. azedarach* on soils with different salt content and the metabolome changes of *M. azedarach* in response to different severities of salt stress.

2. Materials and methods

2.1. Study area

The field experiment was conducted in Xuwei New District (34°37'N, 119deg29'E), Lianyungang City, Jiangsu Province, where the annual average rainfall and evaporation were 901 mm and 855 mm, respectively. The experimental site was divided into three parts: low salinity (< 3 g/kg), medium salinity (5˜8 g/kg), and high salinity (> 10 g/kg). Before*M. azedarach* was cultivated, the experimental site was plowed to a depth of 40 cm, and the row spacing was 2.0 x 3.0 m.

2.2. Sample collection

In August 2020 (after 40 months of *M. azedarach* growth), the samples of soil and roots of*M. azedarach* were collected from each plot [high (H), medium (M) and low (L) salinity]. Samples of rhizosphere soil (soil attached to *M. azedarach* roots) and bulk soil (soil away from *M. azedarach* roots) were collected in each plot. Three replicates were collected in each plot, with each replicate consisting of six sampling points. All soil samples were air-dried and then sieved through a 0.147 mm sieve for soil chemical and enzymatic activities analysis.

Six replicates of roots were sampled in each plot, and each replicate comprised three sampling points. The samples were stored at -80 for analysis of root metabolome. Root samples (R) collected in different salinities were labelled: RH, RM and RL for high, medium and low salinity, respectively.

2.3. Measurement of soil properties

Soil salinity was determined by the conductivity method (Yue et al., 2020). Soil pH was assessed by a calibrated pH meter (FE28-Standard, Mettler Toledo, Greifensee, Switzerland) (Zhang & Pang, 1999). Soil water-soluble Na^+, K^+, Ca^{2+}, and Mg^{2+}were measured by ICP-OES (Optima 2100DV, Perkin-Elmer, Waltham, Massachusetts, USA) (Yang et al., 2016). Soil water-soluble Cl^- content was determined by the silver nitrate titration method (Asakai, 2018).

Soil organic matter was determined using the potassium dichromate method (Osman et al., 2013), available phosphorus by the molybdenum-antimony colorimetric method (Wang et al., 2011) and available potassium by flame photometry (Bilias & Barbayiannis, 2019).

Soil enzyme activities were measured using aprpriate kits (Solarbio, Beijing, China). Activity of soil alkaline phosphatase (S-AP) was measured by the determination of phenol produced during the hydrolysis of the substrate (Li et al., 2021b). Activity of soil urease activity (S-UE) was obtained by measuring NH_3-N produced by the urease hydrolysis of urea based on the indophenol blue colorimetric method (Huang et al., 2014).

2.4. Characterizations of M. azedarach metabolome

2.4.1. Metabolite extraction

An amount of 50 mg of fresh *M. azedarach* roots was weighed accurately ground in 1000 μL of extractant (methanol-acetonitrile-water, 2:2:1, v/v) containing internal standard (l,2-chlorophenylalanine, 2 mg) at 45 Hz for 10 min in a Tissuelyser-32; the suspension was allowed to stand at -20 for 1 hour, followed by centrifugation at 16,114 *g* for 15 min (Sun et al., 2022). The supernatant was collected and dried in a vacuum concentrator. The dried metabolites were reconstructed in the extractant (acetonitrile-water, 1:1, v/v), sonicated in an ice-water bath for 10 min, and centrifuged at 16,114 *g* for 15 minutes; the supernatant was used for metabolomic analysis (Li et al., 2022).

2.4.2. UPLC-Q-TOF-MS for metabolite analysis

Extracts were analyzed by UPLC (Waters UPLC Acquity I-Class PLUS) and QTOF system (Waters UPLC Xevo G2-XS QTof) with a chromatographic column Acquity UPLC HSS T3, mobile phase A (0.1% formic acid aqueous solution), and mobile phase B (0.1% formic acid acetonitrile) (Fan et al., 2022). Metabolites need to be ionized before they can be detected by mass spectrometry. The ESI source operation parameters were as follows: capillary voltage at 2000 V (positive ion mode) or -1500 V (negative ion mode), a cone voltage at 30 V, a source temperature of 150 °C, desolvation to 500 degC and a desolvation gas flow to 800 $L*h^{-1}$ (Liu et al., 2022).

2.4.3. Metabolome data processing

The raw data were collected by MassLynx (v4.2) and processed by Progenesis QI software (Huang et al., 2022), before identified based on Progenesis QI software, the online METLIN database, and Bemec's proprietary library. The criteria for differential metabolites (DEM) were FC[?]2.0 or FC[?]0.5,p -value<0.05, and VIP>1.0.

2.5. Statistical analyses

An independent sample t -test was done by IBM SPSS Statistics 20 (IBM, Armonk, NY, USA), with $p < 0.05$ considered statistically significant. Graphs were drawn by Graphpad prism v8.0.1 and R package ggplot 2 (v3.2.0). The Venn diagrams were constructed by the R package Venn diagram (v1.6.20). The network diagram was produced by Cytoscape (v3.6.1).

3. Results

3.1. Soil chemical properties and enzyme activities

The soil salinity (Figure 1a) and soluble ions (Na^+, Mg^{2+}, K^+, Ca^{2+}, and Cl^-) (Figure 1c-g) in rhizosphere soil were significantly lower than those in bulk soil under medium- and high- salinity. The salinity of rhizosphere soil decreased by 57% and 62% compared with that of bulk soil under medium and high salinity conditions, respectively.

Regardless of soil salinity, the rhizosphere soil had higher organic matter content than the bulk soil (Figure 1h). The same changes were found in the activities of S-AP and S-UE (Figure 1k, 1l). Under medium salinity, the contents of available phosphorus and available potassium in the rhizosphere soil were 2.13- and 1.09-fold greater than in the bulk soil, respectively (Figure 1j-k).

3.2. Metabolomic analysis of M. azedarach under salt stress

3.2.1. Venn diagram and volcanic plot analysis of DEMs

There were 382 (ESI+) (Figure 2a) and 277 (ESI-) DEMs (Figure 2b). Venn diagram analysis of DEMs showed there were more common DEMs between RH vs. RL and RM vs. RL than in other combinations. *M. azedarach*had most of unique DEMs in RH vs. RL, including 72 DEMs in ESI+ and 90 DEMs in ESI-.

The analysis of the volcano plots showed there were more up-regulated than down-regulated DEMs under salt stress. The DEMs significantly up-regulated in RM vs. RL were homoveratric acid, 2'-deoxyuridine and 7,8-dihydroneopterin (Figure 3a-b). The significantly up-regulated DEMs in RH vs. RL were 13-S-hydroxyoctadecadienoic acid, 20-hydroxyleukotriene B4 and 7,8-dihydroneopterin (Figure 3c-d). The significantly up-regulated DEMs in RH vs. RM were anhwiedelphinine, hyperoside and histidinyl-lysine (Figure 3e-f).

3.2.2. Sugars, amino acids, and flavonoids

Nine sugars (D-mannose, maltotetraose, maltohexaose, stachyose, 1-kestose, raffinose, 3-alpha-mannobiose, GDP-glucose, and D-(-)-lyxose) were identified in DEMs, most sugars were up-regulated in RM vs. RL and RH vs. RL (Table 1).

Six amino acids were detected in DEMs, including L-theanine, L-proline, L-lysine, L-isoleucine, DL-leucine and aspartic acid. Five out of six amino acids in DEMs (except L-Theanine) were up-regulated in RM vs. RL (Table 2).

Flavonoids have antioxidant activity. Twenty-seven types of flavonoids were detected in DEMs. Among them, 19 were up-regulated in RM vs. RL and 23 were up-regulated in RH vs. RL (Table 3).

3.2.3. KEGG pathway analysis

The number of DEMs enriched in the biosynthesis pathway of flavonoids and amino acids ranked the top five among KEGG pathways (Figure 4). The expressions of L-proline, L-lysine, L-isoleucine, S-adenosylmethionine, and L-saccharopine in the biosynthesis of amino acids were higher in RM than in RL (Figure 5a), while citrate and L-saccharopine were higher in RH than in RM (Figure 5c). With soil salinity increased (RH vs. RL), three of the four DEMs in the biosynthesis of amino acids (L-proline, aspartic acid, and citrate) were up-regulated (Figure 5b). Among the DEMs involved in flavonoid biosynthesis, the expressions of (-)-naringenin, vitexin, (+)-gallocatechin, pinocembrin, (-)-epicatechin and epigallocatechin were higher in RM vs. RL (Figure 5a) and RH vs. RL (Figure 5b).

3.3. Combined analysis of soil properties and M. azedarach DEMs

Six soil chemical properties (salinity, Na^+, Cl^-, K^+, available phosphorus and available potassium) were correlated negatively with four DEMs of*M. azedarach* : prunasin, androstendione,*o* -succinyl-L-homoserine and traumatic acid (Figure 6). By contrast, alkaline phosphatase activity was positively correlated with these four DEMs. Soil pH was negatively correlated with prunasin, and Ca^{2+} was positively correlated with prunasin and corticosterone.

4. Discussion

Coastal saline soil contains excess salinity, with Na^+ and Cl^- being the main base ions (Zhang et al., 2022). Soil organic matter (SOM) is dominant to global carbon cycle (Bhattacharyya, 2022), S-AP activity is an indicator of soil phosphorus cycle (Liberti et al., 2013), and S-UE plays a crucial role in influencing the content of soil nitrogen (Albiach et al., 2000). In this paper, salinity, contents of Na^+ and Cl^- in the rhizosphere soils were significantly lower than bulk soils, whereas SOM and activities of S-AP and S-UE showed the opposite trend (Figure 1). These results indicated that *M. azedarach* played a critical role in reducing soil salinity and promoting soil nutrient cycling.

Excess salinity in saline-alkali land can cause osmotic stress, ion toxicity, oxidative damage, nutrient deficiency and even cell death in plants (Elkelish et al., 2019; Ghazali, 2020). Plants can cope with salt stress by regulating biosynthesis of metabolites such as sugars, amino acids and flavonoids (Batista-Silva et al., 2019).

Sugars can be bound to inorganic solutes (e.g. K^+) to maintain cell expansion and are active in osmotic regulation under salt stress (Arbelet-Bonnin et al., 2018). Similar results were reported in other studies, with sugars acting not only as osmoprotectants and cell membrane stabilizer, but also as scavengers of reactive oxygen species (Nishizawa et al., 2008). The contents of various sugars were up-regulated under medium and high salinity (Table 1), indicated that *M. azedarach* could produce more sugars to regulate osmotic pressure when exposed to a gradient of salt stress.

Besieds, amino acids are important osmoprotectants and antioxidants, and their accumulation can enhance the stability of cell membrane and improve salt tolerance of plants (Slama et al., 2015; Widodo et al., 2009). Hosseinifard et al. (2022) discovered that proline could inhibit the accumulation of toxic ions under salt stress. With the increase in the severity of salt stress, the amino acid DEMs were up-regulated, and more amino acids were synthesized under medium compared with low salinity (Figure 5a-b, Table 2). The result showed that *M. azedarach* increased its salt tolerance by up-regulating amino acid as the severity of salinity progressed from low to medium.

Flavonoids have antioxidant activity, protecting cell structures from oxidative damage and enhance the stress resistance of plants (Saeed et al., 2012; Sumner et al., 2003). More than 70% of the flavonoids were up-regulated in *M. azedarach* roots with soil salinity increased (Table 3). And DEMs involved in flavonoid biosynthesis were largely up-regulated with salinity increased (Figure 5). These results indicated that*M. azedarach* could enhance the antioxidant activity and salt tolerance of plants by up-regulating the expression of flavonoids.

Among *M. azedarach*metabolites, traumatic acid was shown to participate in the plant adaptation and tolerance to salinity by diminishing the negative effects of sodium and chloride ions and increasing the monosaccharide content (Pietryczuk et al., 2014). Alkaline phosphatase can release inorganic phosphorus for plants growing on phosphorus-deficient saline soils (Li et al., 2021a). In the present study, alkaline phosphatase activity was positively correlated with traumatic acid concentration (Figure 6), indicating that there should be a synergistic effect between soil alkaline phosphatase activity and traumatic acid in *M. azedarach* , facilitating*M. azedarach* tolerance to salt stress.

5. Conclusion

In conclusion, the cultivation of*M. azedarach* could reduce soil salinity and promote soil nutrient cycling. *M. azedarach*alleviated salt stress by up-regulating the expression of metabolites such as sugars, amino acids and flavonoids. There was a synergistic effect between alkaline phosphatase and traumatic acid in improving the salt tolerance of *M. azedarach* . This research provided a solid basis for clarifying the adaptation and amelioration mechanism of*M. azedarach* to salt stress on saline-alkali land.

Aacknowledgments

This work was supported by funding from the following projects: National Key Project of Scientific and Technical Supporting Programs funded by the Ministry of Science & Technology of Jiangsu Province (BE2022304), Forestry Science and Technology Innovation and Extension Project in Jiangsu Province (LYKJ[2019]07), and the Programs funded by the Ministry of Science & Technology of Inner Mongolia (2020CG0057).

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Figure and Table captions

FIGURE 1 Chemical properties and enzyme activities in the rhizosphere and bulk soils with different salinities. (a) Salinity, (b) pH, (c) Na^+, (d) Mg^{2+}, (e) K^+, (f) Ca^{2+}, (g) Cl^-, (h) organic matter, (i) available phosphorus, (j) available potassium, (k) alkaline phosphatase, and (l) urease. Note: Asterisks denote significant differences (p [?]0.05). Means +- SE (n = 3). L, low-salinity soil; M, medium-salinity soil; H, high-salinity soil.

FIGURE 2 Venn diagrams of DEMs in *Melia azedarach* roots: (a) ESI+ mode, (b) ESI- mode. Note: RL- roots in low salinity soil, RM- roots in medium salinity soil, RH- roots in high salinity soil.

FIGURE 3 Volcano plots of DEMs in *Melia azedarach* roots. Note: RL- roots in low salinity soil, RM- roots in medium salinity soil, RH- roots in high salinity soil. Red represents up-regulated and blue represents down-regulated DEMs.

FIGURE 4 Enrichment diagram of *Melia azedarach* root DEMs in KEGG pathways. Note: RL- roots in low salinity soil, RM- roots in medium salinity soil, RH- roots in high salinity soil.

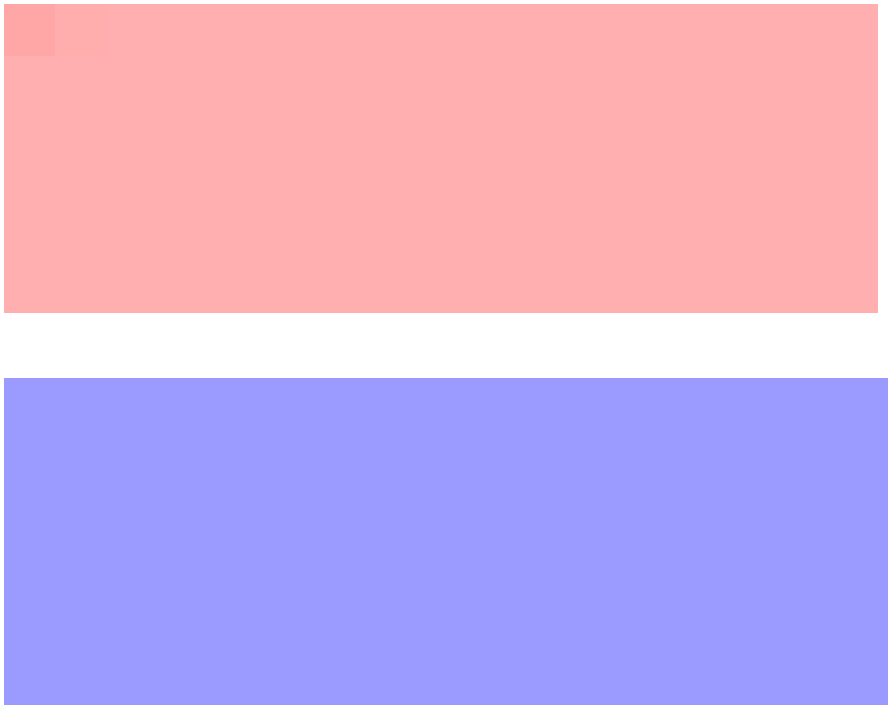

FIGURE 5 DEMs enrichment network diagram of biosynthesis of amino acids and flavonoid biosynthesis. (a) RM vs. RL, (b) RH vs. RL, (c) RH vs. RM. Note: RL- roots in low salinity soil, RM- roots in medium salinity soil, RH- roots in high salinity soil. - upregulated DEMs, - downregulated DEMs. The small nodes connected to the large node (KEGG pathway) are DEMs annotated to the pathway. The thickness of the line indicates the fold change.

FIGURE 6 Combined analysis of soil properties and DEMs in *Melia azedarach* roots. Green circles represent soil properties, and orange circles represent DEMs. The color of connecting lines indicates the correlation between nodes (red- positive correlation, blue- negative correlation).

TABLE 1 Regulation of sugars in DEMs

TABLE 2 Regulation of amino acids in DEMs

TABLE 3 Regulation of flavonoids in DEMs

TABLE 1 Regulation of sugars in DEMs

Flavonoids	RM vs. RL log_2FC	RM vs. RL *p*-value	RM vs. RL regulated	RH vs. RL log_2FC	RH vs. RL *p*-value	RH vs. RL regulated	RH vs. RM log_2FC	RH *p*-v
D-Mannose	2.49	0.005	up	4.00	0.000	up	1.50	0.0
Maltotetraose	1.79	0.000	up	2.88	0.000	up	1.09	0.0
Maltohexaose	0.96	0.000	unchanged	1.32	0.000	up	0.35	0.0
Stachyose	2.34	0.000	up	2.15	0.000	up	-0.19	0.3
1-Kestose	1.44	0.001	up	2.97	0.000	up	1.52	0.0
Raffinose	1.55	0.005	up	1.08	0.039	up	-0.47	0.1
3-alpha-Mannobiose	1.27	0.000	up	1.27	0.001	up	0.01	0.9
GDP-glucose	0.85	0.018	unchanged	1.21	0.002	up	0.36	0.0
D-(-)-Lyxose	2.35	0.025	up	4.08	0.030	up	1.73	0.0

TABLE 2 Regulation of amino acids in DEMs

Flavonoids	RM vs. RL log_2FC	RM vs. RL *p*-value	RM vs. RL regulated	RH vs. RL log_2FC	RH vs. RL *p*-value	RH vs. RL regulated	RH vs. RM log_2FC	RH vs. R *p*-value
L-Theanine	0.92	2.3E-05	unchanged	-1.24	4E-04	down	-2.15	2E-07
L-Proline	3.31	1E-06	up	2.93	1E-07	up	-0.38	0.0004
L-Lysine	1.16	8.1E-05	up	1.00	4E-05	unchanged	-0.16	0.1736
L-Isoleucine	1.72	2.1E-05	up	0.96	1E-04	unchanged	-0.76	6E-05
DL-Leucine	1.95	3.8E-05	up	0.62	0.019	unchanged	-1.33	4E-05
Aspartic acid	1.93	8.5E-05	up	-0.03	0.958	unchanged	-1.96	8E-05

TABLE 3 Regulation of flavonoids in DEMs

Flavonoids	RM vs. RL log_2FC	RM vs. RL *p*-value	RM vs. RL regulated	RH vs. RL log_2FC	RH vs. RL *p*-value	RH vs. RL regulated	RH log_2
5,7,3',4',5'-Pentahydroxyflavanone	1.37	0.002	up	1.43	6E-09	up	0.07
Prodelphinidin A1	1.64	2E-04	up	1.75	2E-08	up	0.11
(+)-Gallocatechin	2.61	0.001	up	2.39	2E-06	up	-0.2
5,7-Dimethoxy-6-C-methylflavone	0.94	4E-05	unchanged	1.04	5E-04	up	0.10
Phellodensin F	1.70	2E-05	up	1.61	3E-06	up	-0.0
Licoagrodin	1.04	8E-06	up	0.85	5E-05	unchanged	-0.1
(-)-Catechin gallate	1.58	0.002	up	1.54	1E-06	up	-0.0
Taxifolin 3-arabinoside	1.67	3E-04	up	1.37	7E-05	up	-0.3
Nympholide A	1.22	2E-05	up	1.62	1E-06	up	0.40
Munsericin	0.97	2E-05	unchanged	1.21	2E-04	up	0.24
(-)-Epicatechin	1.82	2E-04	up	2.15	9E-04	up	0.33
Vitexin	3.03	0.012	up	3.23	1E-03	up	0.20
3-Hydroxyflavone	1.75	0.003	up	2.03	4E-04	up	0.28
Pinocembrin	1.18	0.001	up	1.45	8E-05	up	0.27
Rutin	0.77	0.001	unchanged	1.03	2E-07	up	0.26
6-O-Malonyldaidzin	1.33	2E-04	up	1.32	4E-06	up	-0.0
(-)-Naringenin	1.14	0.003	up	1.05	5E-06	up	-0.0
Catechin	-4.01	0.049	down	-5.06	0.044	down	-1.0
Epigallocatechin	3.03	0.003	up	2.63	0.008	up	-0.4

Theaflavin	1.82	3E-04	up	1.60	0.011	up	-0.2
Taxifolin	3.10	0.001	up	2.79	0.008	up	-0.3
Biochanin A	0.66	0.077	unchanged	1.03	0.017	up	0.37
Xanthohumol	-1.64	0.060	unchanged	-2.09	0.041	down	-0.4
Baicalin	0.19	0.677	unchanged	2.29	0.004	up	2.1(
Apigenin 7-glucoside	4.39	0.009	up	4.74	0.008	up	0.35
Hyperoside	-0.59	0.224	unchanged	0.92	0.011	unchanged	1.5(
Deguelin	1.80	0.024	up	2.06	0.039	up	0.2(