Mathematical analysis of climatic characteristics of planting areas and biological activity of mulberry branch

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Abstract

In this study, mulberry branch from 12 planting areas in China were collected, and the contents of 12 compositions in these mulberry branch and their differences in bioactivities were compared. The effects of climate characteristics (environmental factors) in the planting areas on the compositions and the bioactivities of mulberry branch were analyzed by partial least square regression and multiple factor analysis. We found that the precipitation and temperature difference in the planting areas were important influencing factors, which could change the content and ratio of key compositions in mulberry branch, which in turn lead to differences in bioactivities. Planting environments with smaller temperature difference will make the anti-apoptotic effect of mulberry branch more prominent, and adequate irrigation will promote anti-oxidative effect of mulberry branch. These research results provide evidence for improving mulberry planting conditions and rational use of resources from the perspective of mathematical analysis.

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Abstract

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KEYWORDS: Mulberry branch; Environmental factors; Bioactivities; Multiple factor analysis

11**Abbreviations** : ESI, electrospray ionization; MFA, multiple factor analysis; PA, palmitic acid; PLSR, partial least square regression; RBMEC, Rat brain microvascular endothelial cells; ROS, reactive oxygen species; SD, standard deviation; 1-DNJ, 1-deoxynojirimycin

1 Introduction

Mulberry (*Morus alba* L.) has a very special significance in the agricultural and food history of China, it is not only important supply for silkworm breeding, but also a source of various foods, teas, and medicines, and has great application potential in the energy, food, and pharmaceutical industries (Chao, Yu, Hou, Liu, & Zhang, 2021). Mulberry has unique advantages in natural resources and widely distributed in China, due to their great adaptability to climate. From Heilongjiang province in the north to Guangxi and Guangdong in the south, it spans the entire geographic latitude of China. However, the geographical environments have huge differences, e.g. in precipitation and temperature difference in these areas. In addition, because mulberry trees have strong germination (Hashemi & Tabibian, 2018), and rapid regeneration after pruning, a large number of pruning's must be carried out every year to facilitate the normal growth of the trees, which will produce a large number of mulberry branches and leaves. However, in the actual utilization process, only the mulberry leaves and fruits, but not the mulberry branches are fully developed and utilized. The mulberry branch are crushed to make fertilizer or even burned directly (Yin, Liu, & Zhang, 2017). This is actually a great waste of mulberry resources.

In terms of functional foods, mulberry leaf and branch are teas and condiments with unique flavors. They have been widely used to treat diabetes in China for thousands of years. Mulberry branch are rich in alkaloids, flavonoids, and polysaccharides and other bioactivities ingredients, which can prevent diabetes and related complications and protect pancreatic cells from oxidative damage (Han et al., 2020; Hou et al., 2020). For example, chlorogenic acid, which is one of the active ingredients in mulberry branch, reduces inflammation and fat deposition in the liver by reducing the energy intake and food efficiency, and also increases the diversity of the gut microbiota, thereby improving the overall metabolism in the body. Long-term consumption of chlorogenic acid can reduce inflammation caused by diet, and change in cardiovascular, liver and metabolism (Bhandarkar, Brown, & Panchal, 2019). Morusin is a kind of flavonoids derived from mulberry, which has strong antioxidant capacity, and can reduce oxidative stress and protect the integrity of pancreatic β-cells by reducing the cell death, and improve hyperglycemia and lipid homeostasis in type I diabetic mice induced by streptozotocin (Choi, Cho, Lee, & Choi, 2020). Cortex Mori water extract, containing mulberroside A, has blood glucose reduction effect, thus alleviates liver and kidney damage caused by hyperglycemia, and ameliorates diabetic endotoxemia (Xu, Guo, Zhao, Fu, & Xu, 2021). Resveratrol has been used in various anti-hyperglycemia studies because of its physiological activities in lowering blood

sugar, improving insulin sensitivity and protecting pancreatic β cells (Huang, Shi, Jiang, Yao, & Zhu, 2020; Öztürk, Arslan, Yerer, & Bishayee, 2017; Zhu, Wu, Qiu, Yuan, & Li, 2017). Studies have found that scopoletin can inhibit postprandial blood glucose levels, and the mechanism of action is related to the inhibition of α-glucosidase and α-amylase activity (Jang, Park, & Han, 2018). 1-deoxynojirimycin (1-DNJ) is a potent α-glucosidase inhibitor, which can not only directly inhibit the increase in blood sugar, but also effectively reduce blood sugar after meals without serious side effects (Thaipitakwong, Supasyndh, Rasmi, & Aramwit, 2020). It can also inhibit hypercholesterolemia induced by high-fat diet (HFD), and regulate the compound of the gut microbiota (Yougui Li et al., 2019).

Mulberry branch have similar compositional basis and bioactivities, as well as similar food and medicinal history as mulberry leaves. The abundant yield makes mulberry branch have obvious advantages in development. However, there is no research that reveals the impact of environmental differences in mulberry planting areas on the similarity of the compositions contained in mulberry branch. The lack of verification of the functional similarity of mulberry branch in different planting areas blocks the development and research of mulberry resources. Therefore, in this study, by determining fingerprint morphology and main compositions content, the compositional similarity of mulberry branches in 12 planting areas of China were compared using mulberry leaf as a control. In vitro hyperglycemia and hyperlipidemia model was used to study the effects of mulberry branch from different planting areas against apoptosis and antioxidant damage. The correlation between the environmental factors of the planting areas, the compositions of the mulberry branch extract and the bioactivities differences were analyzed by the partial least square regression (PLSR) and multiple factor analysis (MFA). This research provides inspiration for planting methods to improve the bioactivities of mulberry branch.

2 Materials and methods

2.1 Medicinal materials and reagents

The mulberry branch and leaf were identified belonging to *Morus alba* L. by the chief pharmacist, Ling-bian Sun, of Air Force Medical University (Xi'an, China).

The purity of all standards was [?] 98 %. Chlorogenic acid, cryptochlorogenic acid, rutin, hyperoside, isoquercitrin, astragalin, quercitrin, morusin, mulberroside A, resveratrol, scopoletin, 1-DNJ, and palmitic acid (PA) were purchased from Sigma Aldrich (Shanghai, China). Methanol, ACN, and formic acid were of chromatography-grade (Thermo Fisher Scientific, Waltham, MA, USA). Ultra-pure water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). All other reagents were of analytical grade. The samples (mulberry branch of different areas) are listed in Table 1. Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and Trypsin-EDTA solution were purchased from HyClone (Shanghai, China). Cell Counting Kit-8 was purchased from EnoGene (CCK-8, Xi'an, China), FITC Annexin V Apoptosis Detection Kit I was purchased from Wuhan Seville Biological Technology (Wuhan City, Hubei, China), and ROS Assay Kit was purchased from Biosharp (Guangzhou, China).

2.2 Preparation of samples

Fresh mulberry branch from 12 areas were cut into thick slices (0.2 – 0.5 cm in diameter), dried until the moisture content was less than 11 % (at 30°C and 30 % humidity), crushed, and passed through a No.3 sieve (pore size 355 ± 13 μm). Take 500g of mulberry branch and mulberry leaf powder, soak with 8 times the amount of 50% ethanol overnight and reflux two times for 3 h each time. The ethanol extracts were concentrated under reduced pressure to yield a 1.5 g/mL extracts.

Methanol was added to 0.1 mL of extracts to yield a volume of 10 mL. The suspensions were filtered through a 0.22 μm micropore membrane to obtain the test solution and used for subsequent composition analysis.

2.3 HPLC chromatographic conditions

The HPLC system fitted with an LC-30AD binary pump (Shimadzu Corporation, Kyoto, Japan), an on-line

vacuum degasser, an autosampler, and a column oven was used for similarity analyses. A Poroshell 120 EC-C_{18} column (4.6 mm × 100 mm; 2.7 μm; Agilent Technologies, Santa Clara, CA, USA) was fitted with an EC-C_{18} pre-column (4.6 mm × 5 mm; 2.7 μm; Agilent Technologies, Santa Clara, CA, USA). The column temperature was stabilized to 26°C and the samples were monitored at a wavelength of 320 nm. The mobile phase consisted of ACN (A) and 0.1 % (v/v) phosphoric acid in water (B). The flow rate was 0.80 mL/min and the injection volume was 20 μL. The gradient elution program was as follows:0 – 3 min, 5 % to 10 % A; 3 – 30 min, 10 % to 15 % A; 30 – 40 min, 15 % to 20 % A; 40 – 75 min, 20 % to 30 % A; 75 – 80 min, 30 % to 40 % A; 80 – 100 min, 40 % to 55 % A; 100 – 110 min, 55 % to 80 % A; 110 – 115 min, 80 % to 95 % A, then 5 % ACN hold for 10 min. (He et al., 2020; Polumackanycz, Wesolowski, & Viapiana, 2021; Shreelakshmi et al., 2021).

2.4 Analysis method of HPLC/MS

The HPLC system fitted a LC-30AD binary pump (Shimadzu Corporation, Japan), an on-line vacuum degasser, an autosampler, and a column oven was used for determination the contents of compositions. A Poroshell 120 EC-C_{18} column (4.6 × 100 mm, 2.7 μm; Agilent, USA) was fitted with an EC-C_{18} pre-column (4.6 × 5 mm, 2.7 μm; Agilent), and the column temperature was stabilized to 26°C. The mobile phase consisted of ACN (A) and 0.1 % formic acid (B), with a flow rate of 0.4 mL/min. The injection volume was 5 μL. The gradient elution program was set as follows: 0 – 5 min, 5 % to 25 % A; 5 – 20 min, 25 % A to 25 % A; 20 – 34 min, 25 % to 95 % A, then 5 % ACN hold for 4 min. The HPLC system was equipped with an API 4000 tandem mass spectrometer (Applied Biosystems/MDS SCIEX, USA) and electro spray ionization (ESI) source. The quantification was performed using the multiple reactions monitoring (MRM) method. The ESI voltage of positive ions was set to 5500V, and the ESI voltage of negative ions was set to -4500V. Chlorogenic acid, cryptochlorogenic acid, rutin, hyperoside, isoquercitrin, astragalin, quercitrin, morusin, mulberroside A, and resveratrol were detected in the negative ion mode. Scopoletin and 1-DNJ were detected in the positive ion mode (D'Urso, Mes, Montoro, Hall, & de Vos, 2019; Hu et al., 2017; Ju et al., 2018; Kim, Doh, & Lee, 2020; Negro, Aprile, De Bellis, & Miceli, 2019). The mass spectrometry parameters were shown in Table 4. We performed methodological validation of the established HPLC/MS method, which showed good resolution (Supplemental Figure S1), linearity (Supplemental Table S1), accuracy and stability (Supplemental Table S2).

2.5 Evaluation of similarity between the mulberry branch and leaf extracts

Using the operating conditions of section 2.3, the similarity between the test solutions under the section 2.2 was evaluated. The Similarity Evaluation System for Chromatographic Fingerprint Profiles of Chinese Medicines (2012.130723 edition, Chinese Pharmacopoeia Commission) was used to evaluate the similarity of HPLC fingerprints.

2.6 Determination of 12 compositions in mulberry branch and leaf

The contents of compositions in the test solutions under the section 2.2 were determined by the operating conditions of section 2.4. Data acquisition and processing were performed using the Analyst 1.6.2 software (Applied Biosystems (AB Sciex)). All results were presented as mean ± standard deviation (SD).

2.7 Environmental and climate factors of different planting areas

Obtain the latitude and longitude, altitude (m), annual temperature difference (°C, the average value of the difference between the highest temperature and the lowest temperature in the past three years in the planting area), total annual precipitation (mm), rain concentrated (month), the temperature difference of one month before harvest (°C, the difference between the highest temperature and the lowest temperature in the month before harvest) and the precipitation of one month before harvest (mm) through the China Meteorological Data Sharing Service System (http://cdc.nmic.cn/home.do) (Table 2). The effects of environmental and climate factors in different producing areas on the compositions of mulberry branch were compared.

2.8 Cell culture and establishment of in vitro model

Rat brain microvascular endothelial cells were obtained from BNCC (RBMEC, Jiangsu, China), a cell model of hyperglycemia and hyperlipidemia was established with PA and high concentrations of glucose (Bai et al., 2021; Tyagi, Mirita, Shah, Reddy, & Pugazhenthi, 2021). RBMEC were cultivated with DMEM containing 10 % FBS, 100 U/mL streptomycin, and 100 U/mL penicillin cultured in an incubator at 37°C and 5 % CO_2. The medium was replaced every 2 days and the cells were allowed to adhere and grow in the culture, covering more than 80 % of the bottle. Sterile glucose powder was added to DMEM to prepare a high-sugar medium with a final concentration of 33 mmol/L. A hyperglycemia and hyperlipidemia medium containing 200 μmol/L PA was prepared in conjunction with the 33 mmol/L high-sugar medium. The cells were cultured in the hyperglycemia and hyperlipidemia medium at 37°C and 5 % CO_2 for 24 h to obtain an in vitro hyperglycemia and hyperlipidemia model.

2.9 Cell viability assay

The cell viability was measured using the CCK-8 test to determine the experimental concentration of mulberry branch extracts from different areas. The cells were inoculated in a 96-well plate, and the number of cells per well was 5×10^3. After culturing in DMEM containing 10 % FBS for 24 h at 37°C and 5 % CO_2, they were divided into blank control group, normal control group, and 12 extract groups, each group with 6 replicate wells. Then, 100 μL of serum-free DMEM was added to each well of the blank control group and normal control group, and 100 μL of DMEM containing different concentrations of extracts (Concentrations of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} mg/mL of mulberry branch extract were prepared with serum-free DMEM) was added to the extract groups incubation for 24 h. The original medium was aspirated followed by the addition of 100 μL serum-free medium and 10 μL of CCK-8 reagent to each well. After culturing for 2 h in the incubator, a microplate reader (Infinite M200 Pro, TECAN, Switzerland) was used to measure the absorbance at 450 nm. The cell viability of different concentrations of mulberry branch extracts was expressed as: (The absorbance of extract group-The absorbance of blank control group) ×100% / (The absorbance of normal control group-The absorbance of blank control group).

2.10 Apoptosis assay and determination of ROS level

Flow cytometry was performed on a NovoCyte 2040R (ACEA, USA). Apoptosis was detected via Annexin-V/PI double staining. The cells were inoculated in 6-well plates, and the number of cells per well was 1×10^6. After culturing in DMEM containing 10 % FBS for 24 h at 37°C and 5 % CO_2, they were divided into the normal control group, hyperglycemia and hyperlipidemia model group, and extract groups (prepared using DMEM without adding serum, and the extract concentration was 10^{-4} mg/mL), each with 6 replicate wells. The cells were cultured in the incubator for 24 h. Washed once with PBS, which was discarded. Subsequently, the cells in each group were digested with trypsin in the absence of EDTA followed by washing twice with PBS. Thereafter, 200 μL of buffer was added to the harvested cells. Next, 5 μL Annexin-V-FITC was added to the cells kept in the dark for 15 min at room temperature followed by the addition of 5 μL of PI staining solution and incubation for 5 min. Apoptosis was detected using flow cytometry. The Novo Express software version 1.2.1 (ACEA Biosciences Inc.) was used to generate scatter plots. The number of Annexin-V-FITC and PI positive cells was used to calculate the cellular apoptotic rate.

Reactive oxygen detection was performed using the fluorescent probe, DCFH-DA, which reacted with intracellular ROS, generating fluorescent products. Cell images were captured using aninverted fluorescence microscope (Olympus IX53+DP73, Japan). The cells were inoculated in a 12-well plate with round coverslips, and the number of cells per well was 1×10^5. After culturing in DMEM containing 10 % FBS for 24 h at 37°C and 5 % CO_2, the cells were washed once with PBS. The cells were divided into the normal control group, hyperglycemia and hyperlipidemia model group, and extract groups (prepared using DMEM without adding serum, and the extract concentration was10^{-4} mg/mL), each with 6 re-wells. The cells were then cultured in the incubator in the dark for 24 h, washed with serum-free medium, followed by incubation with 10 μM DCFH-DA in a cell incubator (maintained at 37°C) in the dark for 30 min. Subsequently, the cells were washed twice with PBS to fully remove the DCFH-DA from the extracellular medium. The fluorescence intensity of the cells was detected using a fluorescence microscope at an excitation wavelength (λ_{ex}) of 488 nm and an emission wavelength (λ_{em}) of 525 nm. One-way analysis of variance (ANOVA) in Prism

(GraphPad, San Diego, CA, USA) was used to analyze the differences between the groups. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered a significant difference.

2.11 Correlation analysis of environmental factors on compositions and efficacy

PLSR analysis was performed on environmental factors in "2.7" and compositions contents in "2.6" to reveal the effects of environmental factors on compositions and similarities. MFA was performed for the compositions contents of "2.6" and the bioactivities of "2.10". The calculation method of Anti-apoptotic and ROS-scavenging is as follows: Anti-apoptotic = (The mean value of apoptosis rate in the model group - The mean of the apoptosis rate in the extract group) / (The mean value of apoptosis rate in the model group - The mean of the apoptosis rate in the normal group); ROS can cause oxidative damage, therefore, anti-oxidation activity can be assessed by ROS-scavenging. ROS-scavenging = (The mean value of fluorescence intensity in the model group - The mean value of fluorescence intensity in the extract group) $\times 100\%$ / (The mean value of fluorescence intensity in the model group - The mean value of fluorescence intensity in the normal group).

3 Results

3.1 The influence of planting environment factors on the similarity and contents of compositions in mulberry branch

The Chinese medicine chromatographic fingerprint similarity evaluation system was used to obtain the similarity matrix of mulberry branch from 12 planting areas (Table 1 and Fig. 1). The geographical environment factors at different producing areas of mulberry branch were listed in Table 2 (Climate data obtained from public information on the internet and research reports). From the comparison of the numbers and retention times of chromatographic peaks, the same compositions in the mulberry leaf and the mulberry branch were mainly in the "1-9" peaks, including mulrosidide A, chlorogenic acid, cryptochlorogenic acid, scopoletin, rutin, isoquercitrin, hyperoside, astragalin, quercitrin and resveratrol, etc. The unique compound contained in the mulberry branch was the "10" peak, that was morusin. We used the established HPLC–MS method to analyze the contents of 12 typical compositions in mulberry branch and leaf (Table 3). Our results revealed that the contents of morusin, mulberroside A, and 1-DNJ in the mulberry branch from almost all producing areas were higher than mulberry leaf, while other compositions such as chlorogenic acid, cryptochlorogenic acid, rutin, hyperoside, isoquercitrin, astragalin, and quercitrin were lower than that in mulberry leaf. But there was no big difference in compound types from mulberry leaf. The above results indicated significant similarity between mulberry leaf and branches, with the exception of a few producing regions, such as Guangxi and Guangdong, which had less similarity.

3.2 Mulberry branch extract can reduce the apoptosis rate and inhibit oxidative damage of Rat brain microvascular endothelial cells (RBMEC) in hyperglycemia and hyperlipidemia environment

As shown in Fig. 2A, when the extract concentration in the medium was 10^{-7} to 10^{-4} mg/mL, there was no significant change on the cell viability of the RBMEC ($p > 0.05$). Therefore, we choose 10^{-4} mg/mL as experimental concentration in vitro. The RBMEC were double stained by Annexin-V-FITC/PI, and then subjected to flow cytometry to test the protective effects of the mulberry branch extracts against the damage induced by the hyperglycemia and hyperlipidemia media. The experimental results showed that the cellular apoptotic rate of the model group (34.43 %) was significantly higher than that of the normal control group (7.07 %) ($p < 0.01$). After treating of mulberry branch extracts, apoptotic rates were significantly decreased ($p < 0.01$ relative to model group) (Fig. 2B, C). Among them, the anti-apoptotic effects of S5, S7, S8, S12, and S13 group were significantly lower than that of mulberry leaves group ($p < 0.01$).

The intracellular reactive oxygen species (ROS) in response to hyperglycemia and hyperlipidemia environment was significantly increased (510 % vs. Normal control, $p < 0.01$), which eventually led to cell oxidative damage. Compared to the model, all mulberry branch extracts (10^{-4} mg/mL) had the capabilities to inhibit the intracellular production of ROS and reduce oxidative damage in the RBMEC with varying degrees after

exposure to hyperglycemia and hyperlipidemia media, and the effect was significantly stronger than that of the mulberry leaves group (Fig. 2D, E, $p < 0.01$). Among them, S3 and S8 had the most significant effects.

3.3 Correlation analysis of environmental factors,

content and bioactivities

PLSR was used to obtain the influence of altitude, annual temperature difference, total annual precipitation, the temperature difference of one month before harvest and the precipitation of one month before harvest on the contents and similarity of those 12 compositions in mulberry branch (Fig. 3A, B). The results indicated that the directions of the three environmental parameters of altitude, precipitation and temperature were basically symmetrical but distinguishable. Among the studied 12 compositions, chlorogenic acid, cryptochlorogenic acid, isoquercitrin, and resveratrol showed similar direction as altitude, suggesting a positive correlation. While, they had weakly negative correlation with temperature difference. Thus, the contents of these compositions were most significantly affected by altitude, which meant mulberry branch harvested from higher altitude tended to be rich of these constituents. However, big temperature difference preferred to reduce the amount of these compositions. For example, the altitudes of S3 and S9 were almost the same, but because the temperature difference of S9 was significantly lower than that of S3, the content of chlorogenic acid, cryptochlorogenic acid, isoquercitrin, and resveratrol in S9 was higher than that in S3. Besides, the directions of these four constituents were almost perpendicular to the precipitation, indicating negligible effect of precipitation on the contents of these compositions. By applying the same analysis method, rutin, hyperoside, and morusin were found to have strong negative correlation with temperature difference and weak positive correlation with altitude and precipitation, revealing that temperature difference had greater impact, and the contents of these three compositions were higher in areas with small temperature difference. For instance, S10, S11 and S13 had large diverse in altitude, but small temperature difference, so the contents of rutin, hyperoside, and morusin were all larger than other places. The precipitation was negatively correlated with mulberroside A and 1-DNJ, but had a strong positive correlation with astragalin, quercitrin, and scopoletin. The precipitation of one month before harvest had an average effect on astragalin and quercitrin, while, astragalin was most significantly affected by total annual precipitation E.g., the annual precipitation in S2 and S4 was similar, but because of the more precipitation in the month before harvested in S4, the content of astragalin and quercitrin in S4 were higher than S2. In general, the precipitation was negatively correlated with the similarity of mulberry branch in different planting areas, which meant it was a very important influencing factor, while, the altitude and temperature difference were almost vertical to the similarity, suggesting little effect of these factors on the similarity. Therefore, we infer that precipitation may be an important climatic factor affecting the similarity of composition in the mulberry branch.

MFA were conducted to analyze the correlation between the amounts of 12 compositions and variations of two bioactivities of mulberry branch extracts from different planting areas (Fig. 3C). The results showed that the 12 compositions contributed to the anti-apoptotic and antioxidant activities of mulberry branch to varying degrees. Among them, mulberroside A, chlorogenic acid, cryptochlorogenic acid, astragalin, resveratrol, isoquercitrin and 1-DNJ had similar contributions to these two bioactivities of mulberry branch. The sum of the contributions of these 7 compositions accounted for more than half of the total efficacy. Their directions were all in the first quadrant, which was basically perpendicular to the direction of anti-apoptosis and anti-oxidative effect, indicating these compositions were in general not related to the effect variation among mulberry branch extracts from different planting areas. In other words, these compositions altogether produced the main bioactivities, but had little effect on the magnitude of bioactive differences of mulberry branch extracts from different planting areas. For example, the contents of mulberroside A, chlorogenic acid, cryptochlorogenic acid, astragalin, resveratrol, 1-DNJ in S10 and S11 were very distinctive, but their anti-apoptosis and anti-oxidative effects were no significant difference.

4 Discussion

The mulberry tree has low survival requirements. If sufficient light is available, it is highly adaptable to temperature differences, and soil pH, which allows successful planting in different geographical regions. Studies

have shown that the secondary metabolism of mulberry trees can undergo huge variations (Sánchez-Salcedo et al., 2016; Yang et al., 2017). These secondary metabolites are the main compositions of mulberry trees that produce bioactivities. Environmental changes may be an important cause in the difference of compositions. Therefore, it is necessary to study the changes of compositions of mulberry branch in combination with environmental factors.

According to literature reports (Metwally et al., 2020), chlorogenic acid showed strong antioxidant activity at 200 mg/kg. Mulberroside A in the water extract of Cortex Mori at 0.58 g/mL also had good antioxidant activity (Xu et al., 2021). The literature revealed that a basic diet containing 500 mg/kg resveratrol could reverse the oxidative damage of hepatocytes (Liu et al., 2021), and 5 mg/L 1-DNJ could enhance the antioxidant activity of tilapia splenocytes (Tang et al., 2017). Similar activities were also found in cryptochlorogenic acid and isoquercitrin (Zhou, 2020). Rutin could alleviate high glucose-induced H9C2 cell damage by inhibiting apoptosis and endoplasmic reticulum stress (Wang, Wang, Li, & Yao, 2021). Hyperoside significantly inhibited apoptosis induced by high glucose in a dose-dependent manner (W. Wu et al., 2020). When the concentration of quercitrin reached 160 μg/mL, it could significantly increase the survival rate of human embryonic kidney 293 T cells (Yanyan Li et al., 2020). Scopoletin prevented palmitic acid and bile acid-induced hepatocyte death by inhibiting endoplasmic reticulum stress and ROS generation, and reducing the phosphorylation of the cell death signaling intermediate JNK (Z. Wu, Geng, Buist-Homan, & Moshage, 2022). These research results showed that various compositions in the mulberry branch had clear anti-apoptotic and antioxidant effects, which were the basis for the bioactivities of mulberry branch. Mulberroside A, chlorogenic acid, cryptochlorogenic acid, astragalin, resveratrol, and 1-DNJ in mulberry branch could act together in a mixed form in the extract through various pathways, resulting in stable anti-apoptotic and antioxidant relatively. Therefore, we believed that these compositions constituted the basic anti-apoptotic and antioxidant effects of mulberry branch, and were the fundamental of the bioactivities function of mulberry branch, but were not the elements that caused the differences in the bioactivities effects of mulberry branch from different planting areas.

The analysis results showed that the anti-apoptotic and anti-oxidative effects of mulberry branch were two vectors with opposite directions. Some compositions of mulberry branch had the same or similar directions as anti-apoptotic or antioxidant effects, indicating these compositions were major contributors to the variations of anti-apoptotic or antioxidant activities observed among mulberry branch extracts from different planting areas. Rutin, hyperoside, and morusin showed the same direction as the anti-apoptotic effect but opposite to anti-oxidative path, which meant they were positively correlated to the variations in anti-apoptosis activity among mulberry branch but negatively correlated with the change of anti-oxidative capability. Thus, we might infer that in mulberry branch extract, these three compositions were beneficial to exert anti-apoptosis function of mulberry branch, but unfavorable for anti-oxidant activity of mulberry branch. Compared to the above three compositions, scopoletin and quercitrin showed an almost fully contrary influence on mulberry branch toward anti-apoptotic and anti-oxidation activities. Due to this relative action trend, the ratio of these 5 compositions probably became the key factor for the differences in mulberry branch function from different planting areas, perhaps more important than the contents of these compositions. Environmental factors can have a direct impact on the ratio between these key compositions. According to the analysis in Fig. 3B, the annual temperature difference and the temperature difference of one month before harvest had greater impact on the contents of rutin, hyperoside, and morusin. In areas where these two temperature differences were both small, the amounts of these three compositions were relatively high, e.g., S13. The total annual precipitation and precipitation of one month before harvest had greater impact on the contents of quercitrin and scopoletin, thus they were relatively richer in areas with higher total annual precipitation and precipitation of one month before harvest. For example, S8 had more precipitation than S6, so the contents of quercitrin and scopoletin in S8 were higher, which in turn affected the bio-activity of mulberry branch in these two areas. Extracts of S8 had certain advantage in anti-oxidation, while S6 showed better effect in anti-apoptosis. It can be seen that environmental factors had a significant impact on the content and ratio of compositions in mulberry branch, which lead to the bioactivities bias in different planting areas. Thus, clarifying the relationship between environmental factors and compositions has special guiding significance

for the utilization of bioactivities resources of mulberry branch after harvesting.

We studied the correlation between environmental factors (altitude, temperature difference and precipitation) in the planting areas and the bioactivities of mulberry branch. Specifically, we found that mulberry branch had similar compositions and bioactivities as mulberry leaf. Comprehensive analysis suggested that the main compositions of mulberry branch in different planting areas were similar, but the bioactivities were biased. Among the 12 compositions tested in this study, seven compositions (chlorogenic acid, cryptochlorogenic acid, isoquercitrin, astragalin, mulberroside A, resveratrol, and 1-DNJ) together probably determined the basal level of medicinal activity of mulberry branch, which also was the fundamental for mulberry branch to be used as bioactivities resources of functional food. Mulberry branch from different planting areas exhibited differences in anti-apoptosis and anti-oxidative damage capacities. These differences were mainly caused by five key compositions (rutin, hyperoside, quercitrin, morusin, scopoletin). The climatic factors, especially precipitation and temperature difference in the planting areas significant affected the contents and ratios of these compositions. The relationship between planting environment, compositions, and bioactivities of mulberry branch was studied by PLSR and MFA. The results showed that planting environments with smaller temperature difference would make the anti-apoptotic effect of mulberry branch more prominent, and adequate irrigation would promote anti-oxidative effect of mulberry branch. These results not only indicate that mulberry branch have potential use as new resource of functional foods, but also guide to the rational utilization of mulberry branch into different medical needs, thus reducing waste of resources. The mathematical analysis method based on this study provides data support for the selection of planting conditions and bioactivities utilization of mulberry.

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DATA AVAILABILITY STATEMENT

All data associated with this study are available in the supplementary materials. **References**

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Table 1 Similarity of mulberry branch from different planting areas

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
S1	1	0.185	0.154	0.153	0.262	0.111	0.094	0.275	0.267	0.153	0.245	0.338	0.367
S2	0.185	1	0.922	0.904	0.672	0.897	0.87	0.864	0.92	0.958	0.955	0.529	0.269
S3	0.154	0.922	1	0.98	0.614	0.987	0.94	0.843	0.856	0.966	0.835	0.444	0.174
S4	0.153	0.904	0.98	1	0.608	0.976	0.938	0.848	0.873	0.951	0.8	0.468	0.168
S5	0.262	0.672	0.614	0.608	1	0.519	0.556	0.834	0.794	0.632	0.768	0.789	0.395
S6	0.111	0.897	0.987	0.976	0.519	1	0.949	0.771	0.806	0.95	0.773	0.329	0.109
S7	0.094	0.87	0.94	0.938	0.556	0.949	1	0.727	0.781	0.907	0.743	0.289	0.205
S8	0.275	0.864	0.843	0.848	0.834	0.771	0.727	1	0.954	0.854	0.902	0.841	0.384
S9	0.267	0.92	0.856	0.873	0.794	0.806	0.781	0.954	1	0.868	0.922	0.756	0.355
S10	0.153	0.958	0.966	0.951	0.632	0.95	0.907	0.854	0.868	1	0.892	0.471	0.207
S11	0.245	0.955	0.835	0.8	0.768	0.773	0.743	0.902	0.922	0.892	1	0.682	0.368
S12	0.338	0.529	0.444	0.468	0.789	0.329	0.289	0.841	0.756	0.471	0.682	1	0.481
S13	0.367	0.269	0.174	0.168	0.395	0.109	0.205	0.384	0.355	0.207	0.368	0.481	1
Reference fingerprint	0.38	0.93	0.922	0.921	0.764	0.875	0.855	0.945	0.95	0.924	0.915	0.687	0.449

Table 2 Environmental factors of mulberry branch from different planting areas

Areas	Latitude and longitude	Altitude (m)	Annual temperature difference (°c)	Total annual precipitation (mm)	Rain concentrated (month)	One month before harvest	One month before harvest
						Temperature difference (°C)	Precipitation (mm)
S2	N43° 25'–53deg33', E121deg11'–135deg05'	171.7	42	554	6–8	28	72
S3	N38° 43'–43deg 26', E118deg53'–125deg46'	41.6	37	662	6–8	25	76
S4	N36° 01'–42deg 37', E113deg04'–119deg53'	80.5	30	540	7–9	31	27
S5	N31° 42'–39deg 35', E105deg29'–111deg15'	396.9	28	565	7–9	29	18

Areas	Latitude and longitude	Altitude (m)	Annual temperature difference (°c)	Total annual precipitation (mm)	Rain concentrated (month)	One month before harvest	One month before harvest
S6	N31° 23'–36deg 22', E110deg21'–116deg39'	110.4	28	617	7–9	31	12
S7	N30° 45'–35deg 20', E116deg18'–121deg57'	8.9	26	1275	6–8	25	91
S8	N29° 41'–34deg 38', E114deg54'–119deg37'	29.8	26	1148	6–8	28	93
S9	N27° 02'–31deg 11', E118deg01'–123deg10'	41.7	25	1639	6–8	28	140
S10	N26° 03'–34deg 19', E97deg 21'–108deg 31'	505.9	20	980	7–9	20	21
S11	N21° 08'–29deg 15', E97deg 31'–106deg 11'	1891.4	12	933	6–9	20	25
S12	N20° 54'–26deg 23', E104deg29'–112deg04'	72.7	16	1357	6–8	21	24
S13	N20° 09'–25deg 31', E109deg45'–117deg20'	6.6	15	2084	5–7	28	130

Table 3 Contents of 12 compositions in extracts (n=3)

Serial number	Origin and batch number	Contents (μg/g) Chlorogenic acid	Contents (μg/g) Cryptochlorogenic acid	Contents (μg/g) Rutin	Contents (μg/g) Hyperoside	Contents (μg/g) Isoquercitrin	Contents (μg/g) Astragalin	Contents (μg/g) Quercitrin	Contents (μg/g) Morusin	Contents (μg/g) Mulberroside a	Contents (μg/g) Resveratrol	C
S1	Mulberry leaf (20180401)	432.68±29	82.97±6.6	47.5±2.53	59.43±0.67	66.27±4.72	63.5±5.39	0.27±0.02	—	11.71±0.38	0.88±0.06	1
S2	Hei long jiang (20180502)	152.59±6.91	12.48±0.35	3.22±0.12	5.11±0.04	5.85±0.45	0.41±0.01	0.01±0	251.04±13.04	—	0.49±0.03	2
S3	Liao ning (20180520)	65.63±4.31	8.97±0.57	1.57±0.06	1.91±0.12	2.68±0.05	0.17±0.01	0.01±0	248.44±16.07	—	0.64±0.03	8
S4	He bei (20180530)	335.84±134	41.28±0.48	2.04±0.12	4.51±0.27	9.45±0.32	1.6±0.13	0.01±0	298.97±212	271.32±15	7.67±0.4	8
S5	Shan xi (20180408)	14±0.68	2.12±0.11	0.43±0.01	0.64±0.04	0.95±0.03	0.23±0.02	0.01±0	27.79±1.23	73.44±3.59	1.29±0.07	9
S6	He nan (20180416)	87.35±3.85	11.37±0.87	1.82±0.07	1.91±0.07	2.32±0.14	0.14±0	0.01±0	277.79±14	362.6±28.1	0.84±0.03	8
S7	Jiang su (20180415)	179.59±9.58	17.01±0.97	1.03±0.04	0.88±0.01	4.58±0.18	0.61±0.01	0.04±0	145.53±10	167.57±66	6.85±0.1	2
S8	An hui (20180417)	59.38±3.6	7.24±0.33	1.4±0.09	1.47±0.11	2.18±0.13	0.39±0.01	0.04±0	273.71±15	241.76±3.7	1.485±0.08	1
S9	Zhe jiang (20180310)	264.47±1.7	30.99±2.24	2.42±0.21	1.83±0.06	3.77±0.2	0.46±0.02	0.02±0	212.03±11	282.98±9.2	1.491±0.13	2
S10	Si chuan (20180408)	152.97±3.62	22.28±0.95	1.26±0.1	2.07±0.14	3.67±0.3	0.47±0.02	0.01±0	357.67±18	378.63±15	1.029±0.12	1
S11	Yun nan (20180413)	389.08±256	63.97±5.26	2.83±0.14	4.38±0.38	8.13±0.35	0.66±0.03	0.01±0	276.68±6.64	197.21±62	1.36±0.36	1
S12	Guang xi (20180401)	25.69±1.29	4.64±0.23	0.84±0.05	2.76±0.23	3.39±0.09	1.04±0.04	0.03±0.01	260.7±7.72	—	1.83±0.09	1
S13	Guang dong (20180420)	149.11±8.12	15.29±0.96	3.65±0.3	6.29±0.01	6.44±0.33	1.2±0.03	0.01±0	308.63±20	194.88±10	1.681±0.09	1

Table 4 MS parameters and retention times (RTs) of compositions including declustering potential (DP) and collision energy (CE)

Compositions	Precursor ion (m/z) -product ion (m/z)	DP(V)	CE(V)	RT(min)
Chlorogenic acid	353.0-191.0	-61.0	-31.0	7.11
Cryptochlorogenic acid	352.9-172.7	-60.0	-23.0	7.30

Compositions	Precursor ion (m/z) -product ion (m/z)	DP(V)	CE(V)	RT(min)
Rutin	609.0-300.8	-141.0	-46.0	8.78
Hyperoside	463.0-300.0	-119.1	-38.9	9.29
Isoquercitrin	463.0-300.8	-101.0	-29.0	9.36
Astragalin	447.0-284.0	-102.0	-38.9	10.51
Quercitrin	447.1-300.0	-103.1	-36.1	10.76
Morusin	419.0-297.0	-138.6	-32.8	33.33
Mulberroside A	567.0-243.0	-118.1	-36.1	6.41
Resveratrol	227.0-185.0	-86.5	-26.2	17.19
Scopoletin	193.0-133.0	88.0	27.0	10.61
1-DNJ	164.0-146.2	82.8	16.7	2.24

Figure legends

Fig. 1 The fingerprints of mulberry branch extracts from 12 planting areas. S1. Mulberry leaf; S2. Heilongjiang; S3. Liaoning; S4. Hebei; S5. Shanxi; S6. Henan; S7. Jiangsu; S8. Anhui; S9. Zhejiang; S10. Sichuan; S11. Yunnan; S12. Guangxi; S13. Guangdong; S14. Mixed standard. 1. mulberroside A; 2. chlorogenic acid; 3. cryptochlorogenicacid; 4. scopoletin; 5. rutin; 6. isoquercitrin and hyperoside; 7. astragalin; 8. quercitrin; 9. resveratrol; 10. morusin.

Fig. 2 Mulberry branch can reduce the damage of rat brain microvascular endothelial cells (RBMEC) in hyperglycemia and hyperlipidemia. A. CCK-8 assay to determine the effect of different concentrations mulberry branch extracts on the viability of cells; B. Annexin-V/PI double staining to detect the apoptosis of RBMEC after 24 h of treatment with different mulberry branch extracts; C. Quantitative analysis of the apoptosis rates in different mulberry branch extracts groups; D. Quantitative analysis of cell fluorescence intensity of different mulberry branch extracts treated for 24 h; E. Fluorescence probe DCFH-DA was used to detect the level of ROS generated due to hyperglycemia and hyperlipidemia in vitro model after treatment with different branch extracts for 24 h.

Fig. 3 The correlation between the environmental factors of the planting areas, the compositions of the mulberry branch extract and the bioactivities variations were analyzed by the partial least squares regression (PLSR) and the "FactoMineR" software. A. The precipitation of one month before harvest and the similarity of mulberry branch from different planting areas. Similarity: red $>$ 0.9 (S2, S3, S4, S8, S9, S10, S11); 0.8 $<$ orange $<$ 0.9 (S6, S7); 0.7 $<$ yellow $<$ 0.8 (S5); 0.6 $<$ green $<$ 0.7 (S12); blue $<$ 0.6 (S13); B. The effects of environmental factors on the contents of compositions and similarity of mulberry branch analyzed by PLSR: blue (environmental factors), red (compositions). The vector length of each composition represents the amount of change in the content. The greater the content change, the longer the vector length; the angle between the vector direction of the composition and the environmental factor represents the strength of the correlation: the more similar, the stronger the correlation is, and opposite direction represents negative correlation; C. Multiple factor analysis (MFA) based on ROS and apoptosis results in line with compositions in mulberry branch extracts: The vector length and color of each composition in the mulberry branch extracts represent the contribution of the composition to the overall efficacy. Color (contribution degree, the larger the value, the greater the contribution is); the angle between the vector direction of composition contribution (solid line arrow) and variation in specific activity (dotted line arrow) represents the strength of the correlation: smaller angle indicates the stronger.

Fig. 1

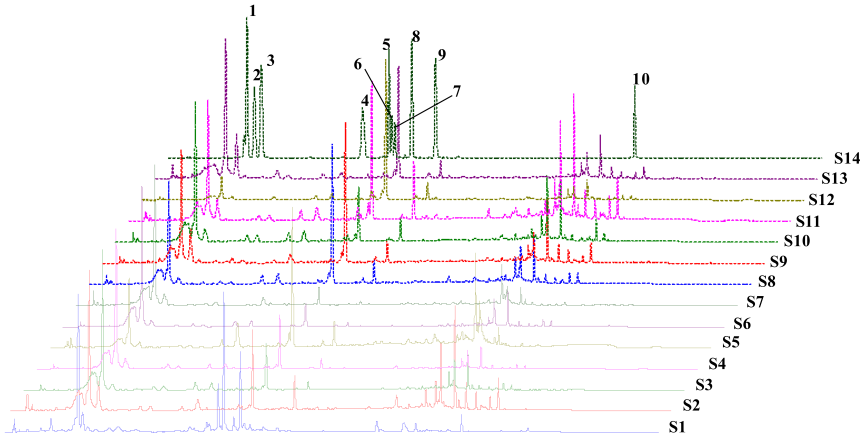

Fig. 2

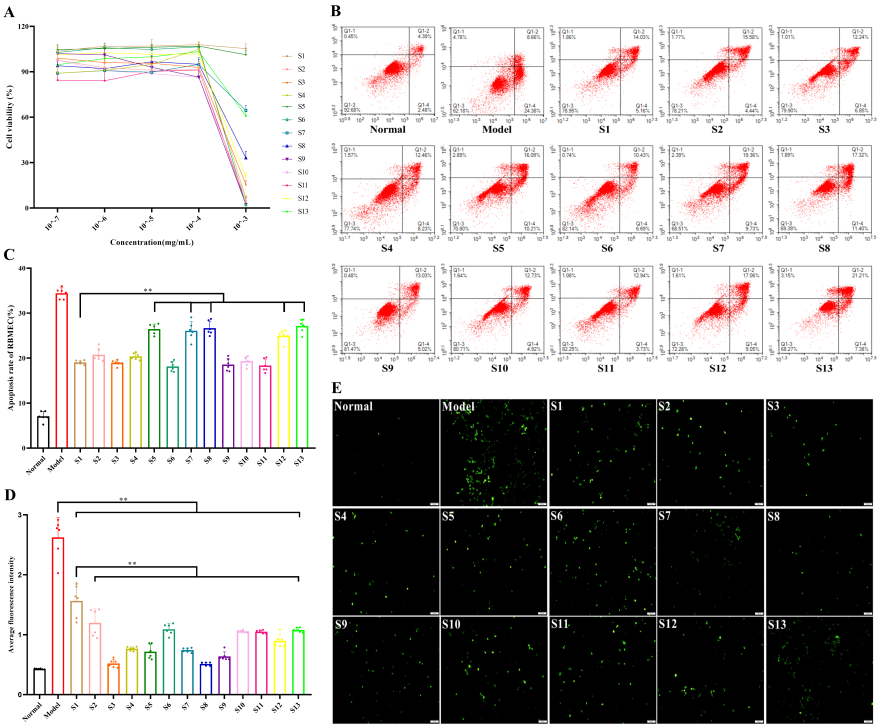

Fig. 3

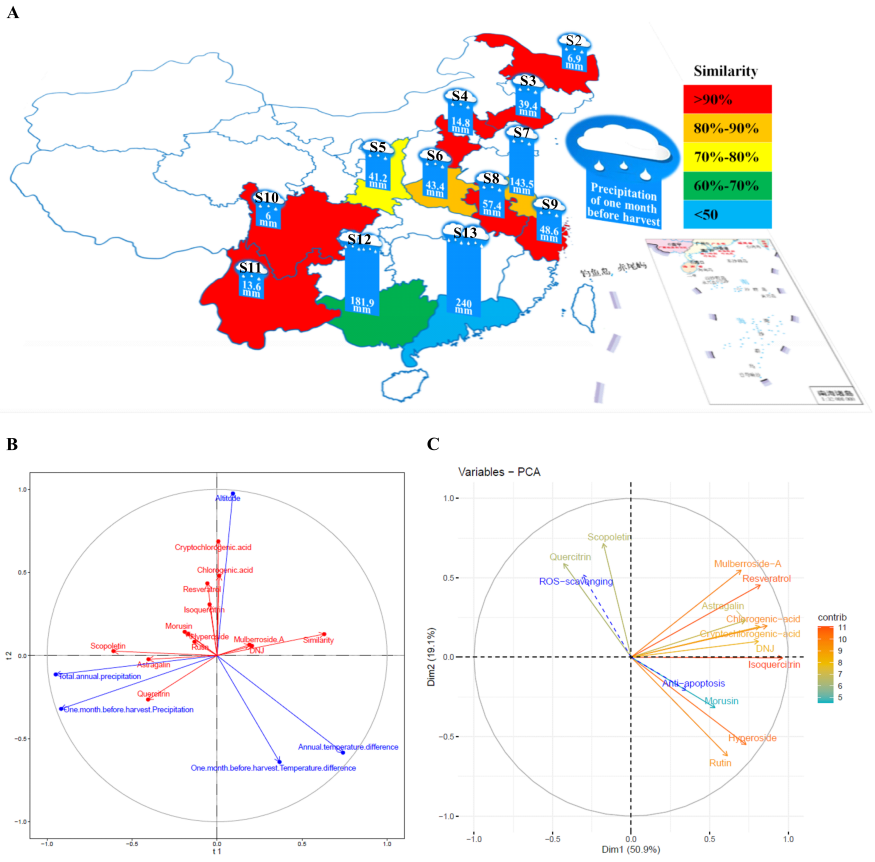

Fig. 1

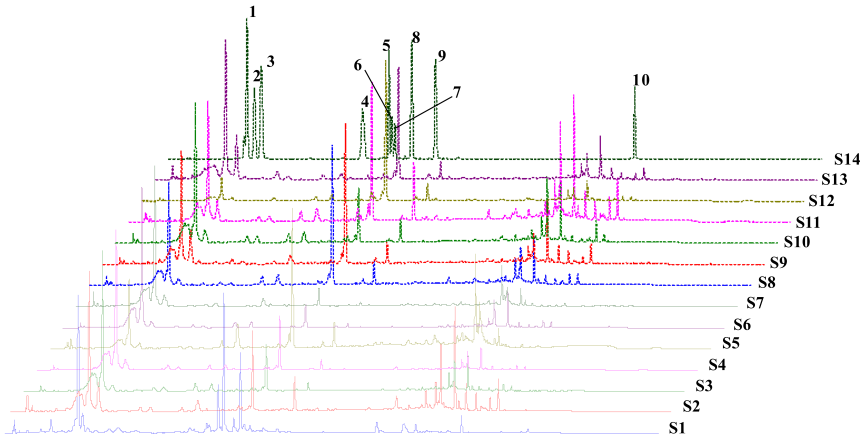

Fig. 2

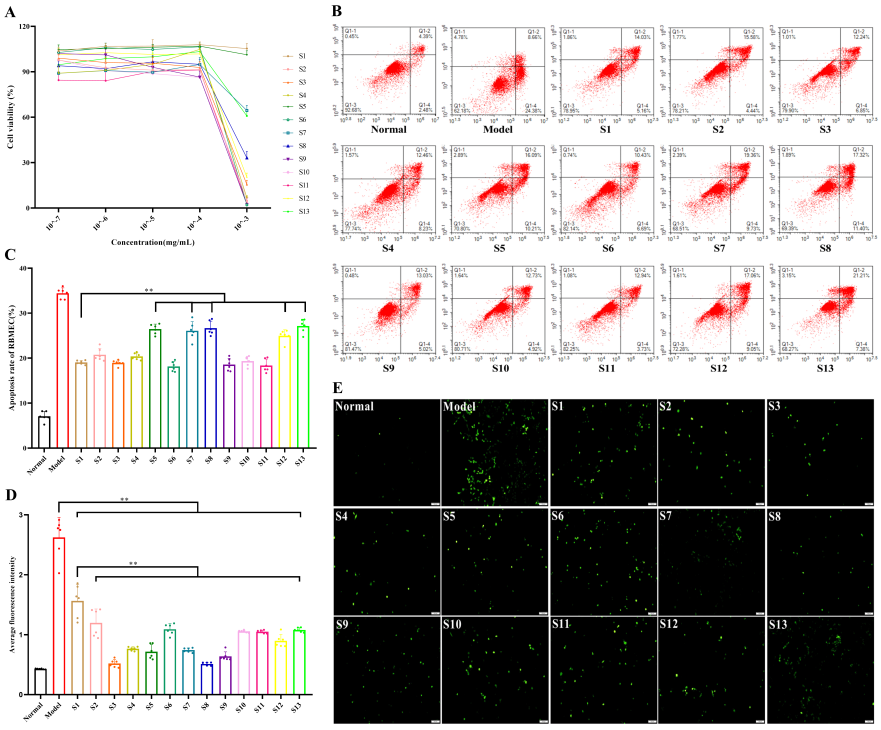

Fig. 3