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Response surface optimization reveals monthly total flavonoid peaks in Ginkgo biloba leaves with corresponding DPPH scavenging activity

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The study investigated the influence of ethanol concentration, reflux time, and reflux temperature on the content of total flavonoids (TFs) in Ginkgo biloba (GB) leaves. The optimal extraction conditions were determined using the response surface (RS) methodology. The fluctuation of flavonoid content in ethanol extracts of GB leaves across different months was compared with these optimized extraction parameters. Additionally, we investigated the antioxidant activity against DPPH free radicals for the GB extract in the month with the highest flavonoid content. Following RS optimization, the optimal extraction conditions for GB flavonoids were identified as follows: extraction rate of 1.175% at 61 °C for 30 min using 77% ethanol. Under these optimized conditions, the total flavonoid content of GB exhibited a bi-peak pattern, peaking in May and August. The extract obtained in May showed superior DPPH free radical-scavenging ability. These findings provide valuable insights for selecting raw materials for future preparations of GB products.

Keywords Monthly peak, Ginkgo biloba leaves, Total flavonoids, Response surface optimization, DPPH free radicals

Ginkgo biloba (GB), a pharmacologically significant medicinal species, has gained global recognition for its leaves containing rich flavonoid compounds with multifaceted bioactivities[1,2]. Contemporary pharmacological studies have revealed that GB flavonoids exhibit a broad spectrum of therapeutic potentials, encompassing antiarrhythmic effects, hematological regulation through blood viscosity reduction and lipid-lowering properties, antitumor activity via inhibition of neoplastic cell proliferation, antiviral efficacy, and hepatoprotective functions[3–5]. Notably, these bioactive constituents demonstrate potent antioxidant capacity through three distinct mechanisms: direct free radical scavenging, metal ion chelation, and preservation of endogenous antioxidant systems.

The remarkable pharmacological profile has driven the global commercialization of GB-derived products, with over 130 countries developing various pharmaceutical formulations. In the Chinese market, GB preparations are available in multiple dosage forms including tablets, capsules, dropping pills, and oral solutions, with capsules and tablets being included in the 2018 National Essential Drug List[2,6]. Nevertheless, a critical quality control issue persists in current GB products: the absence of standardized harvesting time specifications. This oversight is particularly concerning given accumulating evidence of seasonal flavonoid fluctuations. Wang et al. demonstrated that biflavonoid content reaches maximal stability during spring harvests[7], while Chen's team documented significant seasonal variations in total flavonoid content[8]. These findings collectively highlight substantial temporal variations in phytochemical composition throughout the annual growth cycle.

In this experiment, natural drying treatment was adopted for GB leaves to prevent damage to flavonoids caused by high temperatures and light exposure[9,10]. The study optimized the extraction conditions for TFs from GB leaves using ethanol as the solvent, based on both single-factor experiments and the response surface methodology[11]. Furthermore, the study investigated the variation in total flavonoid content in GB leaves across different months. The antioxidant effects of the total flavonoid extracts on DPPH free radicals were also

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evaluated. The aim of this research was to improve the efficient extraction of TFs from GB and promote the safe use of GB preparations[12]. Establishing scientifically validated harvesting protocols requires comprehensive characterization of flavonoid dynamics across different phenological stages. Such temporal profiling is essential for optimizing the therapeutic efficacy, ensuring product safety, and maintaining batch-to-batch consistency in GB-based pharmaceuticals.

Materials and methods

Instruments and chemicals

The equipment used in this study included the OHAUS CP214 electronic balance (Ohaus Instrument Co., Ltd., China), the 752 Pro UV Visible Spectrophotometer (Shanghai Light Technology Co., Ltd., China), the KW series constant temperature water bath (Bangxi Instrument Technology Co., Ltd., China), and the 800Y high-speed multi-functional grinder (Wuyi Haina Electric Co., Ltd., China). GB leaves was obtained from the School of Agriculture, Forestry and Ecology of Shaoyang University. They were harvested on the first day of different months (April to October) and naturally dried. They were identified as dried leaves of Ginkgo biloba L., a plant in the Ginkgo family, by Associate Professor Yang Lin of the School of Pharmacy of Shaoyang University. The samples were stored in the Medicinal Materials Laboratory of the School of Pharmacy of Shaoyang University, and the number is SHY-2022-0410. Rutin standard product and vitamin C (Vc) standard product were obtained from Hefei Bomei Biotechnology Co., Ltd., China. Chemical reagents used included aluminum nitrate (Tianjin Kemiou Chemical Reagent Co., Ltd., China), sodium hydroxide (Hunan Huihong Reagent Co., Ltd., China), sodium nitrite (Sinopharm Group Chemical Reagent Co., Ltd., China), DPPH (Shanghai Yuanye Biotechnology Co., Ltd., China), and 95% ethanol (Tianjin Huagong Technology Co., Ltd., China).

Standard curve

The rutin standard (5.30 mg) was accurately weighed and dissolved in 60% ethanol. The volume was adjusted to 100 mL using a volumetric flask. Subsequently, aliquots of 0.00, 1.00, 2.00, 3.00, 4.00, and 5.00 mL of the standard solution were transferred into six separate 25 mL volumetric flasks. To each flask, 1.0 mL of 5% $NaNO_2$ solution was added and allowed to react for 6 min. Next, 1.0 mL of 10% $Al(NO_3)_3$ solution was added and left to stand for 5 min. Then, 10 mL of 4% NaOH solution was added to each flask. Finally, the volume was adjusted to the mark with 60% ethanol, thoroughly mixed, and allowed to stand for 15 min. The absorbance of each solution was measured at 510 nm wavelength. The concentration of the rutin standard solution was plotted on the x-axis, and the absorbance values were plotted on the y-axis to construct the standard curve for rutin[13].

Single-factor experiment[14]

Drying and storage conditions

Drying Conditions: The freshly harvested GB leaves were spread in a single layer on mesh trays and dried in a shaded, well-ventilated area (avoiding direct sunlight) at an ambient temperature of 25 ± 2 °C and relative humidity of 60 ± 5% for 7–10 days. The moisture content of the dried leaves was determined to be ≤ 8% using a moisture analyzer (MA35, Sartorius, Germany). This method prevents thermal degradation of flavonoids while ensuring uniform drying, as high temperatures and direct sunlight can compromise their stability.

Storage Conditions: The dried GB samples were stored in airtight, light-protected containers at 4 °C in the Medicinal Materials Laboratory to maintain their chemical integrity until analysis. This ensured minimal degradation of flavonoids during storage.

Selection of ethanol concentration

The mixed powder of 1.0 g ginkgo biloba leaves of each month was accurately weighed and extracted for 51 min using 40%, 50%, 60%, 70%, and 80% ethanol solutions in a constant temperature water bath set at 75 °C, maintaining a solid-liquid ratio (SLR) of 1:20. After extraction, the solutions were filtered through a 0.45 μm microporous membrane (Millipore, USA) to eliminate insoluble particles without adsorbing flavonoids, ensuring accurate spectrophotometric analys, and the absorbance was subsequently determined following the procedure outlined in step 2.2.

Selection of SLR

The mixed powder of 1.0 g ginkgo biloba leaves of each month was accurately weighed, and 80% ethanol was chosen as the solvent. The SLRs tested were 1:15, 1:20, 1:25, 1:30, and 1:35. Extraction was carried out in a water bath at 75 °C for 51 min. After extraction, the solutions were filtered through a 0.45 μm microporous membrane (Millipore, USA) to eliminate insoluble particles without adsorbing flavonoids, ensuring accurate spectrophotometric analysis[15], and the absorbance was subsequently determined following the procedure outlined in step 2.2.

Selection of reflux temperature

GB leaves (1.0 gram) from different months were individually weighed, with 70% ethanol selected as the solvent. The mixture was subjected to extraction at temperatures of 40, 50, 60, 70, and 80 °C, corresponding to a SLR of 1:25. The extraction process occurred in a water bath for 50 min. Subsequently, the extraction solution was filtered through a 0.45 μm microporous membrane (Millipore, USA) to eliminate insoluble particles without adsorbing flavonoids, ensuring accurate spectrophotometric analysis[15], and the absorbance was subsequently determined following the procedure outlined in step 2.2.

Selection of reflux time
The mixed powder of 1.0 g ginkgo biloba leaves of each month was accurately weighed, and a solvent of 70% ethanol was chosen for extraction. The extraction process was conducted in a water bath at 75 °C for durations of 20, 30, 40, 50, and 60 min, maintaining a SLR of 1:25. Following extraction, the solution was filtered through a 0.45 μm microporous membrane (Millipore, USA)[15]. Subsequently, absorbance measurements were taken according to Step 2.2 of the experimental protocol.

Optimization of the reflux process
Based on the single-factor results, ethanol concentration (A) (60, 70, 80 /%), reflux time (B) (30, 40, 50 /min), and reflux temperature (C) (50, 60, 70 /℃) were chosen as variables[16], with the extraction rate (ER) of TFs as the response value. The response surface (RS) optimization was conducted using a central composite design, following the principles of RS methodology, to optimize the extraction process of flavonoids from GB leaves. A response regression model was subsequently established.

Comparison of the contents of TFs in GB leaves in different months
GB leaves (1.0 gram) from different months were individually weighed, and the reflux process was carried out according to the optimization process of RS. Under the above conditions, the absorbance of the GB extracts from different months was determined and their contents were calculated accordingly.

The formula for calculating the ER of TFs was as follows: The ER of TFs = concentration × ratio of material to liquid × dilution multiple / quality of GB leaves.

DPPH assay by extractive solution
The DPPH scavenging assay was performed as described by Al-Rimawi F. et al. with minor modifications[17]. Under the optimized conditions of RS methodology, we sequentially add 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of ethanol to the solution without DPPH (0.02 mmol/L). The solution was allowed to stand in a dark environment for 30 min, then the absorbance was determined as A_1. Next, 3 mL of ethanol was taken and DPPH (0.02 mmol/L) was added. The absorbance was determined as A_0. Subsequently, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of DPPH (0.02 mmol/L) were added to the GB extract in turn, and the absorbance was determined as A_2 after standing in a dark environment for 30 min. The rate of DPPH scavenging is calculated as follows:

$$\text{The rate of DPPH scavenging } (\%) = \left(1 - \left(A_1 - A_2\right)/A_0\right) \times 100\%$$

Data analysis
All statistical analyses and calculations were performed using SPSS 22.0 (IBM, Chicago, IL, US), and graphing was generated using Origin 8.5 (OriginLab Corporation, USA). Response surface methodology was used to optimize the process parameters by Designexpert 10.0 (Stat-Ease Inc., Minneapolis, USA). Results of the three-factor, three-level RS tests were analyzed based on the principles of Box-Behnken central composite experimental design.

Results and discussion
Rutin standard curve
Figure 1 shows a good linear relationship rutin concentration (C) and absorbance (A) in Fig. 1 within the range of 0.005–0.025 mg/mL (A = 13.839 C-0.028, $R^2 = 0.9993$). The coefficient of determination ($R^2 = 0.9993$) indicates that 99.93% of the variability in absorbance is explained by the rutin concentration, confirming the curve's reliability for quantification.

Single-factor investigation
Effect of ethanol concentration on the ER of TFs from GB leaves
As shown in Fig. 2A, as the ethanol concentration increased, the ER of TFs increased. However, the ER reached a plateau when the ethanol concentration exceeded 70%, indicating that 70% ethanol might be optimal for extraction. The plateau in extraction rate (ER) beyond 70% ethanol aligns with previous studies[18], suggesting that higher ethanol concentrations may reduce flavonoid solubility due to decreased polarity.

Effect of reflux time on the ER of TFs from GB leaves
As shown in Fig. 2B, as the extraction time increased, the ER of TFs increased. However, the ER decreased when the extraction time was longer than 40 min. The peak extraction rate occurred at 40 min, suggesting it to be the optimal reflux time for extracting flavonoids from GB leaves. The decline in ER after 40 min could be attributed to thermal degradation of flavonoids, as reported by Dai et al.[19]. This underscores the need for optimized extraction durations.

Effect of reflux temperature on the ER of TFs from GB leaves
As shown in Fig. 2C, the extraction of TFs initially increased with higher reflux extraction temperatures. However, beyond a certain point, further increases in temperature led to a decline in the extraction rate. The optimal reflux temperature, where the ER peaked, was determined to be 60 °C.

Effect of SLR on the ER of TFs from GB leaves
As depicted in Fig. 2D, the ER of TFs increased as the SLR increased, reaching its peak at a ratio of 1:30. Comparison of Fig. 2A–D demonstrated that the SLR factor had a less pronounced effect on the ER of TFs

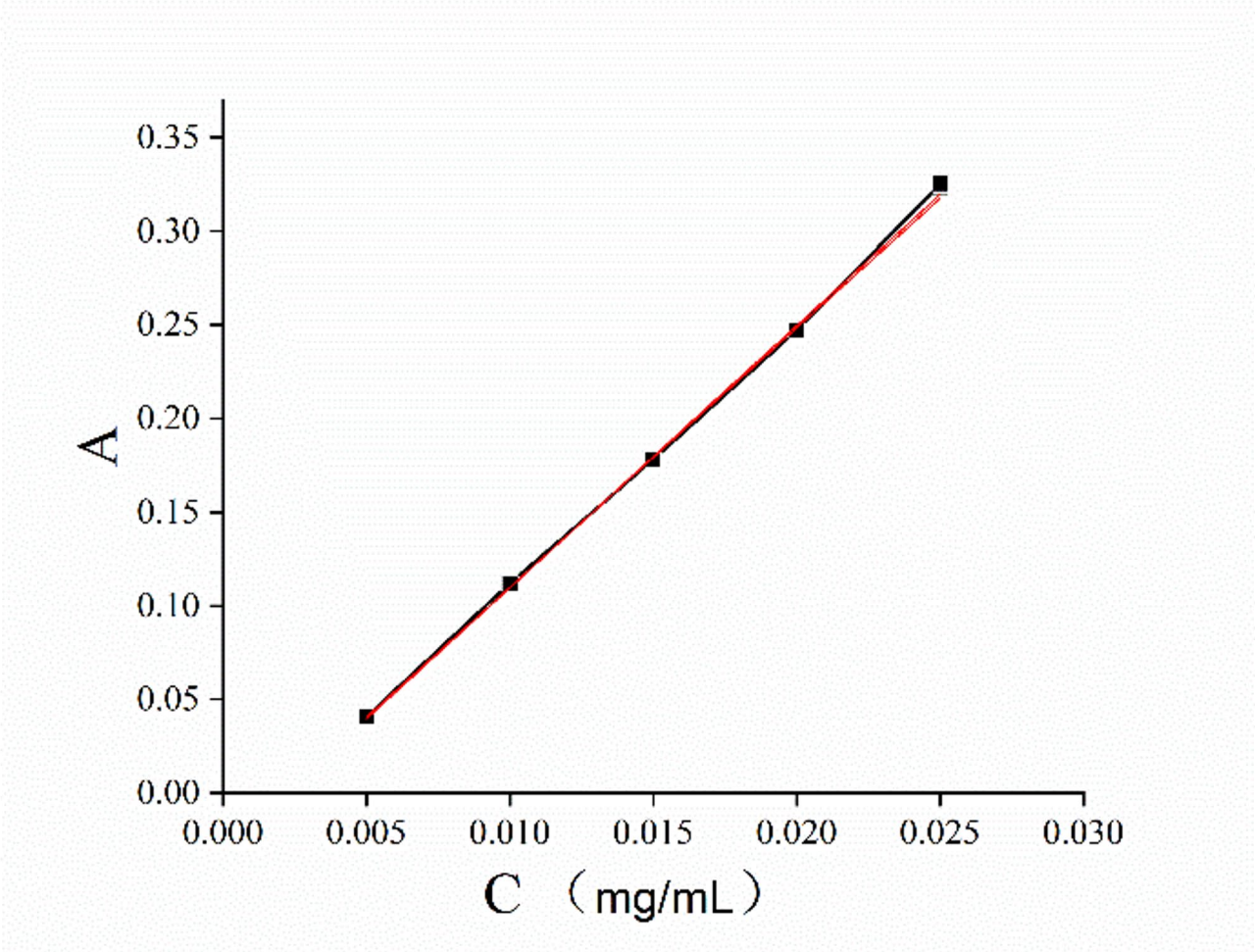

Fig. 1. Rutin standard curve.

compared to ethanol concentration, reflux time, and reflux temperature. Therefore, in the RS experiment, the primary factors selected were ethanol concentration, reflux time, and reflux temperature.

RS optimization experimental results

Scheme and results of RS design (Table 1)

Results of regression analysis and ANOVA (Table 2)

The experimental data were analyzed using Designexpert 10.0 to derive a quadratic equation for the ER:

$$Y = 11.78 + 0.056A + 0.042B + 0.011C + 0.005AB + 0.0075AC + 0.0001BC - 0.13A^2 - 0.0048B^2 - 0.062C^2$$

Where A refers to ethanol concentration; B represents reflux time, and C denotes reflux temperature. The coefficients in the equation indicated the degree and direction of the influence of each factor on extraction efficiency.

The P-value of the model was 0.0022, indicating its significance and good fit with the experimental data. The coefficient of determination (R^2 was 0.9350, indicating a small experimental error. The quadratic model's high significance ($p = 0.0022$) and R^2 value (0.9350) confirm its reliability. The dominance of ethanol concentration over other factors is consistent with findings by Jiang et al.[20], highlighting its critical role in flavonoid solubility. The difference between the adjusted and predicted correlation coefficients was less than 0.2, demonstrating reasonable agreement between them. Precision, defined as the ratio of signal to noise, was 9.029, exceeding the threshold of 4.0, demonstrating the reliability of the dataset. The P values of ethanol concentration (A) and reflux time (B) were less than 0.05, indicating their significant effect on extraction efficiency. In contrast, reflux time (C) showed a P value of more than 0.05, suggesting that it had not significant influence on ER. The lack of fit term was 0.8986, which was greater than 0.05, indicating no significant difference between the quadratic surface and experimental data[20,21]. Based on the results of ANOVA and coefficients in the formula, the significance of factors affecting ER were as follows: ethanol concentration > reflux time > reflux temperature.

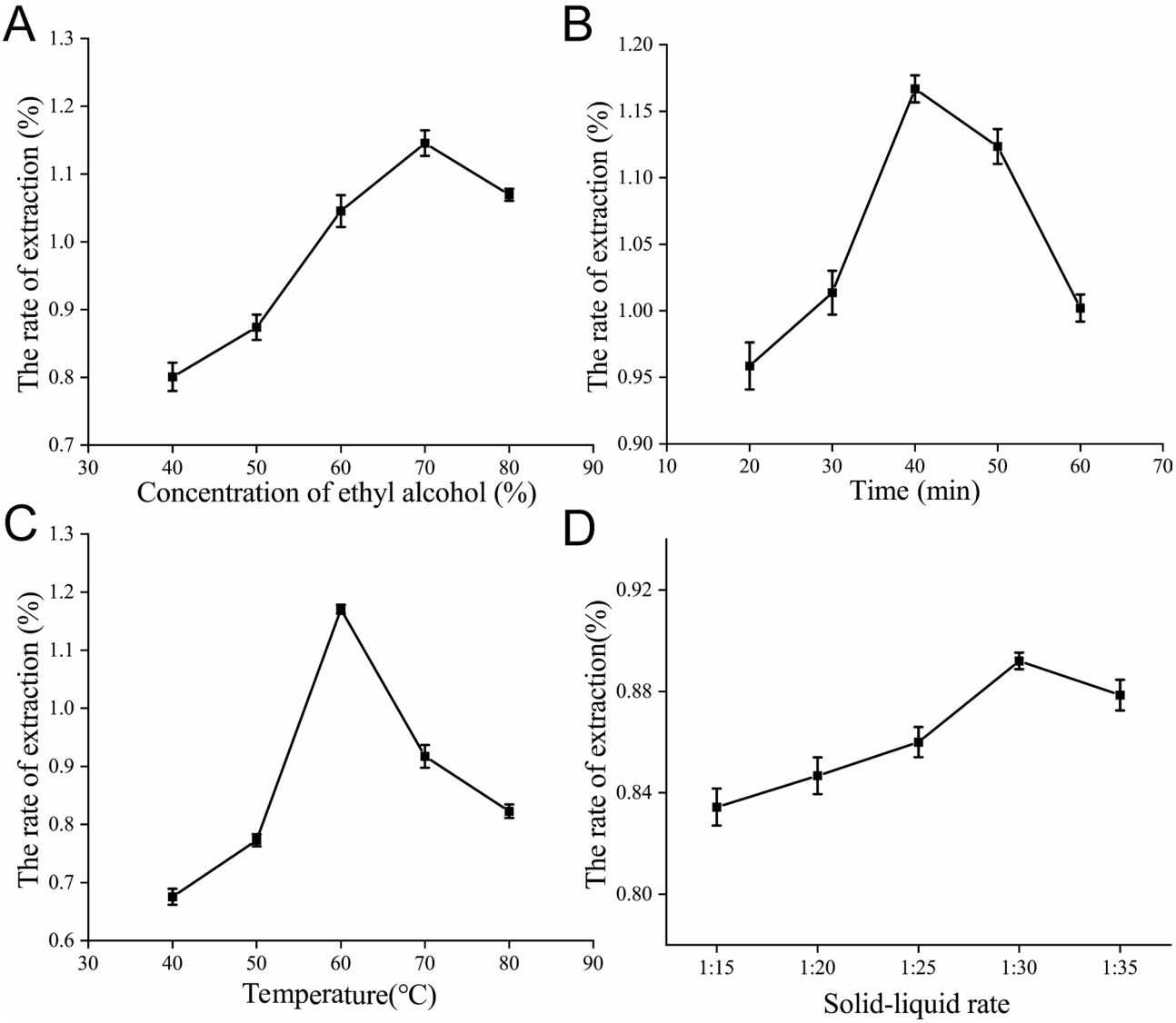

Fig. 2. Results of the extraction process of total flavonoids from Ginkgo biloba leaves: (A) Effect of ethanol concentration on the extraction rate; (B) Effect of reflux time; (C) Effect of reflux temperature; and (D) Effect of solid-liquid ratio.

RS analysis

Ethanol concentration, reflux time, and reflux temperature were analyzed using Designexpert 10.0 software. The resulting two-by-two interaction plots and contours are shown in Fig. 3.

The RS plot clearly reflected the effect of the interaction between the factors on the response values. It was evident that the slope of the ethanol concentration surface was more obvious compared to that of the reflux time surface, and the reflux time surface was steeper relative to the reflux temperature surface. The significant difference between the ethanol concentration curve and the reflux time surface indicated that ethanol concentration might have the most significant impact on the ER. The RS analysis identified the optimal conditions for extracting TFs from GB: 77% ethanol concentration, reflux temperature of 61 °C, reflux time of 30 min, resulting in an ER of 1.175%.

Analysis of TF content of GB in different months

As shown in Fig. 4A, the TF content of GB peaked in May and August, with May showing the highest content among the months analyzed. The bimodal peak in May and August correlates with GB's active growth phases under Shaoyang's subtropical monsoon climate. Increased photosynthesis during these months likely enhances flavonoid biosynthesis, as noted by Guo et al.[22].

Analysis of DPPH free radical-scavenging activity based on different amounts of extracts

As shown in Fig. 4B, the DPPH free radical-scavenging rate exhibited a rapid increase from 63% to about 87% with the increase in the concentration of TFs in GB, indicating that GB extract might have a significant capability

Experimental groups	A	B	C	Extraction rate/%
1	70	30	70	1.177
2	80	30	60	1.175
3	70	50	70	1.170
4	60	40	50	1.154
5	80	40	50	1.163
6	70	40	60	1.183
7	60	30	60	1.164
8	70	40	60	1.180
9	70	40	60	1.172
10	70	40	60	1.181
11	70	30	50	1.173
12	70	50	50	1.166
13	60	40	70	1.153
14	80	50	60	1.166
15	80	40	70	1.165
16	60	50	60	1.153
17	70	40	60	1.175

Table 1. Response surface design scheme.

Sources of variance	Sum of squares	Degrees of freedom	Mean square	F-Value	*p*-Value
Model	0.14	9	0.015	11.19	0.0022
A	0.025	1	0.025	18.74	0.0034
B	0.014	1	0.014	10.70	0.0137
C	0.0011	1	0.0011	0.75	0.4553
AB	0.0001	1	0.0001	0.074	0.7934
AC	0.000225	1	0.000225	0.17	0.6954
BC	0.0004	1	0.0004	0.32	0.5845
A^2	0.074	1	0.074	54.52	0.0002
B^2	0.000095	1	0.000095	0.070	0.7985
C^2	0.016	1	0.016	12.08	0.0931
Residual	0.0095	7	0.0014		
Lack of Fit	0.0012	3	0.0004	0.19	0.8986
Pure Error	0.0083	4	0.0021		
Total variation	0.15	16	Precision	9.029	
R^2	0.9350	Adjusted coefficient of determination	0.8514	Predicted multiple correlation coefficient	0.7818

Table 2. Results of variance analysis. Note: $P > 0.05$: not significant; $P < 0.05$: significant different; $P < 0.01$: extremely significant different.

to combat DPPH free radicals. The concentration-dependent antioxidant activity aligns with literature on flavonoid-rich extracts[23], supporting GB's potential as a natural antioxidant source.

Conclusions

In conclusion, the experimental study found that the highest TF content in shade-dried GB occurred during May and August. Shaoyang, situated in a central subtropical monsoon climate, usually experiences its hottest and rainiest period in July and August, which is beneficial for ginkgo growth. During this period, intensified photosynthesis in GB leads to increased production and accumulation of secondary metabolites, thereby increasing the TF content[22–24]. Subsequently, as temperatures decrease, photosynthesis slows down, metabolic activity in leaves diminishes, and TF content gradually declines. Variations in TF content across other months may be attributed to factors such as climate and geography. The antioxidant experiments conducted on these optimally extracted TFs from GB demonstrated their significant ability to scavenge DPPH free radicals. Besides, their antioxidant activity was found to increase with the increasing concentration. This study might provide a scientific basis for the strategic harvesting and utilization of GB, ensuring its quality and safety in future applications.

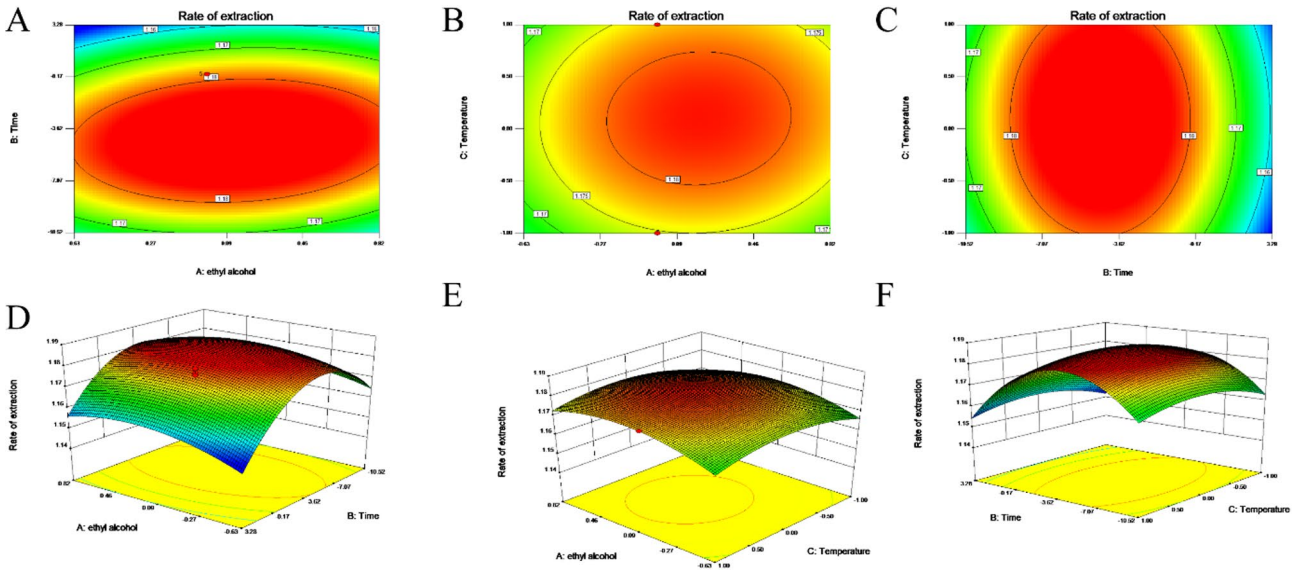

Fig. 3. Response surface plots of the interaction of factors. Note: A, B, and C correspond to 2D contour maps of AB, BC, and AC, respectively; D, E, and F correspond to 3D contour maps of AB, BC, and AC.

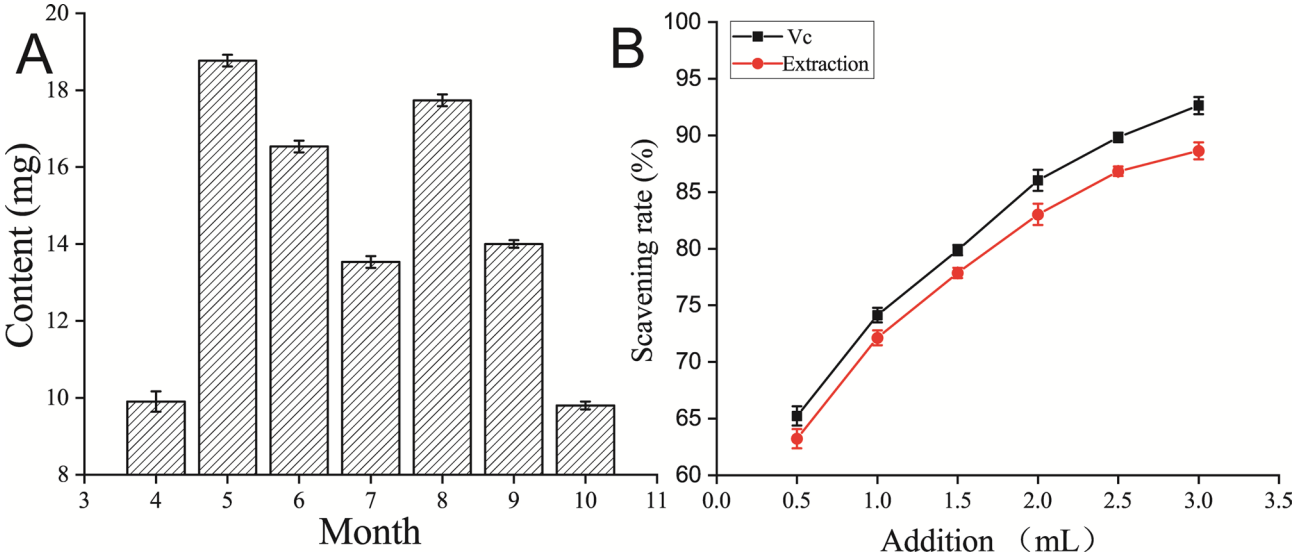

Fig. 4. A: Total flavonoid concentration of Ginkgo biloba in different months; B: Effect of different amounts of extracts on the scavenging rate of DPPH free radicals.

Data availability

Data supporting the results of this study can be obtained by contacting the corresponding author via email.

Received: 19 January 2025; Accepted: 7 May 2025
Published online: 13 May 2025

References

1. Zu, Z. et al. Mechanism of melatonin promotion of flavonoid biosynthesis InGinkgo Biloba. *Russ. J. Plant Physiol.* **71**, 49 (2024).
2. Liu, L., Wang, Y., Zhang, J. & Wang, S. Advances in the chemical constituents and chemical analysis of Ginkgo biloba leaf, extract, and phytopharmaceuticals. *J. Pharm. Biomed. Anal.* **193**, 113704. https://doi.org/10.1016/j.jpba.2020.113704 (2021).
3. Gohil, K. & Packer, L. In *Diet—Brain Connections: Impact on Memory, Mood, Aging and Disease* (eds. Mattson, M. P.) 125–134 (Springer US, 2002).
4. Belwal, T. et al. Ginkgo biloba. *Nonvitamin Nonmineral Nutritional Supplements* 241–250 (Springer, 2019).
5. Xiaoyan, L. et al. Chemical analysis, pharmacological activity and process optimization of the proportion of bilobalide and ginkgolides in Ginkgo biloba extract. *J. Pharm. Biomed. Anal.* **160**, 46–54. https://doi.org/10.1016/j.jpba.2018.07.037 (2018).

6. Wang, C. Z., Yuan, J. J., Li, W. J., Zhang, H. Y. & Ye, J. Z. In vivo and in vitro toxicity evaluation of polyprenols extracted from Ginkgo biloba L. Leaves. *Molecules* **20**, 22257–22271 (2015).
7. Xiaxia, W., Ruiting, X. & Wanglin, H. Effects of season and light on the contents of bifflavone in Ginkgo biloba leaves. *Chin. Hortic. Abstracts* **33**, 39– (2017).
8. Zaixing, C. et al. Changes of total flavonoids, total flavonoid glycosides and terpenoid lactones in Ginkgo biloba leaves in different seasons. *Chin. J. Hosp. Pharm.* **30**, 1067–1069 (2010).
9. Lancaster, J. E., Reay, P. F., Norris, J. & Butler, R. C. Induction of flavonoids and phenolic acids in Apple by UV-B and temperature. *J. Pomology Hortic. Sci.* **75**, 142–148 (2000).
10. Park, N., Cho, S. D., Chang, M. S. & Kim, G. H. Optimization of the ultrasound-assisted extraction of flavonoids and the antioxidant activity of Ruby S Apple Peel using the response surface method. *Food Sci. Biotechnol.* **31**, 1667–1678. https://doi.org/10.1007/s10068-022-01150-8 (2022).
11. Zhu, T., Shen, Q., Xu, Y. & Li, C. Ionic liquid and ultrasound-assisted extraction of chestnut shell pigment with good hair dyeing capability. *J. Clean. Prod.* **335**, 130195 (2022).
12. Sun, Y., Ma, L., Fu, Y., Dai, H. & Zhang, Y. Fabrication and characterization of myofibrillar microgel particles as novel Pickering stabilizers: effect of particle size and wettability on emulsifying capacity. *LWT-Food Sci. Technol.* **151**, 112002 (2021).
13. Zeng, Q. et al. Quantitative analysis of the quality constituents of Lonicera japonica Thunberg based on Raman spectroscopy. *Food Chem.* **443**, 138513. https://doi.org/10.1016/j.foodchem.2024.138513 (2024).
14. Ao, F. et al. Preliminary research on the flavor substance and antioxidant capacity of beers produced with baking Qingke. *Food Chem.: X.* **22**, 101394. https://doi.org/10.1016/j.fochx.2024.101394 (2024).
15. Wang, Q., Zhang, S. & Li, Z. Design of hyper-cross-linked polymers with tunable Polarity for effective preconcentration of aflatoxins in grain. *Chem. Eng. J.* **453**, 139544 (2023).
16. Kong, F. et al. Study on the compositional analysis, extraction process, and hemostatic and Anti-Inflammatory activities of Cirsium japonicum Fisch. Ex DC.-Cirsium setosum (Willd.) MB extracts. *Molecules* **29**, 1918. https://doi.org/10.3390/molecules29091918 (2024).
17. Al-Rimawi, F. et al. Anticancer, antioxidant, and antibacterial activity of chemically fingerprinted extract from Cyclamen persicum mill. *Sci. Rep.* **14**, 8488. https://doi.org/10.1038/s41598-024-58332-z (2024).
18. Gong, Q., Guo, Z., Sun, Z., Gong, J. & Wei, F. Graphene oxide-assisted ethanol reflux extraction of total flavonoids from Ginkgo biloba leaves: study of kinetics and mechanism. *Chem. Pap.* **74**, 971–984. https://doi.org/10.1007/s11696-019-00934-3 (2020).
19. Dai, C. Y. et al. Optimization of flavonoids extraction process in Panax Notoginseng stem leaf and a study of antioxidant activity and its effects on mouse melanoma B16 cells. *Molecules* **23**, 2219. https://doi.org/10.3390/molecules23092219 (2018).
20. Jiang, L. et al. Optimization of ethanol extraction technology for Yujin powder using response surface methodology with a Box-Behnken design based on analytic hierarchy Process-Criteria importance through intercriteria correlation weight analysis and its safety evaluation. *Molecules* **28**, 8124. https://doi.org/10.3390/molecules28248124 (2023).
21. Liu, X., Liu, C. H. & Li, Y. The effects of computer-assisted learning based on dual coding theory. *Symmetry* **12**, 701 (2020).
22. Guo, Y., Wang, T., Fu, F. F., El-Kassaby, Y. A. & Wang, G. Temporospatial flavonoids metabolism variation in Ginkgo biloba leaves. *Front. Genet.* **11**, 589326. https://doi.org/10.3389/fgene.2020.589326 (2020).
23. Shi, D., Wei, X., Chen, G. & Xu, Y. Changes in photosynthetic characteristics and antioxidative protection in male and female Ginkgo during natural senescence. *J. Am. Soc. Hortic. Sci. Am. Soc. Hortic. Sci.* **137**, 349–360 (2012).
24. Wang, Q. et al. Integration of morphological, physiological, cytological, metabolome and transcriptome analyses reveal age inhibited accumulation of flavonoid biosynthesis in Ginkgo biloba leaves. *Ind. Crops Prod.* **187**, 115405 (2022).

Acknowledgements

This work was financially supported by innovative training program for college students of Hunan Province, China [No. 202210547025]; Aid Program for Science and Technology Innovative Research Team in Higher Educational Institutions of Hunan Province, China (Xiangjiaotong [2023] No. 233); the teaching reform research project of Hunan Province, China [No. 202401001313]; Horizontal project of Shaoyang University [No. 2024HX118]; and Natural Science Foundation Project of Hunan Province, China [No. 2025JJ70227].

Author contributions

S.X. and L. Y. completed the experiment and wrote the first draft of the paper, Z. Q. and Zh. Y. analyzed the data and made diagrams, and Z. L.conceived the study and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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