Extraction Optimization and Tyrosinase Inhibitory Activity of Total Flavonoids from *Hamamelis mollis* Oliv.

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Abstract This study optimized the extraction of total flavonoids from *Hamamelis mollis* Oliv. and evaluated their tyrosinase inhibitory activity. After petroleum ether defatting, flavonoids were extracted with ethanol. Single-factor experiments and an $L_9(3^4)$ orthogonal design assessed the effects of ethanol concentration, extraction time, solvent-to-material ratio, and temperature on flavonoid yield. Optimal conditions were determined as 50% ethanol, 70 ℃, 1.5 h, and a 1∶40 (g∶ml) ratio, yielding 5.93 mg/g, verified by replicate tests. The crude extract was concentrated and freeze-dried. Tyrosinase was extracted from potato tubers, and inhibition assays using L-tyrosine as substrate and arbutin as positive control showed that the flavonoids inhibited tyrosinase in a concentration-dependent manner, with 58.2% inhibition at 2 mg/ml, exceeding arbutin at certain concentrations. Additionally, the total flavonoids were tested for cytotoxicity and melanin synthesis inhibition in B16 cells. Data were analyzed by SPSS. The results demonstrate significant tyrosinase inhibition, supporting the potential of *H. mollis* flavonoids as a natural whitening agent for cosmetic applications. This work provides a foundation for industrial scaleup and further development.

Key words *Hamamelis mollis* Oliv.; Total flavonoids; Extraction optimization; Tyrosinase inhibitory activity

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Hamamelis mollis Oliv., a member of the Hamamelidaceae family, is a medicinal plant rich in flavonoids, phenolics, and tannins, which are predominantly found in its flowers, leaves, and bark. This species exhibits multiple bioactivities, including anti-inflammatory, astringent, sebum-regulating, and antioxidant effects[1], rendering it a valuable natural ingredient in both cosmetic and pharmaceutical applications. Tyrosinase, the rate-limiting enzyme in melanin biosynthesis, serves as a primary target for skin-whitening product development. Plant-derived natural inhibitors of tyrosinase have attracted increasing research interest due to their favorable safety profiles and minimal side effects[2].

Despite the evident potential of *H. mollis*, systematic investigations into the optimization of total flavonoid extraction and its tyrosinase-inhibitory properties remain limited. In the present study, flowers of *H. mollis* were used as the raw material, which were first defatted with petroleum ether and subsequently subjected to ethanol extraction for total flavonoid recovery. A combination of single-factor experiments and orthogonal design was employed to optimize the extraction parameters. Tyrosinase was extracted from potato tubers, and the crude enzyme activity was measured. The inhibitory effects of the extracted total flavonoids on tyrosinase were evaluated *in vitro* using a spectrophotometric assay. The findings provide valuable experimental data and technical support for the industrial exploitation of *H. mollis* total flavonoids and their potential incorporation into natural whitening cosmetic formulations[3].

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Materials and Methods

Materials and reagents

Flowers of *H. mollis* Oliv. were obtained from Anhui Bozhou Dongfang Bencao Tang Co., Ltd. The dried flowers were ground into powder, defatted with petroleum ether, and stored at room temperature until further use. Rutin standard (purity 98%) was supplied by Beijing Beina Chuanglian Biotechnology Research Institute. L-Tyrosine and arbutin standards were purchased from Beijing Solarbio Science & Technology Co., Ltd. Petroleum ether, anhydrous ethanol, sodium hydroxide, aluminum chloride, sodium nitrite, and other reagents were of analytical grade and acquired from Tianjin Fuyu Fine Chemical Co., Ltd. and Tianjin Fengchuan Chemical Reagent Technology Co., Ltd. Disodium hydrogen phosphate and sodium dihydrogen phosphate (biochemical grade) were used for buffer preparation.

The following reagents and equipment were employed for cell-based assays: DMEM highglucose basal medium (500 ml), premium fetal bovine serum (Punose, Wuhan Punose Technology Co., Ltd.), an upright microscope, an automatic cell counter, an electric heating incubator, a Memmert temperature-controlled unit (Germany), low-temperature storage cabinets (4 and 25 ℃), and a multifunctional microplate reader.

Extraction and content determination of total flavonoids

Dried flowers of *H. mollis* Oliv. were pulverized after drying at 50 ℃ for 1 h. The resulting powder was immersed in petroleum ether for 48 h, then vacuum-filtered to remove lipids, and dried in an oven at 45 ℃. The treated material was then cooled to room temperature, and sealed for subsequent use.

A rutin reference standard (5.0 mg) was accurately weighed and dissolved in ethanol to prepare a series of standard solutions

with mass concentrations of 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, and 6.0 μg/ml. According to the sodium nitrite-aluminum chloride colorimetric method[4,8], the absorbance of each standard was measured at the maximum absorption wavelength. A calibration curve was constructed by plotting absorbance (y) against concentration (x), and the corresponding regression equation was obtained for the calculation of total flavonoid content.

For extraction, defatted *H. mollis* powder (1.0 g) was precisely weighed and subjected to ethanol extraction under specified conditions in a water bath. The extract was vacuum-filtered to collect the filtrate. An appropriate aliquot of the filtrate was taken and its absorbance was determined using the same colorimetric procedure. The total flavonoid content was calculated from the calibration curve, and the extraction yield was determined using the following formula:

$$\text{Flavonoid content (mg/g)} = C \times V_1 \times V_2 / (V_3 \times M)$$

where C represents the total flavonoid concentration (μg/ml), V_1 is the dilution volume (ml), V_2 is the sample solution volume (ml), V_3 is the volume of the aliquot taken (ml), and M is the sample mass (g).

Optimization of extraction conditions for total flavonoids

Single-factor experiments To determine the optimal range for each extraction parameter, single-factor experiments were performed with the total flavonoid yield as the response variable. The base conditions were set as follows: ethanol concentration 60%, solid-to-liquid ratio 1 ∶ 30 (g ∶ ml), extraction temperature 50 ℃, and extraction time 1.5 h. On this basis, four factors were individually varied: extraction time (0.5 – 3.0 h), solid-to-liquid ratio (1 ∶ 10 – 1 ∶ 40, g ∶ ml), extraction temperature (40 – 80 ℃), and ethanol concentration (20% – 80%). The effect of each factor on the extraction yield was examined, and the appropriate range for each was determined accordingly[10-11].

Orthogonal experiment Based on the single-factor results, four factors, namely ethanol concentration (A), extraction time (B), solid-to-liquid ratio (C), and extraction temperature (D), were selected for further optimization. Each factor was tested at three levels, and an $L_9(3^4)$ orthogonal array was employed (Table 1). The total flavonoid yield served as the evaluation index. The optimal extraction conditions were identified through range analysis and analysis of variance (ANOVA), and the reproducibility was confirmed by three parallel verification experiments.

Table 1 Factors and levels in the orthogonal design for total flavonoid extraction from *H. mollis*

Level	Factor			
	A//%	B//h	C//(g ∶ ml)	D//℃
1	30	1.0	1 ∶ 20	60
2	40	1.5	1 ∶ 30	70
3	50	2.0	1 ∶ 40	80

Preparation of total flavonoid extract

Total flavonoids were extracted under the optimal conditions derived from the orthogonal experiments. The combined filtrates were concentrated under reduced pressure using a rotary evaporator, frozen overnight at −80 ℃, and then freezedried in vacuum to obtain the total flavonoid powder. The resulting powder was stored in a sealed container, protected from light, until further use.

Enzyme extraction and solution preparation

Fresh potato tubers were peeled, diced, and frozen at −20 ℃ overnight. The frozen pieces were homogenized with pre-chilled phosphate-buffered saline (PBS, 0.2 mol/L, pH 6.8) at a 2 ∶ 1 (w/v) ratio on ice, allowed to stand for 4 h, and then centrifuged at 4 000 rpm for 10 min. The supernatant was collected as the crude tyrosinase solution and stored at −20 ℃ until use.

L-Tyrosine substrate solution (1 mmol/L) was prepared by dissolving 18.2 mg of L-tyrosine in a small volume of 0.1 mol/L NaOH and diluting to 100 ml with distilled water. The freeze-dried total flavonoid powder of *H. mollis* was dissolved in distilled water to obtain a stock solution at 2.0 mg/ml, which was then serially diluted to 1.0, 0.5, and 0.25 mg/ml for inhibition assays. Arbutin, used as the positive control, was prepared similarly. All solutions were kept at 4 ℃.

Tyrosinase inhibition assay

The assay was performed following a reported spectrophotometric method[7,9] with slight modifications. The reaction mixture (total volume 5.0 ml) contained PBS buffer (pH 6.8), the test sample (or positive control), crude enzyme solution, and L-tyrosine substrate. For the test group, 0.5 ml of PBS, 1.0 ml of sample solution, 1.5 ml of enzyme solution, and 2.0 ml of substrate were mixed. In the blank control, the sample was replaced with PBS. In the negative control, the substrate was replaced with distilled water. In the positive control, arbutin was used instead of the sample. The mixture was preincubated at 35 ℃ for 10 min, and the reaction was initiated by adding prewarmed L-tyrosine. After incubation at 35 ℃ for 30 min, the absorbance was measured at 475 nm. Each concentration was tested in triplicate.

The inhibition rate was calculated as:

$$\text{Inhibition rate (\%)} = [(A_0 - A_1)/A_0] \times 100\%$$

where A_0 is the absorbance of the control (without inhibitor) and A_1 is the absorbance of the test sample or positive control. Higher values indicate stronger tyrosinase inhibitory activity.

Cell culture and viability assay

Mouse melanoma B16-F10 cells (obtained from a commercial supplier) were maintained in RPMI-1640 complete medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 ℃ in a 5% CO_2 humidified incubator. Cells in the logarithmic growth phase were used for all experiments.

Cell viability was assessed using the CCK-8 assay. Logarithmic-phase cells were seeded into 96-well plates at 1×10^5 cells/ml (100 μl per well) and allowed to adhere for 12 h. The cells were then treated with various concentrations of *H. mollis* total flavonoids (0.031 25 – 4.0 mg/ml) for 24 h, with five replicates per concentration. After treatment, the medium was replaced with 100 μl of fresh RPMI-1640 containing 10% CCK-8, and the

plates were incubated in the dark at 37 ℃ for 1 h. Absorbance was recorded at 450 nm. Cell viability (%) was calculated as $(A_t - A_0)/(A_n - A_0) \times 100\%$, where A_t is the absorbance of the treated group, A_n is that of the untreated control, and A_0 is the blank. Concentrations yielding >80% viability were considered non-cytotoxic and used for subsequent melanin assays[13].

Melanin content measurement

B16-F10 cells were seeded into 24 well plates at 5×10^4 cells/ml (500 μl per well) in phenol red-free DMEM complete medium and incubated for 24 h to allow adhesion. The medium was then replaced with fresh medium containing 100 nM α-MSH[14] and either the test flavonoids (at the safe concentrations determined above) or 500 μM arbutin as a positive control, each in triplicate. After 48 h, half of the medium was renewed and the treatment continued for an additional 48 h (total 96 h). The cells were then lysed in situ by adding 100 μl of 2 mol/L NaOH per well. The lysates were heated at 80 ℃ for clarification, centrifuged at 12 000 rpm for 5 min, and the supernatants were measured at 405 nm[15]. The background absorbance of the medium and drugs was subtracted. Relative melanin content (%) was expressed as $(A_s/A_m) \times 100\%$, where A_s is the absorbance of the treated group and $A_m$is that of the model control (α-MSH only).

Statistical analysis

All data are presented as mean ± standard error (SE). For orthogonal extraction experiments, range and variance analyses were performed to determine the optimal conditions and the significance of each factor ($P < 0.05$ considered statistically significant). For cell assays, comparisons between groups were analyzed by ANOVA or Student's *t*-test as appropriate, using SPSS software.

Results and Discussion

Standard curve of rutin

The absorbance of rutin standard solutions at various concentrations was determined using the $NaNO_2$-$AlCl_3$ colorimetric method at 510 nm. A calibration curve was constructed by plotting absorbance against concentration (Fig. 1), yielding the regression equation $y = 4.000x + 0.043\ 13$ with a correlation coefficient $R^2 = 0.999$. This indicates an excellent linear relationship within the tested concentration range, which enabled the accurate quantification of total flavonoid content in *H. mollis* extracts.

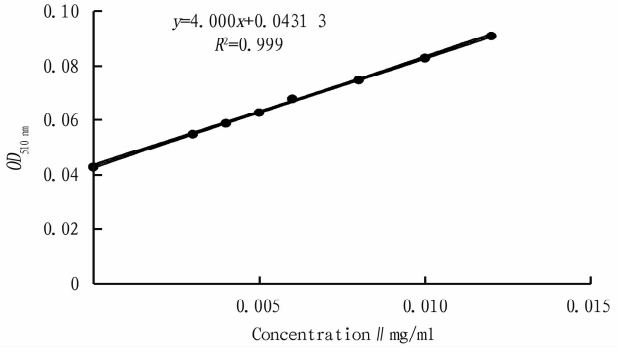

Fig. 1 Rutin standard curve

Analysis of single-factor experimental results

Effect of extraction time The influence of extraction time on the total flavonoid yield was investigated under constant solid-to-liquid ratio, temperature, and ethanol concentration. As shown in Fig. 2, the yield increased with time up to 1.5 h, reaching a maximum of 9.36 mg/g, and then declined with further extension. Prolonged extraction may cause oxidation and degradation of flavonoids, leading to loss of active components. Therefore, 1.5 h was selected as the optimal extraction time.

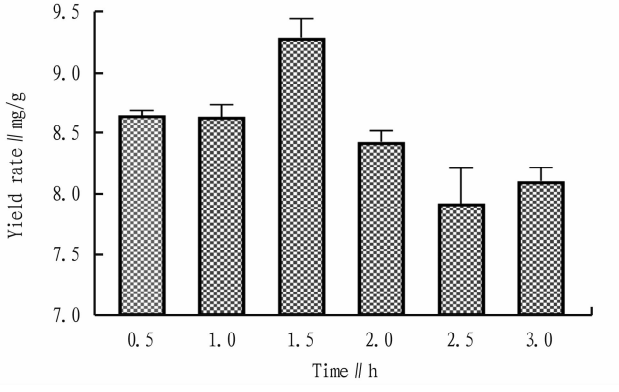

Fig. 2 Effect of time on the extraction rate of total flavonoids from *H. mollis*

Effect of solid-to-liquid ratio The effect of solvent volume on extraction yield was examined under fixed conditions, with results presented in Fig. 3. The yield increased progressively as the ratio rose from 1∶10 to 1∶40 (g∶ml), reaching the highest value of 7.92 mg/g at 1∶40. Beyond this ratio, no significant improvement was observed, and excessive solvent consumption was considered uneconomical. Thus, a solid-to-liquid ratio of 1∶40 was chosen as optimal.

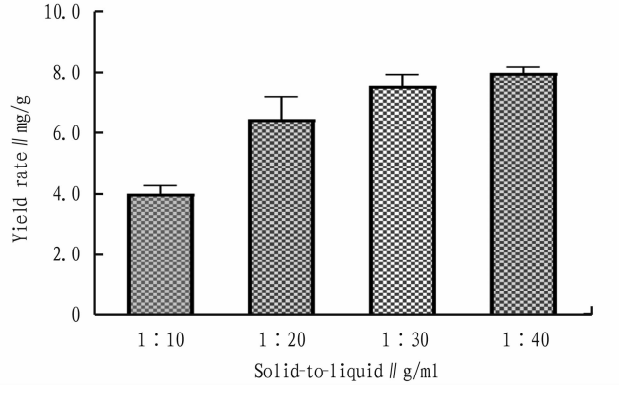

Fig. 3 Effect of Soild-liquid ratio on the extraction rate of total flavonoids from *H. mollis*

Effect of extraction temperature The temperature dependence of the extraction yield was evaluated over the range of 40 – 80 ℃ (Fig. 4). The yield peaked at 70℃, with a value of 8.19 mg/g. Elevated temperature enhances flavonoid dissolution, but temperatures above 70 ℃ may degrade heat-labile constituents, thereby reducing the yield. Consequently, 70 ℃ was adopted as the optimal temperature.

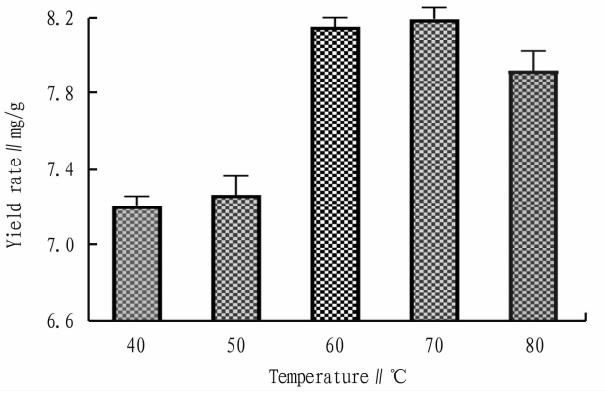

Fig. 4 Effect of temperature on the extraction rate of total flavonoids from *H. mollis*

Effect of ethanol concentration Extraction was performed at ethanol concentrations from 20% to 80%, and the results are illustrated in Fig. 5. The maximum yield (3.02 mg/g) was obtained at 40% ethanol. Lower concentrations failed to fully solubilize flavonoids, while higher concentrations might co-extract more impurities, reducing the overall efficiency. Therefore, 40% ethanol was determined as the optimal concentration.

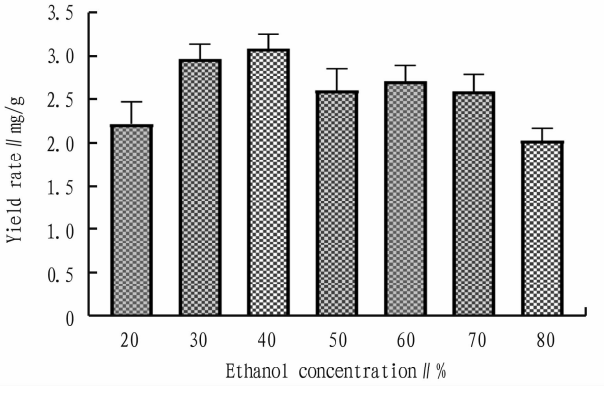

Fig. 5 Effect of ethanol concentration on the extraction rate of total flavonoids from *H. mollis*

Orthogonal optimization of extraction conditions

Based on the single-factor results, four factors, namely ethanol concentration (A), extraction time (B), solid-to-liquid ratio (C), and extraction temperature (D), were further optimized using an $L_9(3^4)$ orthogonal array. The experimental design and results are presented in Table 2.

Range analysis revealed that the factors affected the extraction yield in the following order: C (solid-to-liquid ratio) > B (time) > A (ethanol concentration) > D (temperature). Analysis of variance (ANOVA) indicated that the solid-to-liquid ratio had an extremely significant effect on the yield ($P<0.01$), making it the dominant factor.

The optimal combination was determined as $A_3B_2C_3D_2$, *i. e.*, ethanol concentration of 50%, extraction time of 1.5 h, solid-to-liquid ratio of 1∶40 (g∶ml), and extraction temperature of 70 ℃.

Table 2 Orthogonal optimization of extraction conditions

No.	A	B	C	D	Extraction rate // %
1	1	1	1	1	5.57
2	1	2	2	2	10.89
3	1	3	3	3	15.69
4	2	1	2	3	7.26
5	2	2	3	1	15.57
6	2	3	1	2	6.18
7	3	1	3	2	15.68
8	3	2	1	3	6.18
9	3	3	2	1	10.59
K_1	32.15	28.51	18.22	31.73	
K_2	29.01	32.93	28.74	32.75	
K_3	32.74	32.46	46.94	29.42	
k_1	3.572	3.168	2.024	3.526	
k_2	3.223	3.659	3.193	3.639	
k_3	3.638	3.607	5.216	3.269	
R	0.414	0.491	3.191	0.37	

Verification experiments

Three parallel extractions were performed under the optimized conditions, yielding an average total flavonoid content of 5.93 mg/g. This value was higher than those obtained from all nine orthogonal runs, confirming the reliability of the optimization and the stability of the established process.

Evaluation of tyrosinase inhibitory activity

Tyrosinasa activity assay The crude tyrosinase activity was determined to be 5.1 KU/ml at 280 nm according to the Sigma method. A time-dependent increase in absorbance at 475 nm indicated continuous dopaquinone accumulation (Fig. 6), confirming that the crude enzyme was suitable for subsequent inhibition assays.

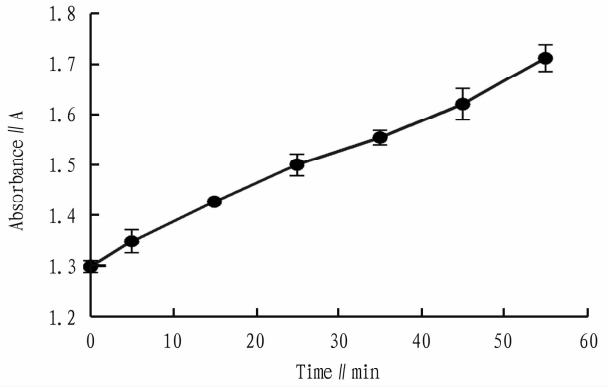

Fig. 6 Validation of tyrosinase activity by absorbance at 475 nm

Inhibitory effect of total flavonoids on tyrosinase The inhibitory activity of *H. mollis* total flavonoids against tyrosinase was evaluated at concentrations ranging from 0.25 to 2.0 mg/ml, with arbutin as the positive control. As shown in Fig. 7, the inhibition rate increased in a concentration dependent manner, reaching 58.2% at 2.0 mg/ml, which exceeded that of arbutin at the same concentration. These results demonstrate that the total flavonoids from *H. mollis* possess significant tyrosinase-inhibitory activity and exhibit promising potential for application in whitening cosmetic products.

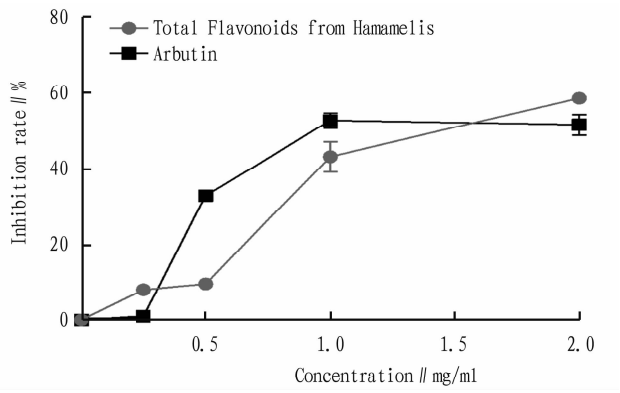

Fig. 7 Inhibition of tyrosinase by total flavonoids of *H. mollis*

Effects of total flavonoids on B16-F10 cell viability The CCK-8 assay results demonstrated that cell viability remained above 80% across all treatment groups within the concentration range of 0.031 25 – 4.0 mg/ml, indicating no significant cytotoxicity of *H. mollis* total flavonoids toward B16-F10 cells under the tested conditions. Notably, compared with the blank control, cell viability was significantly increased at 1.0 ($P < 0.001$) and 2.0 mg/ml ($P < 0.01$), suggesting a moderate proliferative effect at these concentrations (Fig. 8).

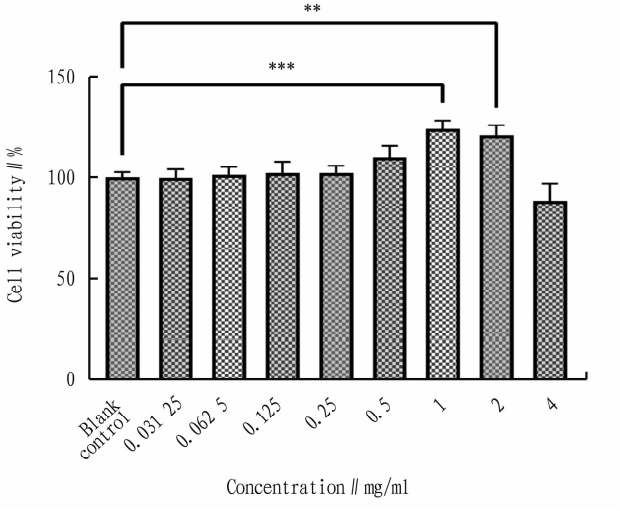

Fig. 8 Effect of different concentrations of total flavonoids of *H. mollis* on the viability of B16-F10 cells

Effects of total flavonoids on melanin content in B16-F10 cells

As shown in Fig. 9, the positive control, arbutin, significantly reduced intracellular melanin content to 48.41% of the model control level ($P < 0.01$), confirming the reliability and efficacy of the experimental system. Among the *H. mollis* total flavonoid treatment groups, the strongest inhibitory effect was observed at 1.0 mg/ml, which decreased melanin content to 76.30% of the control ($P < 0.05$). In contrast, the 0.125 mg/ml group significantly promoted melanin synthesis ($P < 0.05$), while the 0.25 and 0.5 mg/ml groups showed no significant effect. At 2.0 mg/ml, the inhibitory activity was weaker than that at 1.0 mg/ml, and the difference from the control was not statistically significant.

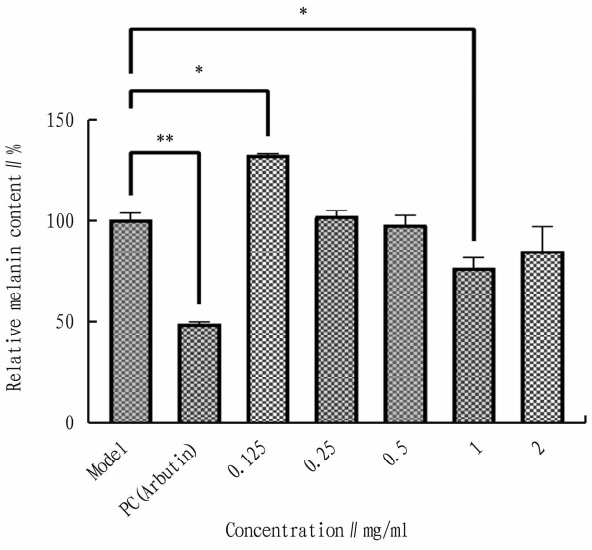

Fig. 9 Effect of different concentrations of total flavonoids of *H. mollis* on melanin content in B16-F10 cells

The orthogonal optimization highlighted the solid-to-liquid ratio as the dominant extraction parameter, reflecting the critical role of solvent accessibility in releasing flavonoids from the plant matrix. The selected ethanol concentration and temperature suggest that the target compounds are moderately polar and thermally stable, which is consistent with the flavonoid profiles of related species.

The tyrosinase inhibition assays confirmed the anti-melanogenic potential of the extract, with activity exceeding that of the reference positive control, implying a strong interaction with the enzyme's active site—likely via copper chelation or substrate competition. In B16 melanoma cells, the extract showed no cytotoxicity across a broad concentration range, supporting its safety for topical applications. Notably, a biphasic effect on melanin synthesis was observed: inhibition at higher concentrations, but stimulation at the lowest dose. This nonmonotonic response may arise from the coexistence of multiple bioactive constituents with opposing effects on melanogenesis pathways, and warrants further fractionation to identify the individual active compounds.

Overall, the established extraction process is simple and reproducible, and the bioactive extract demonstrates a favourable safetyactivity balance. These findings position *H. mollis* as a promising candidate for natural whitening ingredients. Future studies should focus on elucidating the molecular mechanisms—particularly the involvement of MITF and downstream enzymes, and validating the efficacy in more physiologically relevant skin models.

Conclusion

In this study, total flavonoids were extracted from *H. mollis* Oliv. via ethanol extraction. Through single-factor experiments and orthogonal optimization, the optimal extraction conditions were determined as follows: ethanol concentration of 50%, extraction time of 1.5 h, solid-to-liquid ratio of 1∶40 (g∶ml), and extraction temperature of 70 ℃. Under these conditions, the total flavonoid yield reached 5.93 mg/g.

The tyrosinase inhibitory activity of the extracted flavonoids

was evaluated *in vitro*. At a concentration of 2.0 mg/ml, the inhibition rate reached 58.2%, which was higher than that of the positive control arbutin at the same concentration, indicating significant inhibitory potential. In B16-F10 melanoma cell assays, the flavonoids exhibited no cytotoxicity within the tested concentration range (0.031 25 – 4.0 mg/ml) and showed a concentration-dependent inhibition of melanin synthesis, with the strongest effect observed at 1.0 mg/ml (melanin content reduced to 76.30% of the control). Notably, a promoting effect on melanin synthesis was observed at the lowest concentration (0.125 mg/ml), suggesting a biphasic regulatory profile that warrants further investigation.

Statistical analyses were performed using SPSS software. Collectively, these findings demonstrate that *H. mollis* total flavonoids possess notable tyrosinase-inhibitory and melanin-reducing activities, with favorable safety profiles in B16 cells. This work provides valuable experimental data and a technical foundation for the potential application of *H. mollis* as a natural whitening ingredient in cosmetic formulations, and supports its further development toward industrial utilization.

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with the frequency of microbial agent application increasing. From an economic perspective, multiple applications of the microbial agent throughout the entire growth period (T_2) achieved the greatest total increases in yield, soil fertility, and income, while a single basal application of the compound enzyme preparation at the sowing stage (T_1) exhibited the highest input-output efficiency. The corresponding application method can be selected and promoted according to production objectives.

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