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Comparative Study of Three Raspberry Cultivar (*Rubus idaeus* L.) Leaves Metabolites: Metabolome Profiling and Antioxidant Activities

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Abstract: Raspberry (*Rubus idaeus* L.), known as one of the famous healthy fruits an d are consumed fresh or processed products all over the world. The antioxidation activity of raspberry fruits as well as leaves have been widely investigated. To better understand the metabolite accumulation mechanisms and to develop different functional cultivars, we performed a non-targeted metabolomics analysis using LC-MS/MS to investigate the contents of existing components from three raspberry cultivars, Autumn Britten, Autumn Bliss, and Red Autumn leaves, respectively. The results show multiple differentially accumulated metabolites among three cultivars, especially for the lipids (α-linolenic acid and eicosatetraenoic acid), amino acids and their derivatives (L-cysteine, Phenylalanine), flavonoids (Kaempferol 3-O-rhamnoside-7-O-glucoside, Quercetin 3-glucoside), and vitamins (Biotin, Thiamine, Vitamin K2), etc. The in vitro cellular antioxidant activities of three raspberry cultivars leaves ethanol extracts (RLEE) were also characterized. Through comparison the superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and reactive oxygen species (ROS) levels before or after RLEE protection of L929 fibroblast cells upon excessive UVB exposure, we evaluated the antioxidation potentials for all three cultivar RLEEs. It turns out the raspberry Autumn Britten leaf extract holds the greatest potential for protecting the L929 fibroblast cells from UVB induced damage. Our study provides theoretical support for screening of active metabolites from three raspberry cultivars leaves, spanning metabolites' accumulation to cell damage protection, which could be used to refine bioactivity assessment for different raspberry cultivars suitable for antioxidant products extraction.

Keywords: raspberry; metabolomics; LC-MS/MS; L929 fibroblasts; UVB damage; antioxidant

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1. Introduction

The raspberry (*Rubus idaeus* L.) is a Rosaceae plant that belongs to the *Rubus* genus. Raspberry farming has a long history, and after 100 years of continual breed selection and optimization, it has become widely planted over the world [1]. Raspberry is a popular fruit among customers because of its sweet taste and high nutritional content. Raspberry leaves have attracted increased interest for their diverse applications as well as their global market expansion [2]. Raspberry leaves have been found to have a wide range of active compounds, including terpenoids, flavonoids, phenols, and other anti-aging and antioxidant components, according to several studies [3,4]. The use of raspberry leaves, on the other hand, is significantly less than that of raspberry fruits. A large number of raspberry leaves are disposed as waste as a by-product of raspberry production, and fertilizer resources are used inefficiently [5,6].

Pradeepa et al. (2014) synthesized and characterized silver nanoparticles using water extract with strong healing force from the wounds of raspberry leaves as reducing agent, their antibacterial activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* showed that the synthesized silver nanoparticles had a clear inhibition zone and could be used as an effective antibacterial agent for wound healing; Another study from Na et al. (2019) showed that kaempferol, quercetin, and vitiligo glycoside isolated from 70% ethanol group extracted from water extract of raspberry leaves had significant delayed effect on plasma calcium rehydration time (PRT) [7,8]. Phenolic compounds are useful components in a variety of beverages and processed foods, and their health-related qualities have made them a research hotspot [9,10]. In vitro antioxidant and anti-inflammatory activities of raspberry leaf extract were discovered by Luo et al. (2020) with the presence of phenolic compounds being particularly relevant [11]. Ellagic acid is a polyphenolic dilactone that is a dimer derivative of gallic acid. Pavlovi et al. (2016) discovered that ellagic acid is also the most abundant phenolic acid in berry plants [12]. Ellagic acid has been shown to be effective against microbial infections and neurological illnesses, owing to its antioxidant capabilities. A vast body of literature also suggests that ellagic acid possesses anticancer qualities [13].

At present, the deep processing of raspberries mainly focuses on fruits. As a by-product of raspberry production, the raw materials of leaves are easy to collect. Moreover, various components with antioxidant effects in the leaf extract also make it a potential natural material for developing product for protecting of skin photo-damage. At the same time, there are also differences in the active ingredients in different cultivars of raspberry leaves. In order to evaluate raspberry leaves more efficiently, it is particularly important to compare and screen the active ingredients in leaves among different cultivars. Excessive UVB irradiation causes the skin to produce too many reactive oxygen species (ROS), which activates the oxidative stress reaction in cells, producing oxidative damage and resulting in dermal fibroblast apoptosis, collagen, and elastin degradation, and skin light damage [14,15]. Collagen, elastic fibers, and extracellular matrix components found in fibroblasts are essential for the skin's normal physiological state. Antioxidant enzymes like SOD (Superoxide Dismutase), CAT (Catalase), and GSH (Glutathione) form a crucial mechanism in the body's battle against oxidative stress. These enzymes will clear excessive ROS produced by fibroblasts, hence their antioxidant activity is tightly connected to the degree of oxidation in the body [16]. The antioxidant capacity of skin cells is dependent on the activity of antioxidant enzymes, according to studies on skin UV damage prevention [17]. There are few studies on the protective effect of extracts from different cultivars of raspberry leaves on photo damage, and the mechanism of action is still unclear.

This work aims to distinguish the original differences in the composition and content of metabolites in leaves of different varieties of raspberry, and for further evaluating the protective effects of leaf extracts of different varieties on photo damage of fibroblasts caused by UVB. In order to achieve this goal, firstly, the metabolites in the leaves of three raspberry cultivars, Autumn Britten, Red Autumn, and Autumn Bliss, were extracted using an ultrasonic extraction method, and LC-MS/MS based untargeted metabolomics profiling was used to compare the accumulated metabolites and metabolic pathways between the three raspberry cultivars. Then, the protective effect of ethanol extract (RLEE) from raspberry leaf on UVB induced skin photo damage were investigated in L929 and the effect on SOD, CAT, GSH antioxidant enzyme activity was studied. This study can be used as a reference for screening higher value-added raspberry varieties, and provide a basis for the application of raspberry leaf extract in antioxidant damage, and improving the value of raspberry by-products.

2. Materials and Methods

2.1. Extraction and Sample Preparation

Raspberries (*Rubus idaeus* L. Autumn Britten, Autumn Bliss and Red Autumn) seedlings were purchased from XiLing Agricultural Development Co., LTD. (Jilin, China) and culti-

vated in the experimental field of Northwest Normal University (Lanzhou, Gansu Province, China, N 34°14′41.68″, E 108°54′38.05″, altitude: 1650 m, an annual average temperature: 6–19 °C). The identification of the raspberry variety resources was done by Professor Xuelin Chen, a plant taxonomist at Northwest Normal University.

The leaves of three different cultivars of raspberry were collected and dried by freezing with liquid nitrogen and ground, then stored at −80°C for further use. For LC-MS/MS sample preparation, 1 g of tissue were homogenized in liquid nitrogen and extracted total metabolites using 400 µL of methanol-acetonitrile-water solution (2:2:1 *v*:*v*:*v*) with ultrasound for 10 × 3 min in a cold bath (120 W, 4 °C, 40 kHz), then standing for 10 min at −20 °C and centrifuged (14,000× *g*, 20 min, 4 °C) to extract the supernatant. The supernatant was freezer dried and stored at −80 °C for LC-MS analysis [3,18].

2.2. Instrumentation and Condition for LC-MS/MS Analysis

Sample was analyzed on Thermo Scientific™ Vanquish™ UHPLC system (Thermo). AC18 column (Thermo Hypersil Gold C18, 100 mm × 2.1 mm, 1.8 µm) coupled to Q Exactive mass spectrometer (Thermo) was used for the separation and identification of metabolites. The mobile phase consisted of 0.1% formic acid in water (A) for positive mode, 5 mM ammonium acetate in water (A) for negative mode and acetonitrile (B) use the following gradient condition: 0 min–1 min, 1% B; 1 min–8 min, 1–99% B; 8 min–10 min, 99% B; 10 min–10.1 min, 99–100% B; 10.1 min–12 min, 1% B. The flow rate was 0.3 mL/min, the column temperature was set as 35 °C and the sample injection volume was 4 µL [19,20]. Mass spectrometric analysis was performed with Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), using electrospray ionization sources in positive and negative ionization modes. The operating parameters were operated as follows: Positive polarity; spray voltage 4.0 kV (positive) or −3.6 kV (negative), funnel RF lens value at 50, capillary temperature of 400 °C, the flow rates for sheath gas, aux gas and sweep gas were set to 45, 15, and 0, respectively. Except otherwise noted, data dependent acquisition (DDA) using the Full MS-ddMS2 setup was used. Full MS resolution was set to 70,000, Mass range was set to 100–1500. For MS2 spectra, Resolution was set to 17,500.

2.3. In Vitro Bioactivity Screening

2.3.1. Culture Condition and Cytotoxicity Assay of RLEE

Mouse fibroblast L929 (ATCC, Washington, DC, USA) cells were maintained in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Corporation, Logan, UT, USA) 100 µg/mL streptomycin and 100 µg/mL penicillin, cultured in flask at 37 °C and 5% CO_2 [21]. Cells were transferred into 96-well culture plates and divided into different groups, with 6 replicates in each group.

The effect of RLEE on the viability of mouse fibroblasts was examined by Cell Counting Kit-8 (CCK-8 kit Shanghai Yeasen Biotechnology Company, Shanghai, China). Freeze-dried RLEE was dissolved in 0.1% DMSO solution and diluted into different concentrations by culture medium. L929 cells were seeded in 96-well plates at a density of 2 × 10^4/well and divided into 10 groups of 6 replicates each: blank group (medium only), control group (medium and L929 cells), spiked group (medium and L929 cell solution and final concentrations of 1, 5, 10, 20, 50, 75, 100, 200 µg/mL RLEE). After 12 h of incubation, 10 µL of CCK-8 solution was added to each well and incubated for 2 h at 37 °C with 5% CO_2 in a cell culture incubator [22]. The absorbance values were measured using a microplate reader (Tecan Company, Männedorf, Switzerland) at 450 nm and cell viability was calculated according to the instruction of the CCK-8 kit.

2.3.2. Bioactivity Assessment of RLEE against UVB Induced Damage

L929 cells were cultured in 96-well plates at a density of 2 × 10^4/well and the medium was changed to PBS for treatment, then divided into 6 groups of 12 replicates each: control group, UVB irradiated group; RLEE treated groups (pre-protected with different varieties

50 μg/mL of RLEE, then UVB irradiated). The cell culture irradiation treatment was achieved by exposure of plates at a distance of 10 cm from the UVB lamp (TL40W, Philips, Amsterdam, The Netherlands) for 120 s (irradiation dose 600 mJ/cm^2), where the control group was covered with tin foil to block the UVB radiation [22,23]. After irradiation, PBS was discarded and DEME complete medium was added for 12 h culturing. The absorbance of each well was measured using the CCK-8 cell viability assay kit for cell viability calculation after culturing.

The grouping and treatment were same as above, after treatment the cells in culture plate were washed with phosphate-buffered saline (PBS). Then, the lysis buffer (Solarbio, Beijing, China) was added to the cell, after scraping off and crushed with an ultrasonic cell crusher, the lysate cells were centrifuged for 3 min (10,000× g rpm) at 4 °C, and the supernatant was collected. The levels of SOD, GSH, and CAT were determined according to the instructions of the respective kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The ROS level was assessed on an inverted fluorescence microscope (Olympus China Company, Beijing, China) after treatment according to the instruction of the ROS kit.

2.4. Metabolome Data Analysis

The matching peak data were retrieved from the original LC-MS data using composite discoverer 3.0 software, and the peak area data were normalized in Microsoft Excel for metabolomics data analysis. The data was then analyzed using the MetaboAnalyst 5.0 online server (http://www.metaboanalyst.ca (accessed on 20 October 2021)) using principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS -DA), and a volcano map. Finally, the variable importance in the projection (VIP) value (VIP > 1) and t-test ($p < 0.05$) were used to determine the difference metabolites [24]. MetaboAnalyst 5.0 and the Kyoto Encyclopedia of Genes and Genomes (KEGG database) were used to assess prospective biomarker metabolic pathways, and a comprehensive metabolic network was built based on the relationships of identified potential biomarkers in the metabolic pathways.

2.5. Statistical Analysis

Statistical significance was determined using single-factor analysis of variance (one-way ANOVA) and Tukey's range test via the SPSS statistical software (version 21; 158 SPSS Corp., Chicago, IL, USA). The results were considered significant when the p-value was lower than 0.05.

3. Results

3.1. Metabolomics Analysis of Three Raspberry Cultivars Leaves

A non-targeted UPLC-MS/MS-based metabolomics technique was used to discover the differentially accumulated metabolites of three raspberry cultivars leaves. In the leaves of the three kinds, according to the VIP value (VIP > 1) and t-test ($p < 0.05$) screening conditions, a total of 399 metabolites with significant differences were found (Supplement Table S1). Figure 1A,B show PCA analysis of detected metabolites in different cultivar leaves and quality control (QC) samples. QC samples were clustered closely in the PCA diagram of metabolites, indicating that the experimental stability and repeatability are reliable. The values of Autumn Bliss and Autumn Britten are focused in the negative half of the Y-axis, whereas the values of Red Autumn are concentrated in the positive half. PCA analysis is an unsupervised analysis method that struggles to explain the relationship between the contents of the principal components. OPLS-DA analysis was utilized to further evaluate the metabolic data in order to better discern the differences between samples. The metabolites of Autumn Bliss, Red Autumn, and Autumn Britten leaves were clustered in three different groups by OPLS-DA, showing that the three cultivars' metabolites profiles were significantly different (Figure 1C,D). The OPLS-DA was validated using 10-fold cross validation, and the evaluation parameters Q2 (predictive power) and R2 (fitting power)

values were both larger than 0.5. (Figure 1E,F). Differential metabolites might then be examined and filtered based on the variable importance in the projection (VIP) values greater than one.

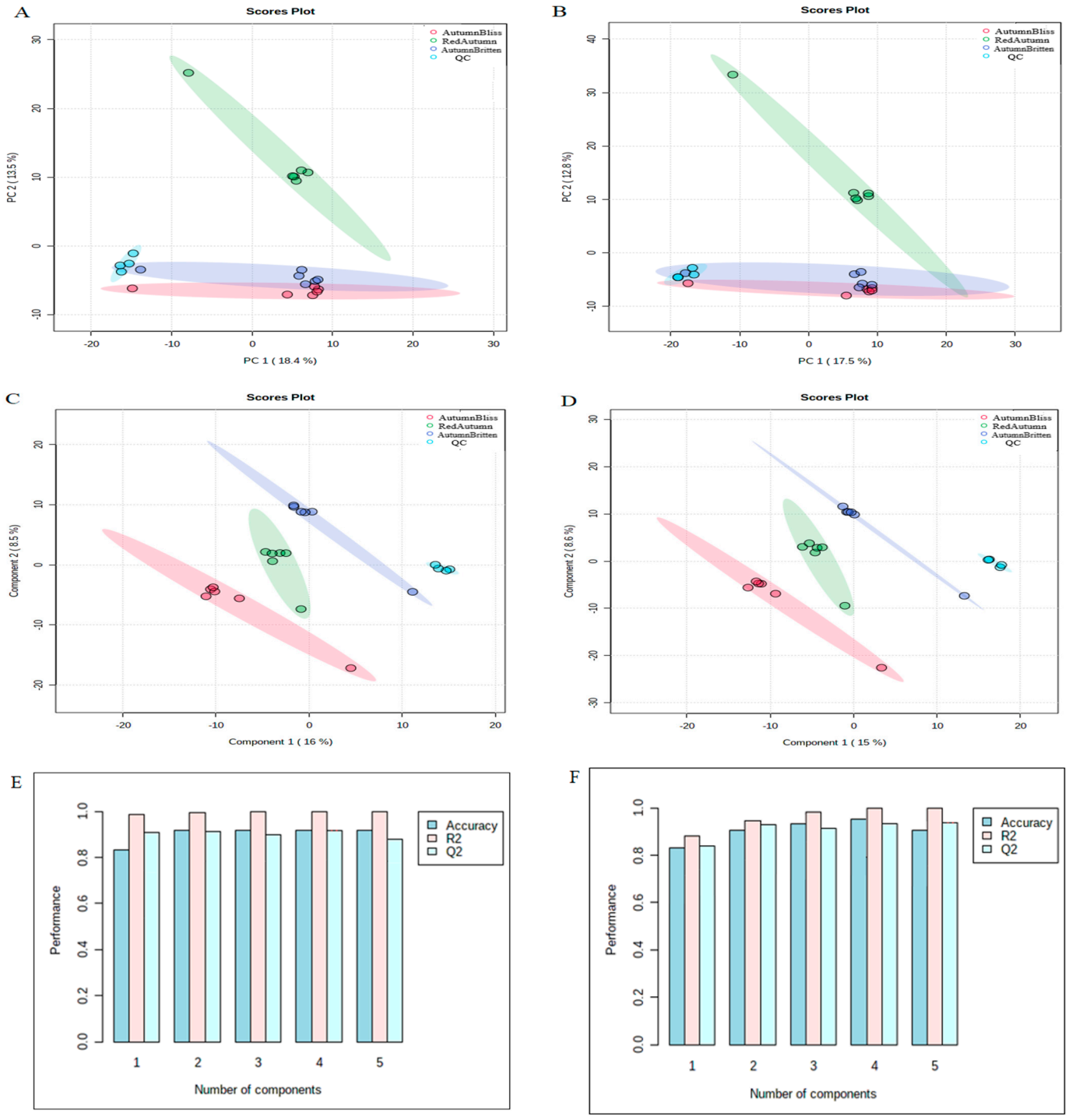

Figure 1. Principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA) of metabolite accumulation levels in three raspberry cultivars. Three cultivars groups are shown in their names, respectively. The X and Y axes represent the first principal component (PC1) and the second principal component (PC2) in positive ion mode (**A**), negative ion mode (**B**) for PCA, positive ion mode (**C**), negative ion mode (**D**) for OPLS-DA, 10-fold cross validation of negative ion mode (**E**) for OPLS-DA, 10-fold cross validation of positive ion mode (**F**) for OPLS-DA, respectively.

Differential accumulated metabolites in three cultivars were compared using multi-dimensional and one-dimensional analysis. The VIP value was utilized to determine the impact of each metabolite on group discrimination. Autumn Britten vs. Autumn Bliss (Table 1) had 36 up-regulated metabolites and 65 down-regulated metabolites; Autumn Britten vs. Red Autumn (Table 2) had 48 differentially up-regulated metabolites and 108 down-regulated metabolites; Autumn Bliss vs. Red Autumn (Table 3) had 88 differentially up-regulated metabolites and 54 down-regulated metabolites, indicating that leaf metabolites were significantly different. The significance of difference accumulated metabolites between groups (VIP > 1, $p < 0.05$) was verified using a *t*-test, which was represented by volcano plots between groups, as shown in Figure 2. The metabolites differentially accumulated among the groups were indicated by pink dots in the figures, and the average accumulation levels were calculated using log2 (fold change) values.

Table 1. Differential accumulated metabolites top 10 (Autumn Britten vs. Autumn Bliss, negative ion mode).

Up-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted	Down-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted
Leucodelphinidin	4.0004	5.2115	0.000353	(1R,6R)-6-Hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate	1.981	−9.7775	0.000364
Citicoline	2.0738	4.4126	0.000378	Quercetin 3-glucoside	1.8664	−3.7997	0.004807
Pantetheine 4′-phosphate	2.0679	4.3753	0.000335	2-Methyl-6-phytylhydroquinone	1.5523	−3.2782	0.0000000004
1-O-Sinapoyl-beta-D-glucose	1.8218	3.5535	0.0000004	(S)-3-Hydroxybutyryl-CoA	1.4495	−2.7599	0.001836
4-(4-Deoxy-alpha-D-gluc-4-enuronosyl)-D-galacturonate	1.6364	2.8387	0.032985	15(S)-HPETE	1.435	−2.6728	0.036865
Prunasin	1.5326	2.8185	0.012186	Arachidic acid	1.3926	−2.4542	0.0000004
Leucopelargonidin	1.3803	2.6083	0.012042	8-Methylthiooctyl-desulfoglucosinolate	1.3725	−2.4453	0.000724
Argininosuccinic acid	1.3016	2.5508	0.000039	Adenosine phosphosulfate	1.3038	−2.2126	0.018818
3,6,8-Trimethylallantoin	1.2061	2.4081	0.016974	Amygdalin	1.2666	−2.1232	0.026546
D-Erythro-imidazole-glycerol-phosphate	1.3016	1.3016	0.027269	3-alpha(S)-Strictosidine	1.2234	-2.0788	0.007547

Table 2. Differential accumulated metabolites top 10 (Autumn Britten vs. Red Autumn, negative ion mode).

Up-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted	Down-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted
SAICAR	4.0004	5.4584	0.047114	Castasterone	2.316	−8.3039	0.000106
Leucodelphinidin	2.2308	3.3671	0.019403	Sirohydrochlorin	2.0838	−5.248	0.001097
2beta-Hydroxygibberellin 1	1.826	3.2476	0.001585	Kaur-16-en-18-oic acid	1.9624	−5.2391	0.004953
Argininosuccinic acid	1.6775	3.0899	0.000441	Vitamin K2	1.8252	−5.0163	0.003455
Glucoiberverin	1.3803	2.9702	0.005375	Prephytoene diphosphate	1.6364	−4.9073	0.001097
2-Oxo-9-methylthiononanoic acid	1.2666	2.814	0.00000005	7,7′,8,8′-Tetrahydrolycopene	1.5917	−4.7239	0.000134
Galactosylglycerol	1.1467	2.7782	0.000169	Behenic acid	1.5275	−4.5183	0.000060
Leucocyanidin	1.1459	2.7444	0.001885	7,8-Diaminononanoate	1.4495	−4.4145	0.002004
Cis-zeatin-7-N-glucoside	1.1105	2.7402	0.000059	N1-trans-Feruloylagmatine	1.435	−4.3678	0.000037
D-Gal alpha 1->6D-Gal al-pha 1->6D-Glucose	0.018223	2.7328	0.001915	2-Deoxycastaster-one	2.316	−4.2749	0.005609

Table 3. Differential accumulated metabolites top 10 (Autumn Bliss vs. Red Autumn, negative ion mode).

Up-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted	Down-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted
Castasterone	3.533	8.7869	0.000019	(1R,6R)-6-Hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate	2.2308	−7.1632	0.015585
Propinol adenylate	2.316	6.9795	0.032560	Phenylacetaldehyde	1.826	−5.8687	0.000909
Pantetheine 4′-phosphate	2.2522	5.545	0.000002	Adenosine phosphosulfate	1.7621	−4.6282	0.000007
4-Hydroxycinnamic acid	2.2308	5.3486	0.0000000005	SAICAR	1.6775	−4.57	0.017379
Sirohydrochlorin	2.0838	5.2977	0.000039	6-Geranylgeranyl-2-methylbenzene-1,4-diol	1.5523	−3.8981	0.000010
Kaur-16-en-18-oic acid	2.0679	5.0538	0.000150	Prostaglandin H2	1.2666	−3.282	0.016800
Behenic acid	1.9624	4.769	0.000002	15(S)-HPETE	1.2294	−3.1716	0.005833

Table 3. *Cont.*

Up-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted	Down-Regulated Metabolites	VIP	log2(FC)	*p*. Ajusted
4-(4-Deoxy-alpha-D-gluc-4-enuronosyl)-D-galacturonate	1.826	4.7415	0.000038	Flavin Mononucleotide	1.1819	−3.082	0.001200
Prephytoene diphosphate	1.8252	4.665	0.000022	2-Oxo-9-methylthiononanoic acid	1.1571	−2.9249	0.005972
S-(Phenylacetothiohydroxi-moyl)-L-cysteine	0.012543	4.6557	0.000027	Phos-phory-lcholine	0.5808	−2.6394	0.000342

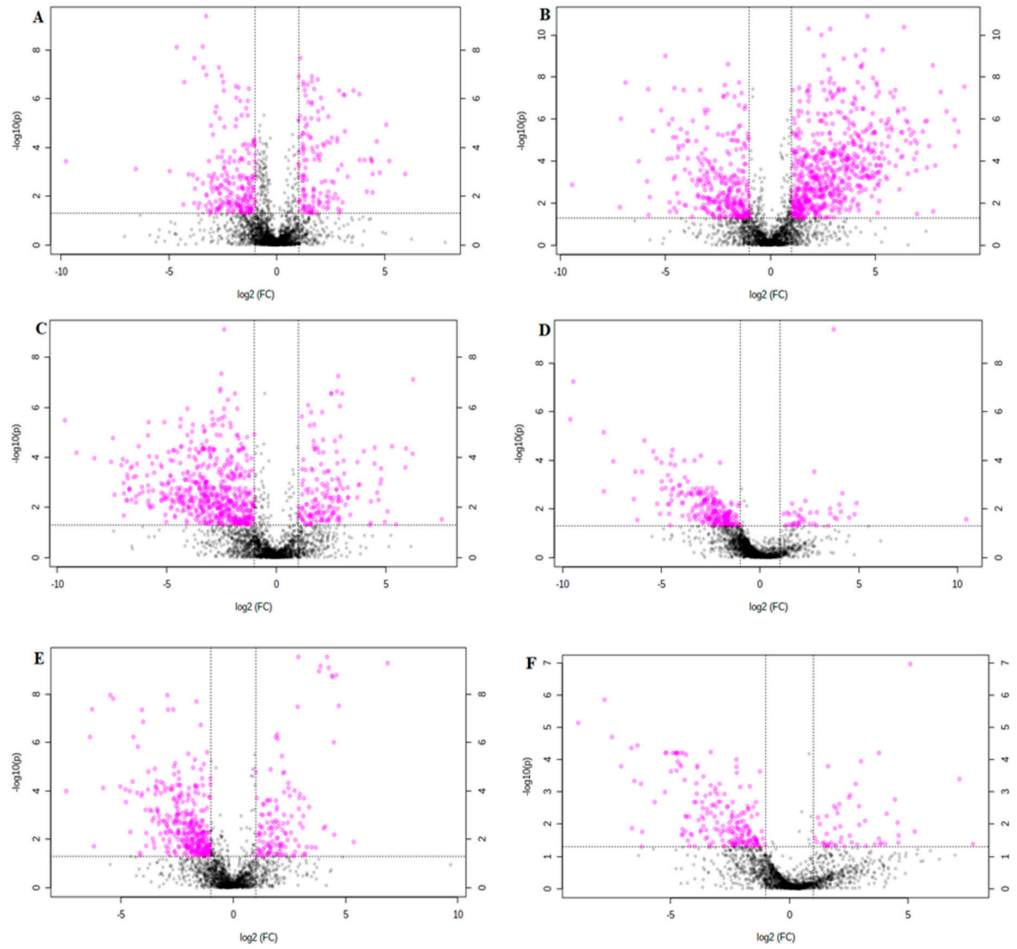

Figure 2. Volcano plots of metabolites detected in cultivars for different comparisons: (**A**) Autumn Bliss vs. Autumn Britten; (**B**) Autumn Bliss vs. Red Autumn; (**C**) Autumn Britten vs. Red Autumn; in negative ion mode: (**D**) Autumn Bliss vs. Autumn Britten; (**E**) Autumn Bliss vs. Red Autumn; (**F**) Autumn Britten vs. Red Autumn; in positive ion mode. The vertical dotted lines indicate fold change of 1.5, and the horizontal dotted line indicates $p = 0.05$.

In order to further distinguish the differentially accumulated metabolites between different cultivars, comparisons between each two different cultivars were employed to identify up or down regulated metabolites. Then the differentially accumulated metabolites in different comparisons were grouped, and the selected differential metabolites in each group were analyzed for KEGG pathway enrichment. The comparison of Autumn Britten vs. Autumn Bliss found 36 up-regulated metabolites enriched in 38 metabolic pathways, and 65 down-regulated differential metabolites enriched in 46 metabolic pathways; Autumn Britten vs. Red Autumn found 48 up-regulated metabolites enriched in 44 metabolic pathways, and 108 down-regulated metabolites enriched in 55 metabolic pathways; Autumn Bliss vs. Red Autumn found 88 up-regulated metabolites enriched in 50 metabolic pathways (Figure 3).

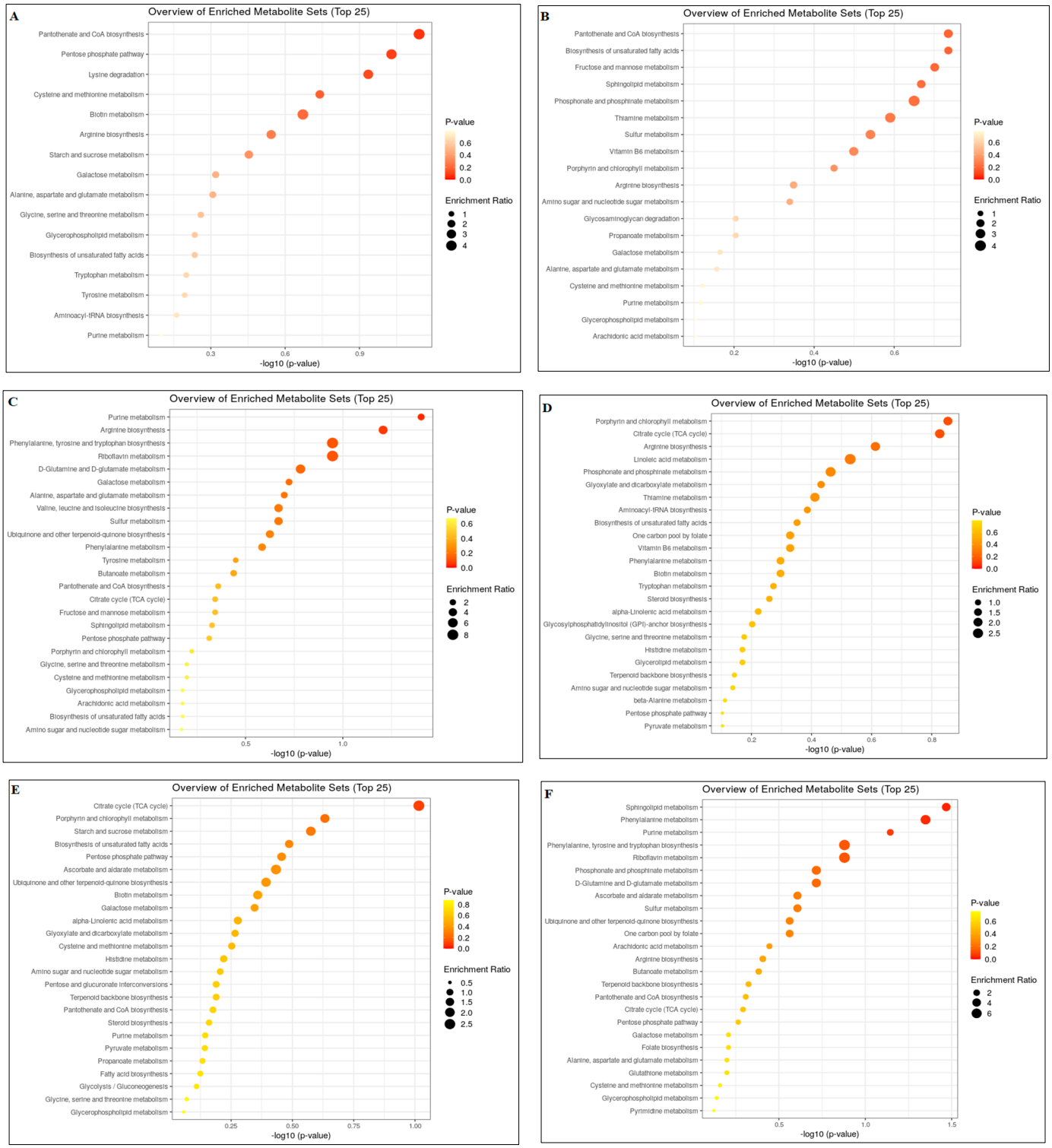

Figure 3. Metabolic pathway analysis of different comparisons of three cultivars. (**A**) Autumn Bliss vs. Autumn Britten (up-regulation metabolites); (**B**) Autumn Britten vs. Red Autumn (up-regulation metabolites); (**C**) Autumn Bliss vs. Red Autumn (up-regulation metabolites); (**D**) Autumn Bliss vs. Autumn Britten (down-regulation metabolites); (**E**) Autumn Britten vs. Red Autumn (down-regulation metabolites); (**F**) Autumn Bliss vs. Red Autumn (down-regulation metabolites).

3.2. Comparison of Differential Metabolite Groupings between Species

A total of 399 differential metabolites were screened out in the three group comparisons (Autumn Bliss vs. Autumn Britten, Autumn Britten vs. Red Autumn and Autumn Bliss vs. Red Autumn). Venn diagram was used to screen specific differential metabolites in different group comparisons, there were 54, 69, 83 specific metabolites in the three comparison groups, respectively (Figure 4A). The metabolites specific to each of the three groups were mainly Lipids, Amino acids, and Sugars. Lipid metabolites were the most abundant in group Autumn Bliss vs. Red Autumn, accounting for 34.91%, while amino acid and sugar metabolites were the most abundant in group Autumn Britten vs. Autumn Bliss, accounting for 25.33% and 16% respectively. In addition, flavonoids were screened in the highest number in group Autumn Britten vs. Autumn Bliss, accounting for 11.57%.

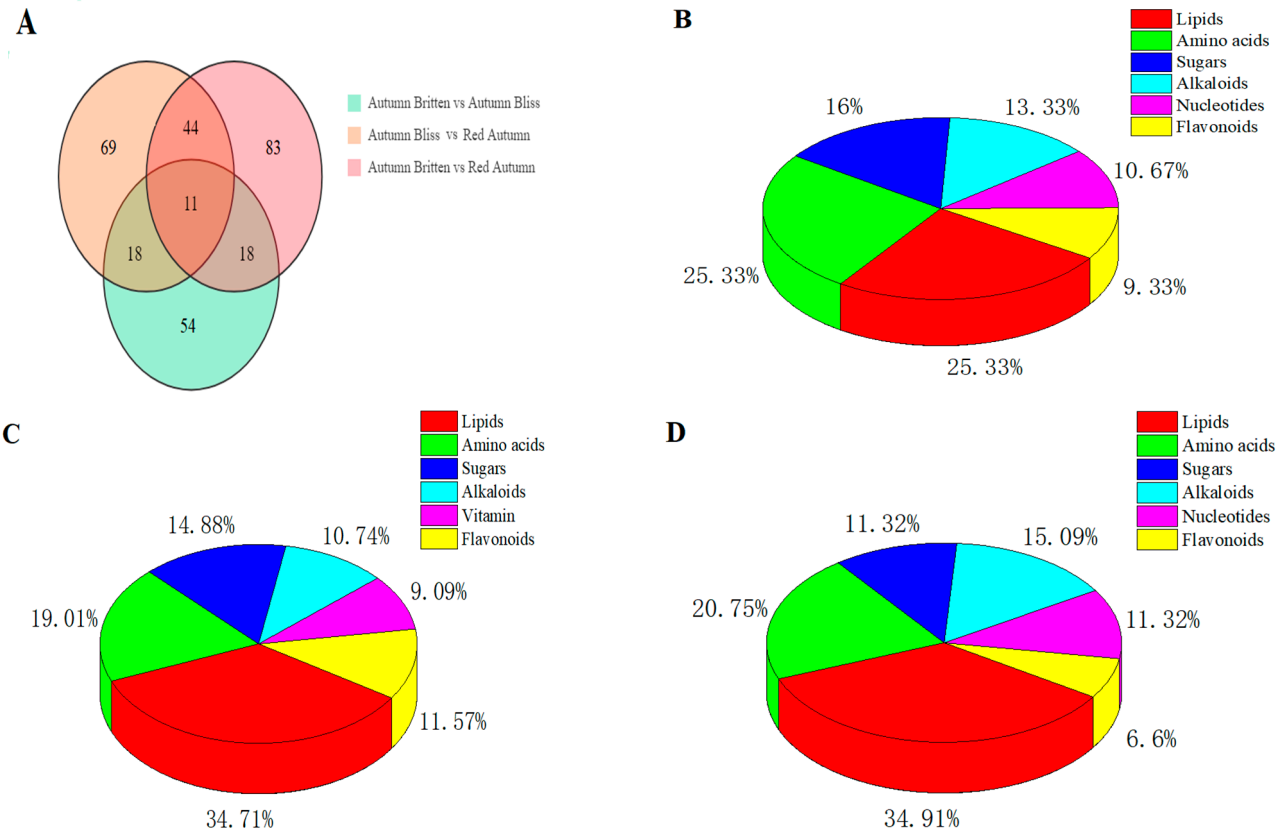

Figure 4. Comparison of classification of identified differential metabolites. (**A**) Venn for metabolites between different varieties. (**B**) Pie graph for specific metabolites in Autumn Britten vs. Autumn Bliss. (**C**) Pie graph for specific metabolites in Autumn Britten vs. Red Autumn. (**D**) Pie graph for specific metabolites in Autumn Bliss vs. Red Autumn.

The up-regulated differential metabolites in the leaves of the three different raspberry varieties were mainly in the three major types of metabolic pathways: lipid metabolism, nucleotide metabolism, and sugar metabolism, while the down-regulated differential metabolites were mainly in the metabolism of cofactors and vitamins, carbohydrate metabolism, nucleotide metabolism and lipid metabolism. Among the differential metabolites of raspberry leaves, lipids such as α-linolenic acid and eicosatetraenoic acid; amino acids and their derivatives such as L-cysteine, Phenylalanine, L-Histidinal, L- glutamate, Glucoiberverin, L-Lysine, L-Arginine; flavonoids such as Kaempferol 3-O-rhamnoside-7-O-glucoside, Quercetin 3-glucoside; vitamins such as Biotin, Thiamine, Vitamin K2, etc. all had significant differences in content (Table 4). Other metabolites such as Phosphorylcholine and organic acids such as Oxoadipic acid showed significant interspecific

differences. The antioxidant activity of the leaf extracts may also vary with the differences in metabolite content.

Table 4. Differential varieties representing intermediate metabolites (mean ± SD, n = 6).

	Autumn Bliss	**Red Autumn**	**Autumn Britten**
Alpha-linolenic acid	$1.0363 \times 10^{8\,a}$ $\pm 4.3826 \times 10^{6}$	$3.8463 \times 10^{7\,b}$ $\pm 2.2716 \times 10^{6}$	$1.0980 \times 10^{8\,c}$ $\pm 5.3622 \times 10^{6}$
L-cysteine	$3.2698 \times 10^{5\,a}$ $\pm 1.5488 \times 10^{4}$	$1.4537 \times 10^{5\,b}$ $\pm 1.9103 \times 10^{4}$	$4.6593 \times 10^{5\,c}$ $\pm 1.5832 \times 10^{4}$
Quercetun-3-glucoside	$3.0711 \times 10^{5\,a}$ $\pm 1.1350 \times 10^{4}$	$3.0067 \times 10^{4\,a}$ $\pm 1.0110 \times 10^{4}$	$5.1269 \times 10^{5\,b}$ $\pm 1.0016 \times 10^{4}$
Vitamin K2	$3.7465 \times 10^{5\,a}$ $\pm 1.6836 \times 10^{4}$	$2.0264 \times 10^{4\,b}$ $\pm 0.0254 \times 10^{4}$	$5.5264 \times 10^{5\,c}$ $\pm 0.0509 \times 10^{4}$

[a b c] Significant differences among different varieties expressed by different letter labels, $p < 0.05$.

3.3. RLEE Cytotoxicity Test on L929 Fibroblasts

L929 cells were cultivated and subjected to different concentrations of RLEE (1–100 μg/mL) for 12 h in order to assess the cytotoxicity of RLEE in L929 fibroblasts, and cell viability was measured using the CCK-8 kit. Figure 5 shows that L929 cells exposed to 1–75 μg/mL RLEE had no cellular damage as compared to the control group. Because 100 μg/mL and 200 g/mL RLEE significantly reduced cell viability when compared to the control group ($p < 0.05$), 50 μg/mL RLEE was used for following tests.

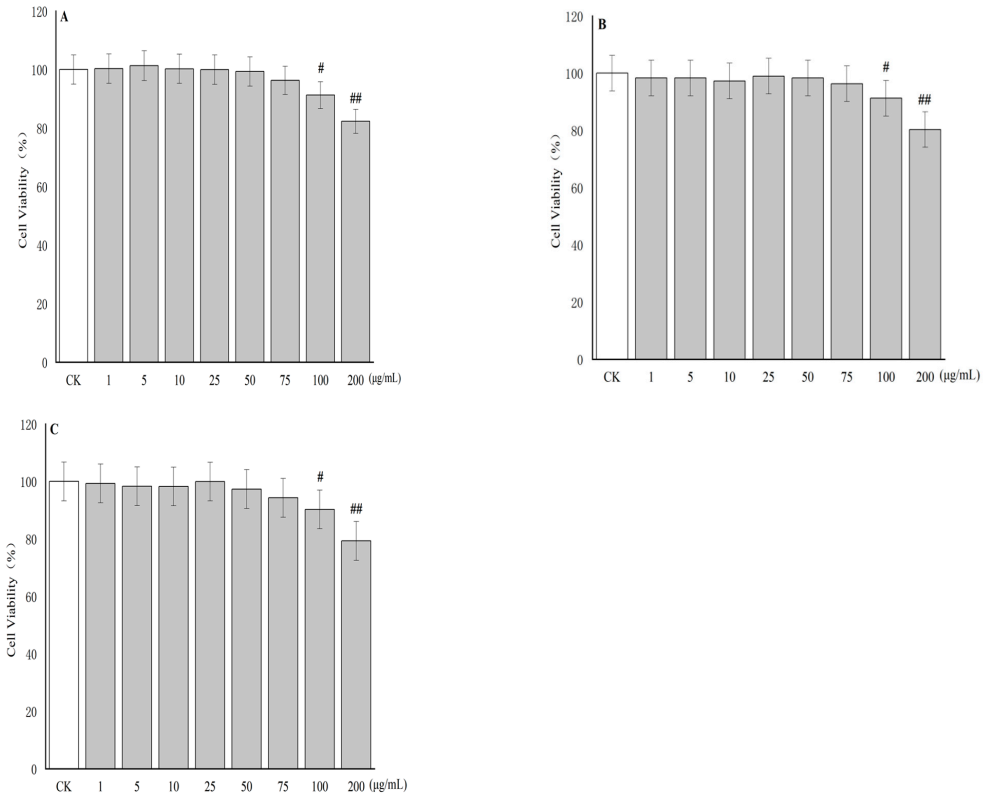

Figure 5. Cytotoxicity assessment of L929 fibroblasts treated with different concentrations of RLEE. The fibroblasts were determined by CCK-8. Readings on a spectrophotometer at 450 nm. ((**A**): Autumn Britten; (**B**): Autumn Britten; (**C**): Red Autumn, CK: control group: untreated cells. RLEE: 1–200 μg/mL RLEE treated cells. [#] $p < 0.05$, [##] $p < 0.01$, significant difference compared to CK).

3.4. Protective Effect of Different Varieties of RLEE on UVB Induced L929 Fibroblast Injury

In order to further investigate the protective effect of RLEE from different varieties of on L929 fibroblasts under UVB irradiation, fibroblasts were treated according to the method section. Firstly, the CCK-8 kit was used to measure the fibroblast viability after UVB exposure with or without 50 μg/mL RLEE pre-protection (Figure 6A). Compared with the control group, UVB exposure could induce dramatic cell viability decreasing caused by oxidative stress and leading to cell injury and apoptosis. When pre-protected by Autumn Britten and Autumn Bliss RLEE, UVB exposure induced fibroblast toxicity was significantly reduced (Figure 6A, $p < 0.001$). Additionally, as shown in Figure 6B, the levels of SOD in L929 fibroblast cells showed significant decrease in UVB groups compared with control counterparts. Moreover, SOD activity was significantly increased in the RLEE pre-protected group compared to the UVB group, Autumn Britten increased SOD activity to 120.32 ± 0.63 U/mgprot compared to the UVB exposed group (Figure 6B). As illustrated in Figure 6C, the levels of GSH in L929 fibroblast cells showed significant reduction in the UVB group, compared with the control group, RLEE pre-protection increased the GSH level of fibroblasts, among which Autumn Britten and Autumn Bliss had the most significant effect ($p < 0.01$). As shown in Figure 6D, the levels of CAT in L929 fibroblast cells exhibited significant inhibition in UVB and UVB + RLEE groups, while cells from the Autumn Britten RLEE and the Autumn Bliss RLEE pre-protection groups showed significant elevation ($p < 0.01$) compared to their control counterparts.

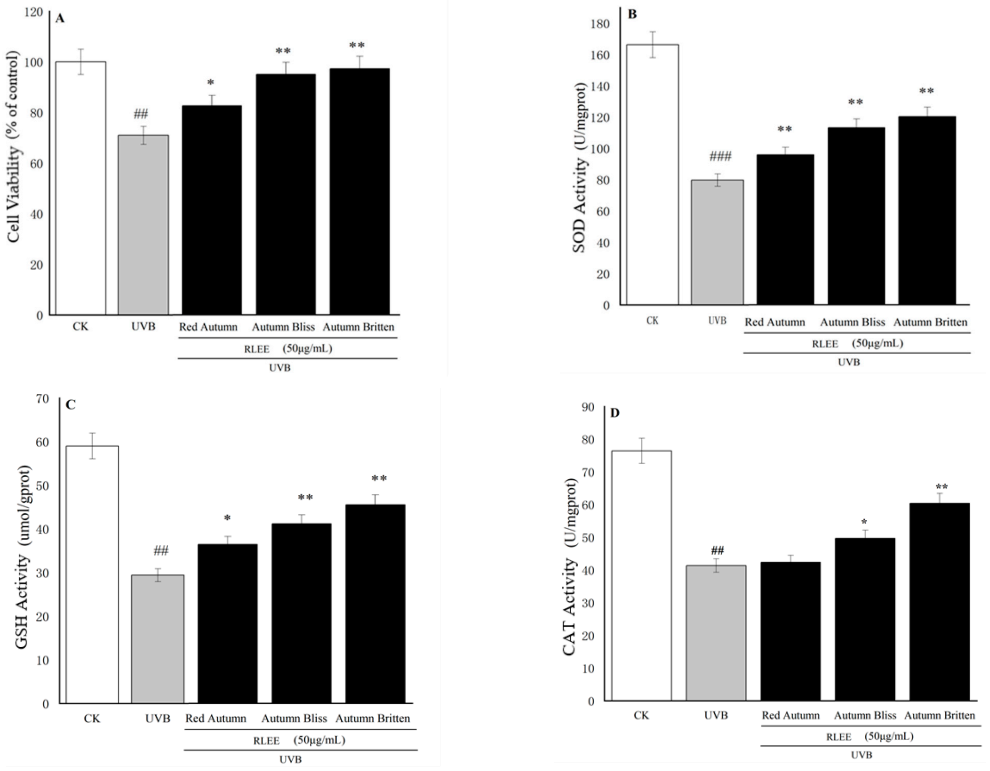

Figure 6. Cell viability and antioxidant enzyme activity of L929 fibroblasts treated with different varieties of RLEE (50 μg/mL); (**A**): cell viability (**B**): SOD Activity (**C**): GSH Activity (**D**): CAT Activity. SOD activities were expressed in terms of U/mg protein, U means that when the SOD inhibition rate reaches 50% in the reaction system, the corresponding enzyme amount is an activity unit (U); GSH activity were expressed in terms of μmol/g protein; CAT activity were expressed in terms of U/mg protein, U means 1 μmol of H_2O_2 per milligram of cellular protein per second is an activity unit (U). (CK: control group: untreated cells; UVB: exposed only to UVB without RLEE protection; $^{\#\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.01$, significant difference compared to CK, * $p < 0.05$, ** $p < 0.01$, significant difference compared to UVB group).

L929 fibroblasts were pre-protected with RLEE of different varieties (50 μg/mL) for 12 h. After UVB exposure, they continued to be cultured for 12 h and then treated with the ROS kit to observe the fluorescence using inverted fluorescence microscopy (Figure 7). Compared with the blank group, the fluorescence intensity of fibroblasts after UVB exposure increased significantly, this indicated the content of ROS in cells increased significantly. The fluorescence intensity of cells treated with different varieties of RLEE decreased gradually, and the fluorescence intensity of cells protected by 50 μg/mL Autumn Britten RLEE decreased most after exposure to UVB.

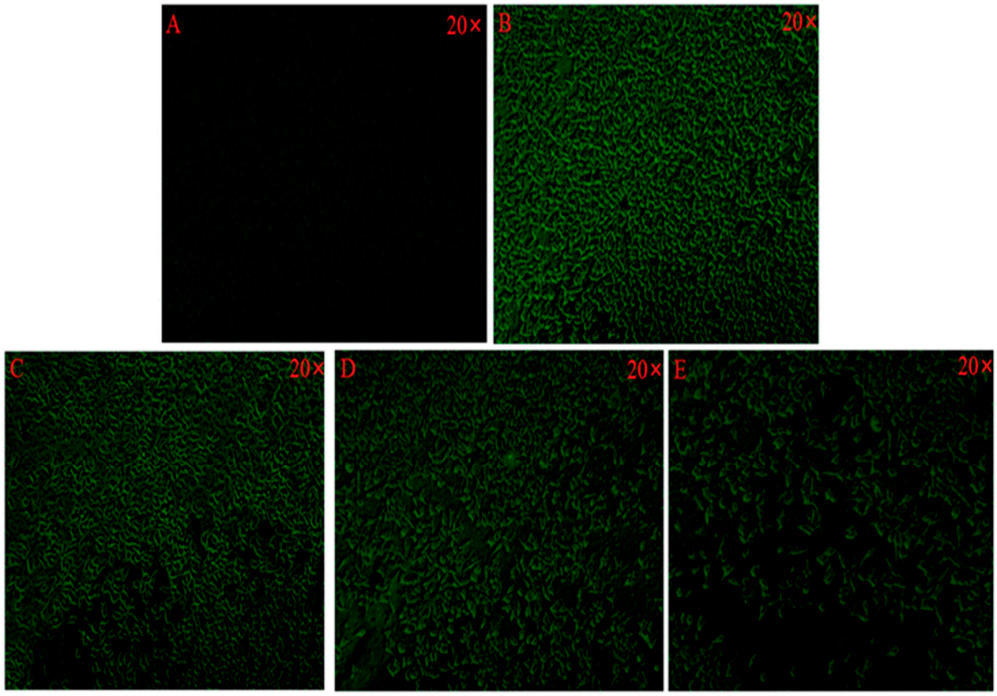

Figure 7. Effects of RLEE on ROS fluorescence intensity in UVB injury protection group (**A**): control; (**B**): UVB; (**C**): UVB + 50 μg/mL Red Autumn RLEE; (**D**): UVB + 50 μg/mL Autumn Bliss RLEE; (**E**): UVB + 50 μg/mL Autumn Britten RLEE.

4. Discussion

Natural plant extracts have been widely used to study the antioxidant capacity of grape seeds, begonia, ginkgo biloba, and other plant and herbal extracts containing polyphenols, flavonoids, vitamins, organic acids, amino acids and other antioxidant components [25–27]. In this study, the lipid metabolites α-linolenic acid and eicosatetraenoic acid in different raspberry leaf varieties were screened as essential fatty acids for humans and animals. α-linolenic acid, as a natural antioxidant, was shown to significantly increase GSH content in porcine oocytes and decrease ROS content in bovine oocytes [28–30]. Amino acids have been shown to regulate skin moisture, balance skin oil and increase skin immunity, reduce or prevent oxidative damage to the skin, as well as reduce ageing caused by the sun, and are very effective in renewing, repairing, and avoiding dehydration of the skin [31,32]. Cysteine is a sulfur-containing amino acid that reacts with free radicals and has greater antioxidant activity [33]. Histidine can enhance the antioxidant activity in grass carp by increasing the GSH content and the activity of antioxidant enzymes such as SOD, CAT, GPx, and their related genes, and by activating the Nrf2/Keap1 and NF-κB signaling pathways [34]. In a chronic mild stress depression model, treatment with the flavonoid quercetin was found to significantly increase the activity of glutathione, SOD, and CAT in the brain and inhibit oxidative stress [35]. Fu et al. (2012) showed that the addition of vitamin K to abalone feed significantly increased the muscle SOD activity [36]. The nature of the action of antioxidant products is the transfer of electrons,

and the antioxidant products will be oxidized and turned into new free radicals, which can be reduced again by other antioxidants, forming a redox cycle system. Phenols, flavonoids, vitamins, organic acids, and other active substances have stronger antioxidant properties than amino acids. The synergistic effect of natural antioxidant products on each other is one of the mechanisms by which amino acids exert their antioxidant activity, which is also known as synergistic antioxidant effect with other antioxidants. Antioxidants such as vitamin C, β-carotene or glutathione can reduce α-tocopherol radicals to vitamin E, and Marcuse found that the addition of a certain concentration of amino acids to tocopherols significantly increased their antioxidant activity, suggesting a significant synergistic effect between amino acids and tocopherols [37,38]. The synergistic effect of various active metabolites in raspberry leaf extracts was found to protect cells by enhancing the activity of SOD, GSH, and CAT antioxidant enzymes in L929 cells to scavenge the excess ROS accumulated by UVB exposure.

As the first important barrier of the body, the skin does not only resist the body from the damage of the external environment, but also maintain the homeostasis by regulating body temperature, protecting water loss, and participating in various metabolic processes [39]. When the body is exposed to excessive ultraviolet radiation, it will lead to the imbalance between oxidation and anti-oxidation in the body, and lead to a large amount of ROS accumulation through a variety of ways. ROS will destroy the DNA structure and combine with the biofilm to form a strong cross-linking agent malondialdehyde (MDA), which can induce cell apoptosis and cause body damage and lesions [40]. In fibroblasts, excessive ROS will activate peroxidation reaction with biological macromolecules in the cells, destroy the structure of collagen fiber and elastic fiber, lead to the degradation of elastin, and cause skin light damage [41,42]. Starting with ROS, the key products of oxidative stress, screening components that can inhibit the production of ROS after UVB induction or remove excessive ROS effect has become an effective approach to protect the skin light injury. Plants contain a large number of active metabolites that are beneficial to human body. In recent years, new plant extracts and natural compounds have been proved to have the effect of improving and preventing skin damage caused by UVB exposure [43]. Recently, multiple plant water or alcohol extracts from Garden Angelica, Rooibos, Honeybush, tea, coffee, and others were proved to be able to reduce the skin light damage by inhibiting the production of ROS and the expression of related inflammatory genes after UVB exposure through the verification of cell and animal models [44–48].

A comparison of the metabolites of different varieties of raspberry leaves shows that there are significant differences in metabolites between varieties and therefore there should also be differences in their RLEE antioxidant effects. The toxicity tests showed that 1–50 μg/mL of RLEE was non-toxic to L929 cells. Next, L929 fibroblasts were then irradiated with a 600 mJ/cm^2 UV lamp to establish an in vitro UVB exposure model. After UVB irradiation, the activity of L929 fibroblasts without RLEE pretreatment was significantly reduced, while the activity of L929 fibroblasts pretreated with different types of 50 μg/mL RLEE was significantly increased, demonstrating that RLEE has a protective effect on UVB-induced damage in terms of cell viability, and there were varietal differences in the protective capacity.

Excessive UVB exposure will cause excessive production of ROS, causing oxidative stress reaction in the body to destroy the internal organs and cause tissue damage [48]. Antioxidant enzymes are the most important substances to remove ROS in the body, such as SOD, GSH, and CAT, which maintain internal homeostasis by fighting against endogenous ROS, the antioxidant reaction chain composed of SOD, GSH, and CAT can catalyze the decomposition of O_2 to H_2O, and remove excess ROS in time to maintain the balance of the redox reaction of the organism [49]. Under UVB radiation, the massive increase of ROS production will significantly reduce the activity of antioxidant enzymes, thus aggravating oxidative stress injury [50]. After 600 mJ/cm^2 UVB exposure, L929 fibroblasts were detected by ROS kit, and the fluorescence intensity in the body was significantly increased compared with the control group, while the fluorescence intensity in the cells

after RLEE pre-protection was decreased to varying degrees. Then, the activity of major antioxidant enzymes in cells treated by different groups was measured, which also proved that RLEE could remove excess ROS generated after UVB exposure by increasing the activity of antioxidant enzymes mechanism, and reduce oxidative stress and photodamage caused by UVB.

Lipids, amino acids, flavonoids, alkaloids, vitamins, organic acids, and other natural metabolites with antioxidant potential are all abundant in RLEE. Because the leaves of raspberry Autumn Britten are higher in natural metabolites than the other two types, the 50 μg/mL RLEE of Autumn Britten can effectively eliminate ROS in L929 fibroblast cells, alleviating UVB-induced cell damage and cell viability. Our findings backed up the theory that RLEE protects the skin barrier function by reducing UVB-induced skin damage. As a result, RLEE can be used as a key raw material for anti-oxidation, skin hydration, and permeability.

5. Conclusions

Screening for differential metabolites and analyzing differential metabolite pathway enrichment in the leaves of three different raspberry varieties revealed that the metabolites of the three varieties of raspberry leaves differed significantly, with the differential metabolites screened primarily consisting of lipids, amino acids and their derivatives, sugars, alkaloids, nucleotides and their derivatives, flavonoids, and organic acids. Lipid metabolism, nucleotide metabolism, sugar metabolism, cofactor, and vitamin metabolism, and carbohydrate metabolism were the main pathways of enrichment for various metabolites. The accumulation of amino acids, lipids, flavonoids, and other metabolites with antioxidant activity in raspberry Autumn Britten leaf extracts was significantly higher than in the other two varieties. In the L929 fibroblast assay, the raspberry Autumn Britten leaf extract considerably boosted the activity of intracellular antioxidant enzymes and gave superior protection to fibroblasts following UVB exposure, according to the results of the L929 fibroblast experiment. The results of the study can be used as a basis for the selection of varieties for the extraction of specific metabolites from raspberry leaves, as well as contributing to the reuse of raspberry leaf resources and enhancing the added value of raspberries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12030990/s1, Table S1: Summary of differential metabolites.

Author Contributions: X.Z. and S.Z. conceived and designed the study, X.Z., Z.L. (Zhao Liu) and M.A.A. collected, analyzed the data and wrote the paper, X.L. (Xu Li), Z.L. (Zhengdou Li) and S.Z. prepared metabolomics samples, X.W., X.L. (Xiaoxiao Liu) and X.C. collected and analyzed metabolomics data, J.Z., X.C. and X.Z. provided funding. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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