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Traditional Chinese Medicine in Skin Depigmentation: Pharmacological Insights and Clinical Potential

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ABSTRACT

Skin-lightening effects of traditional Chinese medicine (TCM) are widely recognized, yet the underlying molecular mechanisms remain unclear. In this study, we investigated ten commonly used TCM extracts for their ability to inhibit tyrosinase from mushrooms, humans, and mouse melanoma B16F0 cells. Among the extracts tested, *Rosa rugosa* Thumb, *Morus alba* L., and *Paeonia lactiflora* Pall displayed strong inhibitory effects on both mushroom and human tyrosinase at 1.0 mg/mL. Several other extracts, including *Bletilla striata*, *Centella asiatica*, *Cynanchum atratum*, *Rosa canina*, *Rhus chinensis*, and *Glycyrrhiza uralensis*, selectively inhibited one of the two tyrosinase sources, whereas *Tribulus terrestris* L. showed negligible activity. In cellular experiments using B16F0 melanoma cells, *M. alba* markedly decreased both tyrosinase activity (by 70%) and melanin production (by 50%) at 250 µg/mL. Overall, nine of the ten tested TCM extracts demonstrated potential as depigmenting agents. Furthermore, the variable responses observed across tyrosinase from different species highlight the importance of multi-source enzyme assays when evaluating candidates for human skin-lightening applications.

words: Traditional chinese medicine (TCM), Tyrosinase, Tyrosinase inhibitor, Skin lightning

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Introduction

Melanin, the primary pigment in human skin, serves a critical protective function against damage induced by ultraviolet (UV) radiation and reactive oxygen species (ROS) [1]. However, excessive melanin production or its uneven distribution can lead to various skin disorders, including hyperpigmentation, lentigines, age spots, freckles, melasma, and post-inflammatory melanoderma, as well as DNA damage, gene mutations, photoaging, and compromised immune function [2-4]. Tyrosinase (TYR), a copper-containing enzyme, catalyzes the initial steps of melanogenesis, converting tyrosine into 3,4-dihydroxy-L-phenylalanine (L-DOPA) and subsequently into dopaquinone, ultimately leading to the formation of eumelanin and pheomelanin [3, 5, 6]. In mammals, TYR is predominantly expressed in melanocytes, which are responsible for pigmentation in the skin, eyes, and hair [7-9]. Inhibiting TYR is considered the most validated approach for reducing melanin synthesis and is therefore a key target in skin depigmentation strategies [10]. Several depigmenting agents, including hydroquinone, arbutin, azelaic acid, kojic acid, and ascorbic acid, are widely employed in cosmetic formulations, yet their clinical use is limited by poor skin penetration, low stability, potential toxicity, and variable efficacy [9, 11].

For decades, mushroom tyrosinase (Agaricus bisporus, abTYR) has been widely used as a reference enzyme in the development of tyrosinase inhibitors due to its availability, affordability, and natural origin. However, studies have revealed structural differences between abTYR and human tyrosinase (hsTYR), resulting in discrepancies in inhibitor efficacy, where compounds effective against abTYR often fail to inhibit hsTYR adequately [12-14]. Consequently, modern screening approaches recommend assessing potential inhibitors against both abTYR and hsTYR to ensure relevance for human applications [15]. Moreover, evaluating TYR inhibitors in cellular systems,

such as mouse melanoma (mmTYR) cells, human melanoma cells, or primary human melanocytes, is advised to confirm their anti-melanogenic activity in more physiologically relevant contexts [16, 17].

Traditional Chinese medicine (TCM) has a long history of use for skin-lightening purposes, and is often favored for its low cost, minimal side effects, and abundance of natural sources [18, 19]. Reviews of the Chinese Pharmacopoeia, Compendium of Materia Medica, and German Plant Drug Analysis indicate that over 120 TCM have been incorporated into cosmetic products for pigmentation control. Among these, ten herbs—*Morus alba* L., *Glycyrrhiza uralensis* Fisch. ex DC., *Rosa rugosa* Thumb, *Rosa canina* L., *Rhus chinensis* Mill., *Paeonia lactiflora* Pall., *Tribulus terrestris* L., *Cynanchum atratum* L., *Bletilla striata* (Thunb.) Rchb. f., and *Centella asiatica* (L.) Urb.—are commonly applied either individually or in [10, 18, 20]. Several studies have demonstrated that some of these extracts can inhibit abTYR, and a subset has shown activity in mouse melanoma assays. However, systematic evaluation against hsTYR remains lacking [21].

In line with our interest in TCM for skin depigmentation, we established enzymatic assays using abTYR and hsTYR, along with cellular assays in B16F0 mouse melanoma cells, to evaluate the activity of the ten commonly used herbs. Considering the role of oxidative stress in melanogenesis, the antioxidant properties of these extracts were also assessed [22-24]. To reflect traditional preparation methods, including water decoctions and wine tinctures, extractions were performed using varying ratios of water and ethanol, and both extraction yield and tyrosinase inhibitory efficacy were examined.

Materials and Methods

General experimental procedure

Ethanol, tyrosine (≥99.4%), mushroom tyrosinase (abTYR, 8503 U/mg), dimethyl sulfoxide (DMSO), potassium dihydrogen phosphate, sodium phosphate dibasic, L-DOPA, kojic acid, and arbutin were sourced from Sigma Aldrich (Merck, Australia). Human tyrosinase (hsTYR, >85%, MW 52 kDa) was obtained from Bioresearch, USA. Fresh solutions of tyrosine, abTYR, and hsTYR were prepared in phosphate buffer 30 minutes prior to use at concentrations of 0.625 mM, 217 U/mL, and 20 mg/mL, respectively. Samples were prepared using 0.1 M sodium phosphate buffer (pH 6.8) or 50 mM potassium phosphate buffer (pH 7.0). Extractions were assisted by sonication using a Branson B2500S-DTH, and solvents were removed with a Shen Sheng rotary evaporator followed by freeze-drying (Fevik). Chemical analyses were performed on a Shimadzu LC-20AD HPLC system, which included a DGU-20A5R degasser, SIL-20AC autosampler, SPD-M40 PDA detector, CTO-20AC column oven, CBM-20A controller, and FRC-10A fraction collector, with data acquisition via LB Solution DB software. UV absorbance for tyrosinase assays was measured using a Bio-Strategy SpectraMax ABS Plus multi-well reader.

TCM materials

The ten herbal materials—*Morus alba*, *Glycyrrhiza uralensis*, *Rosa rugosa*, *Rosa canina*, *Rhus chinensis*, *Paeonia lactiflora*, *Tribulus terrestris*, *Cynanchum atratum*, *Bletilla striata*, and *Centella asiatica*—were obtained from Ferngrove Pharmaceutical. All samples were authenticated by a TGA-licensed analytical research laboratory at Southern Cross University (TGA licence number: MI-01122004-LI-000264-1). The respective authentication reference numbers are as follows: ARL2205667 (*M. alba*), ARL2205668 (*G. uralensis*), ARL2205669 (*R. rugosa*), ARL2205670 (*R. canina*), ARL2205671 (*R. chinensis*), ARL2205662 (*P. lactiflora*), ARL2205663 (*T. terrestris*), ARL2205664 (*C. atratum*), ARL2205665 (*B. striata*), and ARL2205666 (*C. asiatica*).

Extraction of TCM material

Each TCM herbal material was thoroughly rinsed with water, air-dried, and subsequently ground into a fine powder. For extraction, 600 mg of the dried powder was soaked in 15 mL of the designated solvent—95% ethanol, 70% ethanol, 30% ethanol, or water—at room temperature overnight. This was followed by four rounds of sonication (10 minutes each) in 15 mL of the same solvent. The resulting supernatants from all extraction cycles were pooled, concentrated under vacuum using a rotary evaporator, and finally freeze-dried to yield the crude extracts.

HPLC analysis

For chemical profiling, 5 mg of each TCM crude extract was dissolved in 1 mL of methanol, and 200 µL of the solution was injected into a Shimadzu HPLC system equipped with a diode array detector. Separation was

achieved using a Waters XTerra C18 reverse-phase column (250 × 4.6 mm, 5 μm) with a gradient elution consisting of 0.1% formic acid in water (solvent A) and methanol (solvent B). The gradient progressed from 0% to 100% methanol over 70 minutes at a flow rate of 1 mL/min.

abTYR assay

The inhibitory activity of the TCM extracts against mushroom tyrosinase (abTYR) was evaluated using a modified version of a previously reported method [25]. Briefly, 10 mg of each extract was solubilized in 10–50 μL of DMSO according to solubility, then diluted with 0.1 M sodium phosphate buffer (pH 6.8) to achieve a final concentration of 1.0 mg/mL. In a 96-well plate, 100 μL of the extract solution was mixed with 20 μL of abTYR solution (217 U/mL) and 80 μL of freshly prepared L-tyrosine (0.625 mM). The reaction mixtures were incubated at 37 °C for 30 minutes, after which the generation of L-DOPA was monitored by measuring absorbance at 475 nm using a Bio-Strategy SpectraMax ABS Plus microplate reader [26, 27].

Control wells were included to ensure assay reliability: the positive controls used kojic acid and arbutin (1.0 mg/mL); negative control 1 contained L-tyrosine and the extract without abTYR; negative control 2 contained L-tyrosine and abTYR without extract; and the blank consisted of L-tyrosine with buffer alone. The percent inhibition of tyrosinase activity was calculated as follows:

$$\text{Inhibition (\%)}=[1-(A1-A2)(A3-A4)]\times 100\text{Inhibition (\%)} = \left[1 - \frac{(A_1 - A_2)}{(A_3 - A_4)} \right] \times 100\text{Inhibition (\%)}=[1-(A3-A4)(A1-A2)]\times 100 \quad (1)$$

Where A_1 represents the absorbance of the test reaction, A_2 and A_3 correspond to the two negative controls, and A_4 is the blank.

hsTYR assay

The activity of human tyrosinase (hsTYR) in the presence of TCM extracts was assessed using a modified method based on Pomeranz (1963)[28]. The assay was performed similarly to the abTYR protocol, with 50 mM potassium phosphate buffer (pH 7.0) used for sample dilution. For each reaction, 1 mL of extract solution (1.0 mg/mL) was combined with 0.5 mL of hsTYR solution (50 mM in potassium phosphate buffer) and incubated at 37 °C for 5 minutes. Following this pre-incubation, 0.5 mL of freshly prepared L-DOPA (3 mM in potassium phosphate buffer) was added, and the mixture was incubated for an additional 10 minutes at 37 °C. The formation of dopachrome was monitored by measuring absorbance at 484 nm.

The assay included the same controls as the abTYR assay: positive controls (kojic acid and arbutin, 1.0 mg/mL), negative controls (without enzyme or extract), and a blank (substrate with buffer only). Tyrosinase inhibition (%) was calculated using the formula:

$$\text{Inhibition (\%)}=[1-(A1-A2)(A3-A4)]\times 100\text{Inhibition (\%)} = \left[1 - \frac{(A_1 - A_2)}{(A_3 - A_4)} \right] \times 100\text{Inhibition (\%)}=[1-(A3-A4)(A1-A2)]\times 100 \quad (2)$$

Where A_1 is the absorbance of the test sample, A_2 and A_3 represent the negative controls, and A_4 corresponds to the blank.

Total antioxidant capacity assay

The overall antioxidant potential of the TCM extracts was assessed using a commercial total antioxidant capacity (TAC) assay kit (MA334, Sigma) following the kit protocol with minor adaptations [29]. This assay relies on the reduction of Cu^{2+} ions to Cu^{+} by antioxidants in the sample. The Cu^{+} then reacts with a chromogenic dye to generate a colored complex, the intensity of which at 570 nm reflects the total antioxidant activity of the extract [30-32].

A standard curve was constructed using Trolox, prepared from a 1 mM stock solution at concentrations of 0, 300, 600, 800, and 1000 μM. For both standards and extracts, 20 μL was added per well of a 96-well plate, after which 100 μL of the Cu^{2+} reagent was introduced. The plate was gently agitated and incubated at room temperature for 10 minutes. Absorbance readings were taken at 570 nm using a SpectraMax ABS Plus microplate reader (Bio-Strategy, USA).

The total antioxidant capacity of each sample was determined using the equation:

$$\text{TAC } (\mu M) = (A570)\text{sample} - (A570)\text{blank} \text{Slope } (\mu M^{-1}) \times n \quad (3)$$

where (A570)sample corresponds to the sample absorbance, (A570)blank is the absorbance of the blank (water), and nnn represents the dilution factor applied during sample preparation.

Cell culture

B16F0 mouse melanoma cells (ATCC® CRL-6322™) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine, and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL; Life Technologies, CA, USA). Cultures were kept in T-75 flasks at 37 °C in a humidified incubator containing 5% CO_2 and were routinely subcultured every two days to preserve exponential growth.

Normal human dermal fibroblasts (NHDF; PromoCell, Heidelberg, Germany) were cultured in Fibroblast Basal Medium 2, enriched with 2% FBS, 1 ng/mL basic fibroblast growth factor, and 5 µg/mL insulin. Cells were incubated under identical conditions to the melanoma cells and passaged every two days to maintain healthy monolayers.

Resazurin-based cytotoxicity assay

For cell viability assessment, B16F0 and NHDF cells were seeded into 96-well plates at densities of 3,300 and 10,000 cells per well, respectively, and incubated overnight at 37 °C in a humidified atmosphere with 5% CO_2 to allow attachment. The next day, cells were treated with different concentrations of TCM extracts, adjusting the total volume to 100 µL per well, and each condition was tested in triplicate. Wells containing culture medium alone served as blanks, while cells exposed to 10% DMSO acted as positive controls. Plates were maintained under standard incubation conditions for 72 hours.

Following treatment, 5 µL of CellTiter-Blue® (Resazurin) reagent (Promega) was added to each well and gently mixed for 10 seconds. The plate was then returned to the incubator for 3 hours to allow reduction of resazurin to resorufin. Fluorescence, reflecting metabolically active cells, was measured at 560 nm excitation and 590 nm emission using a FLUOstar Omega multi-mode plate reader (BMG Labtech, Ortenberg, Germany).

mmTYR assay

Cellular tyrosinase activity in B16F0 cells was assessed following a modified version of the protocol described by Parvez *et al*. (2007)[33]. Briefly, 1×10^5 B16F0 cells were seeded into 6-well plates and incubated overnight at 37 °C with 5% CO_2 to allow adherence. The following day, cells were treated with TCM extracts for 72 hours in the presence of 0.1 µM α-MSH (Sigma-Aldrich, Sydney, Australia) to stimulate melanogenesis.

After the treatment period, cells were harvested, washed with buffer, and lysed in 100 µL of ice-cold lysis buffer containing 1% Triton X-100 in 50 mM sodium phosphate (pH 6.8). Cell suspensions were sonicated at 20 kHz for 30 seconds and then centrifuged at 7,500 × g for 30 minutes at 4 °C to remove debris. For the assay, 20 µL of each supernatant was transferred into a 96-well plate in triplicate, followed by addition of 150 µL of 15 mM L-DOPA solution. Wells containing lysis buffer alone served as blanks.

Tyrosinase activity was monitored by measuring absorbance at 490 nm every minute for 60 minutes using a FLUOstar Omega multi-mode plate reader (BMG Labtech, Ortenberg, Germany). Enzyme activity was expressed as the change in absorbance per minute per milligram of total protein, with protein concentrations determined using the Lowry assay [33].

Melanin assay

Intracellular melanin levels in B16F0 cells were quantified using a modified protocol based on Chung *et al.* (2019)[34]. Briefly, 1×10^5 cells were seeded into 6-well plates and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. Cells were then treated with TCM extracts for 72 hours in the presence of 0.1 µM α-MSH (Sigma-Aldrich, Sydney, Australia) to stimulate melanin production.

After treatment, cells were harvested, washed, and lysed in 150 µL of 1N NaOH. The lysates were incubated at 60 °C for 1 hour to solubilize melanin. A series of melanin standards (2.5–80 µg/mL) was prepared in 1N NaOH.

For quantification, 100 µL of each standard and 100 µL of a twofold diluted cell lysate were loaded into a 96-well plate in triplicate and duplicate, respectively. The plate was mixed for 30 seconds using a SpectraMax M3 plate reader (Molecular Devices, CA, USA), and absorbance was measured at 405 nm.
Melanin content in each sample was determined using the standard curve and normalized to total protein concentration, which was measured by the Lowry assay. Final results were expressed as micrograms of melanin per milligram of total protein.

Lowry protein estimation
Bovine serum albumin (BSA) was employed as the protein standard. A 10 mg/mL stock solution of BSA (Sigma-Aldrich, NSW, Australia) was serially diluted to generate working standards of 2, 1, 0.5, 0.25, and 0.125 mg/mL. For the standard curve, 10 µL of each dilution was dispensed in triplicate into a 96-well plate. Cell lysates were diluted 1:20, and 10 µL of each sample was added to the plate in triplicate. Protein levels were quantified using the Bio-Rad DC protein assay kit. Reagent S (20 µL) was combined with 1 mL of reagent A, and 25 µL of this mixture was added to each well and incubated at room temperature for 1 minute. Thereafter, 200 µL of reagent B was added, and the plate was shaken for 5 minutes using a SpectraMax M3 plate reader (Molecular Devices), then incubated in the dark for an additional 15 minutes. Absorbance was recorded at 750 nm, and protein concentrations were calculated from the BSA standard curve [35].
All experimental data from cell-based assays were analyzed using GraphPad Prism 9. Each experiment was performed at least three times with independent biological replicates. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was assessed by one-way ANOVA followed by Dunnett's post hoc test, with $p < 0.05$ considered significant.

Results and Discussion

Skin lightning TCM
A review of the Chinese Pharmacopoeia, the Compendium of Materia Medica, and German Plant Drug Analysis indicated that over 120 traditional Chinese medicines (TCMs) have applications in skin lightening, anti-aging, tyrosinase inhibition, and the treatment of hyperpigmentation. From this pool, ten herbs are most frequently used in commercial formulations: Morus alba, Glycyrrhiza uralensis, Rosa rugosa, Rosa canina, Rhus chinensis, Paeonia lactiflora, Tribulus terrestris, Cynanchum atratum, Bletilla striata, and Centella asiatica. Key information about these ten herbs—including their Chinese names, common names, botanical family, medicinal properties, and utilized plant parts—is provided in **Table 1**. These selected herbs were the focus of the present study.

Table 1. 10 TCM commonly used as skin-lightning agents.

No	Chinese name	Common name	Botanical name	Family	Reported property [28]	plant part
1	桑叶	Mulberry leaf	*Morus alba* L.	Moraceae	Skin lightning	Leaf, fruit
2	甘草	Liquorice	*Glycyrrhiza uralensis* Fisch. ex DC.	Leguminosae	Skin lightning, freckle removing	Root
3	玫瑰花	Rose flower	*Rosa rugosa* Thumb	Rosaceae	Skin lightning	Flower
4	玫瑰果	Dog rose	*Rosa canina* L.	Rosaceae	Skin lightning	Fruit
5	五倍子	Chinese gall	*Rhus chinensis* Mill.	Anacardiaceae	Skin lightning products	Fruit
6	芍药	White peony	*Paeonia lactiflora* Pall	Paeoniaceae	Skin lightning	Root
7	蒺藜	*Tribulus*	*Tribulus terrestris* L.	Zygophyllaceae	Anti-*ab*TYR activity	Fruit
8	白薇	Swallowwort	*Cynanchum atratum* L.	Asclepiadaceae	Anti-aging, skin lightning	Root
9	白芨	*Bletilla*	*Bletilla striata* (Thunb.) Rchb.F.	Orchidaceae	Anti-ageing	Stem, leaf
10	积雪草	*Centella*	*Centella asiatica* (L.) Urb	Umbelliferae	Skin lightning products	Whole plant

In traditional practice, TCMs are commonly prepared either by water decoction or by soaking in alcohol-based tinctures, typically 70% ethanol. These methods are favored due to their simplicity, cost-effectiveness, and established therapeutic benefits [36]. To replicate these traditional preparations, plant materials were extracted using solvents with varying ethanol-to-water ratios: 95%, 70%, 30%, and 0% ethanol. The resulting extracts were analyzed via HPLC to profile their chemical composition and subsequently evaluated for their inhibitory activity against mushroom tyrosinase (abTYR).

Figure 1 shows representative HPLC chromatograms of R. rugosa extracts. The chromatograms indicated comparable chemical profiles across the four solvent conditions, as reflected by the number and pattern of peaks. Functional testing revealed that the 95% ethanol extract achieved the highest inhibition of abTYR (99.5%), outperforming the other extracts, which showed 80–85% inhibition. Similar results were observed for M. alba extracts. Based on these findings, 95% ethanol was selected as the optimal solvent for TCM extraction in this study.

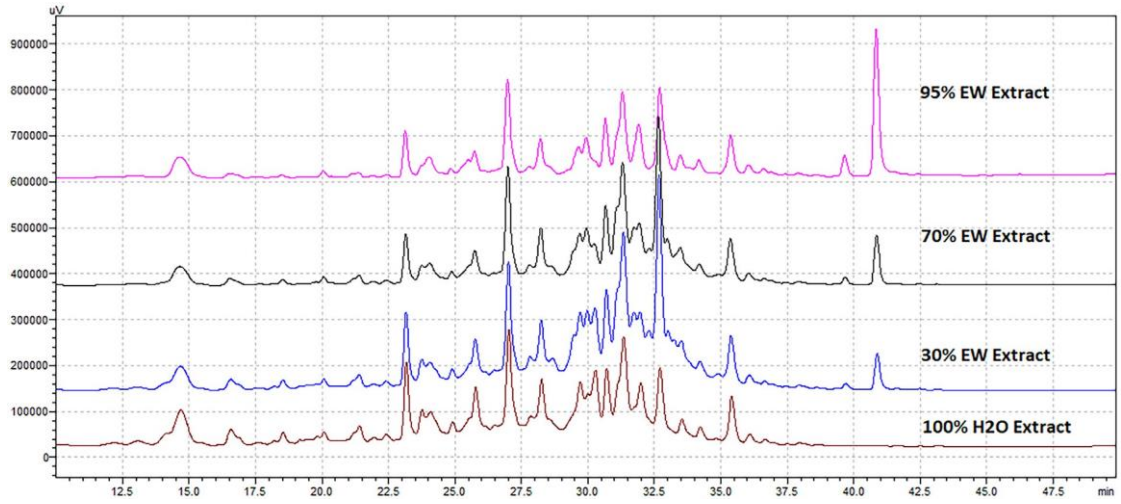

Figure 1. Representative HPLC profiles of R. rugosa extracts prepared using four different solvent ratios. Chromatographic separation was carried out on a Waters Xterra C18 column (250 × 4.6 mm, 5 µm) with a mobile phase consisting of 0.1% formic acid in water and methanol. The methanol proportion was linearly increased from 0% to 100% over 70 minutes at a flow rate of 1 mL/min.

Inhibitory activity against abTYR and hsTYR

The 95% ethanol extracts of the ten TCMs were assessed for their ability to inhibit mushroom (abTYR) and human tyrosinase (hsTYR) at a fixed concentration of 1 mg/mL, with all experiments carried out in triplicate. Due to the high cost and limited availability of hsTYR, dose–response testing was not performed. As shown in **Table 2**, the extracts displayed distinct inhibitory patterns. Against hsTYR, R. rugosa, M. alba, and B. striata showed the strongest inhibition, exceeding 90%, while C. asiatica, P. lactiflora, C. atratum, and R. canina produced moderate effects, ranging from 45% to 67%. The remaining extracts, including G. uralensis, R. chinensis, and T. terrestris, had minimal activity (~20%) or were essentially inactive. For abTYR, R. rugosa, M. alba, G. uralensis, and R. chinensis exhibited robust inhibition (>85%), P. lactiflora showed moderate inhibition at 72%, and the other five extracts were largely inactive (<10%). Overall, seven out of the ten TCMs were effective against hsTYR, while only five displayed notable inhibition of abTYR.

Table 2. Anti-TYR and antioxidant activities of 10 TCM extracts at 1 mg/mL.

TCM	***hs*TYR (% inhibition ±SEM)**	***ab*TYR (% inhibition ±SEM)**	**Total Antioxidant Capacity (µM/mg ± SEM)**
Rosa rugosa Thumb	100.00 ± 0.01	99.50 ± 0.01	3006.70 ± 0.02
Morus alba L.	100.00 ± 0.01	97.50 ± 0.02	232.40 ± 0.001
Bletilla striata (Thunb.) Rchb.F.	94.40 ± 0.06	10.20 ± 0.01	472.01 ± 0.04
Centella asiatica (L.) Urb	66.70 ± 0.01	NA[1]	229.50 ± 0.04
Paeonia lactiflora Pall	59.10 ± 0.11	72.20 ± 0.09	1024.85 ± 0.01

Cynanchum atratum L.	58.30 ± 0.01	NA[1]	427.41 ± 0.02
Rosa canina L.	50.00 ± 0.11	NA[1]	224.10 ± 0.03
Glycyrrhiza uralensis Fisch. ex DC.	23.10 ± 0.01	88.90 ± 0.01	218.35 ± 0.04
Rhus chinensis Mill.	NA[1]	89.50 ± 0.01	7403.45 ± 0.02
Tribulus terrestris L.	NA[1]	5.00 ± 0.01	12.08 ± 0.11
Kojic acid	80.0 ± 0.01	97.7 ± 0.01	NT[2]
Arbutin	60.0 ± 0.01	27.8 ± 0.01	NT[2]

NA[1]: not active; NT[2]: not tested.

When comparing the inhibitory activities of the ten TCM extracts on human and mushroom tyrosinase **(Figure 2)**, only a few extracts showed consistent effects across both enzymes. Specifically, R. rugosa, M. alba, and P. lactiflora inhibited hsTYR and abTYR to a similar extent. In contrast, six extracts—C. atratum, B. striata, G. uralensis, R. chinensis, R. canina, and C. asiatica—demonstrated activity against only one of the two enzymes. Tribulus terrestris displayed negligible inhibition against either enzyme. Overall, R. rugosa and M. alba were the most potent, achieving greater than 95% inhibition against both hsTYR and abTYR at 1 mg/mL.

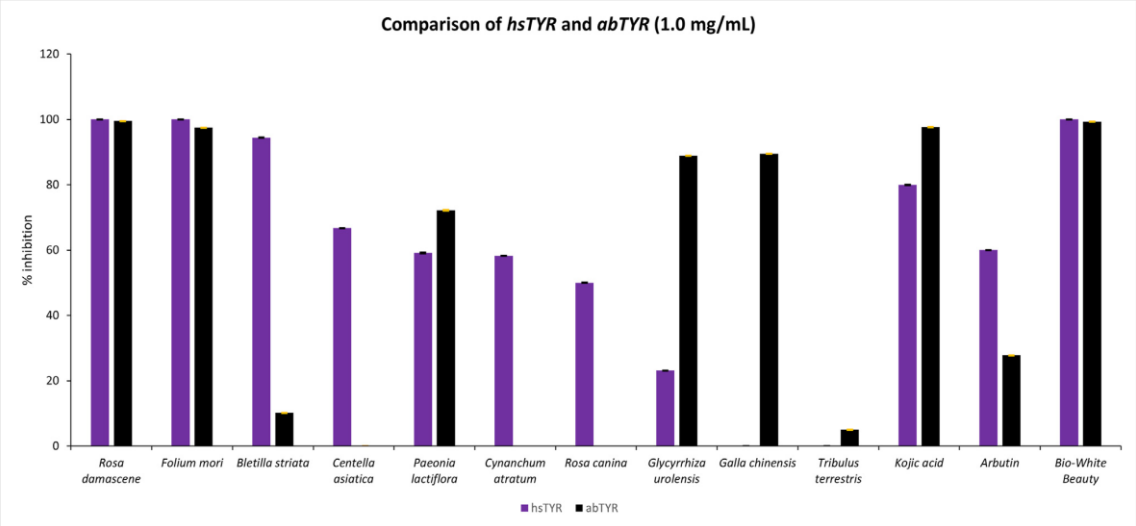

Figure 2. Inhibitory effects of ten TCM extracts on human (hsTYR) and mushroom tyrosinase (abTYR) at a concentration of 1 mg/mL.

Antioxidant activity

Given the established link between oxidative stress and tyrosinase activity [22-24, 37], the antioxidant potential of the ten TCM extracts was evaluated. Nine of the extracts that showed inhibitory effects against either mushroom or human tyrosinase also demonstrated notable antioxidant activity, with total antioxidant capacities ranging from roughly 200 to 7,500 µM/mg. Among them, R. chinensis displayed the strongest antioxidant effect, with a TAC of 7,403.45 µM/mg, correlating with its 89.5% inhibition of abTYR at 1 mg/mL. R. rugosa, which almost completely inhibited both hsTYR and abTYR (100% and 99.5%, respectively), also exhibited a substantial antioxidant effect (TAC = 3,006.5 µM/mg). By comparison, T. terrestris showed minimal antioxidant capacity (12.08 µM/mg) and negligible inhibitory activity against both enzymes.

Inhibitory activity against mmTYR and melanin production

Pigmented B16F0 mouse melanoma cells were employed to assess cellular tyrosinase activity, while normal human dermal fibroblasts (NHDF) were included to evaluate cytotoxicity. Because B16F0 cells tend to lose pigmentation after multiple passages, α-melanocyte stimulating hormone (α-MSH) was used to enhance melanogenesis and increase intracellular melanin. This co-treatment approach with α-MSH in B16F0 cells is widely recognized in the literature as a suitable in vitro model for melanogenic studies [36, 38, 39]. The two extracts showing the highest enzymatic inhibition, M. alba and R. rugosa, were selected for further cellular experiments.

To determine a safe and effective concentration for assays, the impact of M. alba extract on cell proliferation was first examined. B16F0 and NHDF cells were exposed to 0, 31.25, 62.5, 125, 250, and 500 µg/mL of the extract for 72 hours. Cell viability was measured using a resazurin-based proliferation assay. As shown in Figure 3A, concentrations up to 125 µg/mL did not significantly affect cell growth. Moderate growth inhibition (~15%) was observed in B16F0 cells at 250 µg/mL, whereas 500 µg/mL markedly reduced NHDF viability (>70%). Based on these results, 250 µg/mL was selected as the working concentration for subsequent cellular tyrosinase and melanin content assays.

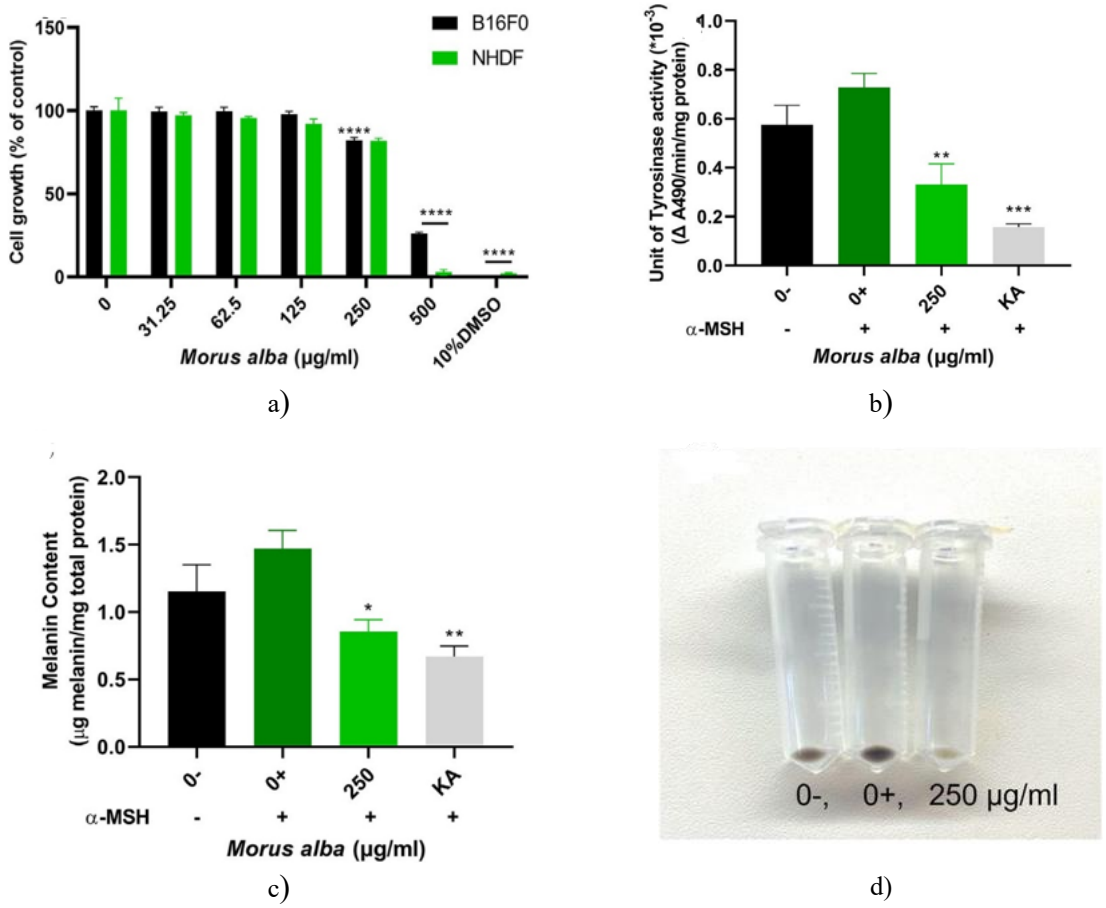

Figure 3. Effects of M. alba on B16F0 and NHDF cells. (a) Cell proliferation assessed by resazurin assay. B16F0 mouse melanoma cells and normal human dermal fibroblasts (NHDF) were exposed to 0, 31.25, 62.5, 125, 250, and 500 µg/mL M. alba extract for 3 days. Cell growth is expressed relative to untreated controls. (b) Tyrosinase activity, (c) melanin content, and (d) representative cell pellet images were determined after co-treatment with 0 or 250 µg/mL M. alba and 0.1 µM α-MSH for 3 days. Untreated cells without α-MSH (0-) and with α-MSH (0+) were included as controls. Kojic acid (500 µg/mL) served as a positive control. Data are presented as mean ± SEM (n = 3), with each experiment performed in triplicate. Statistical significance was evaluated using one-way ANOVA followed by Dunnett's test (∗P < 0.05, ∗∗∗∗P < 0.0001).

B16F0 cells treated with 250 µg/mL M. alba in combination with 0.1 µM α-MSH for 3 days exhibited a significant reduction in cellular tyrosinase activity by approximately 60% **(Figure 3b)** and melanin content by 50% **(Figure 3c)** compared to α-MSH-treated controls (0+). The treated cell pellets appeared visibly lighter in color relative to both untreated (0-) and α-MSH-stimulated (0+) controls **(Figure 3d)**.

Following the same experimental procedure, R. rugosa extract was tested on B16F0 and NHDF cells. Resazurin assays indicated that concentrations of 62.5 and 125 µg/mL did not affect cell proliferation, whereas higher doses caused cytotoxicity **(Figure 4a)**. Therefore, 125 µg/mL was selected for subsequent tyrosinase and melanin measurements. Treatment with this concentration produced a modest, non-significant decrease in tyrosinase activity **(Figure 4b)**, while melanin content showed a slight increase **(Figures 4c and 4d)**.

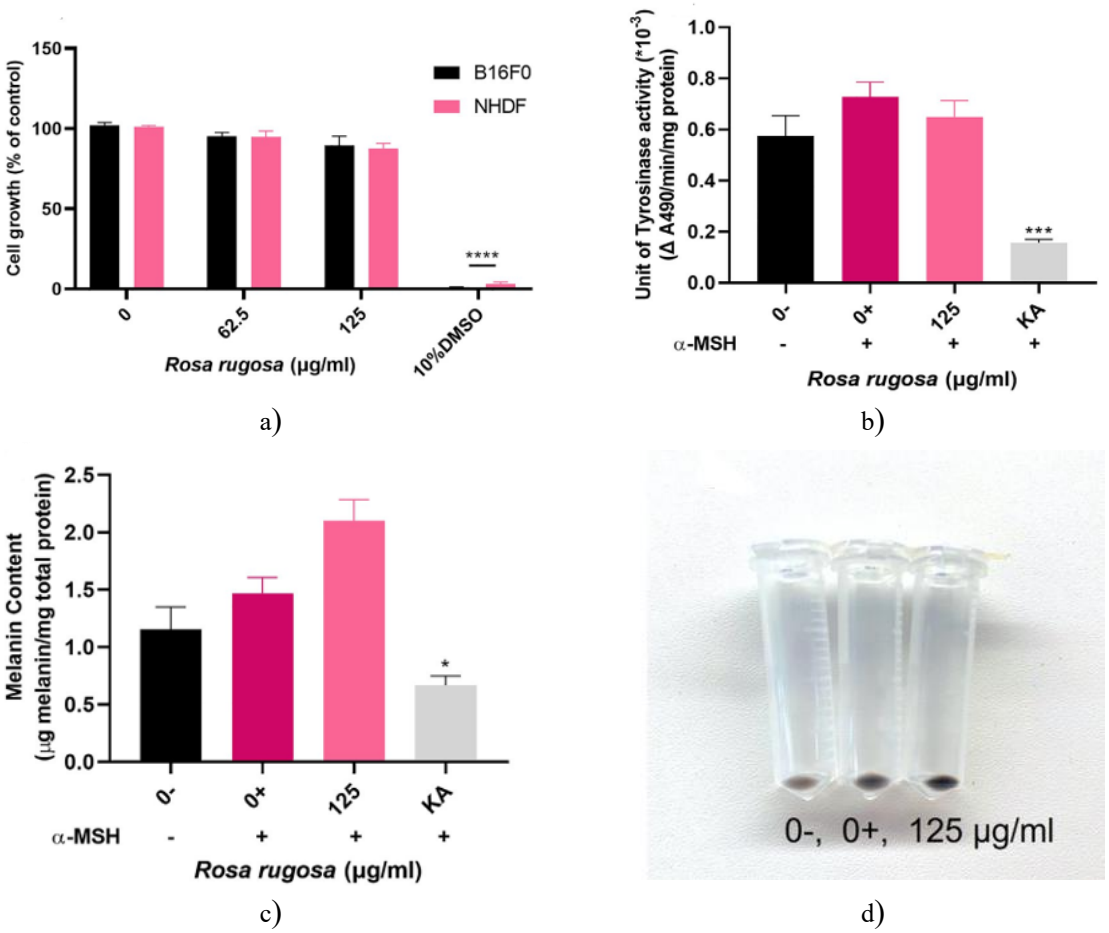

Figure 4. Cellular effects of R. rugosa on B16F0 and NHDF cells. (a) Cell proliferation was evaluated using the resazurin assay after 3 days of treatment with 0, 62.5, and 125 µg/mL of R. rugosa extract. Results are expressed as a percentage relative to untreated controls. (b) Tyrosinase activity, (c) melanin content, and (d) representative cell pellet images were measured following co-treatment with 125 µg/mL R. rugosa extract and 0.1 µM α-MSH for 3 days. Cells cultured without α-MSH (0-) or with α-MSH alone (0+) served as controls. Kojic acid (500 µg/mL) was included as a positive control. Data represent the mean ± SEM from three independent experiments, each performed in triplicate. Statistical analysis was conducted using one-way ANOVA with Dunnett's post hoc test (∗P < 0.05, ∗∗P < 0.01, ∗∗∗∗P < 0.0001).

The traditional Chinese medicine (TCM) system has been extensively documented in the Chinese Pharmacopoeia, with the number of recorded plant species expanding from 78 in its earliest edition to 1,146 by the 2005 version. Drawing on this accumulated knowledge, numerous TCM formulations have been used clinically to manage a wide range of health conditions and continue to hold a significant position in both the Chinese and global healthcare landscapes. Despite their long history of use, the biological efficacy of many TCM herbs has been a subject of ongoing research, with modern assays increasingly employed to validate their health benefits.

Reviews of the Chinese Pharmacopoeia indicate that approximately 120 TCM are commonly incorporated into cosmetic formulations aimed at freckle removal, skin brightening, and anti-aging. Recent literature further emphasizes TCM as a valuable source of anti-pigmentation agents [1, 20]. In this study, we focused on ten widely used skin-lightening TCM herbs. Their depigmentation activity was assessed using enzymatic assays against mushroom (abTYR) and human tyrosinase (hsTYR), as well as a cellular assay employing mouse melanoma B16F0 cells. Seven of the ten extracts demonstrated significant inhibition (>50%) of hsTYR at 1 mg/mL, whereas six showed similar inhibition of abTYR.

Among the extracts, M. alba was particularly effective, exhibiting over 97% inhibition against both hsTYR and abTYR at 1 mg/mL, and it significantly reduced cellular tyrosinase activity and melanin content in B16F0 cells at 250 µg/mL. In contrast, R. rugosa, despite nearly complete enzyme inhibition (~100% against both tyrosinases at 1 mg/mL), produced minimal effects on cellular tyrosinase activity and slightly increased melanin levels at 125

μg/mL. Notably, higher concentrations of R. rugosa proved cytotoxic, restricting further cellular evaluation. These findings provide experimental support for the traditional use of seven of these TCM herbs as skin-lightening agents and highlight a strong alignment between long-established ethnomedical knowledge and modern biological evidence.

Melanogenesis is an oxygen-dependent process involving multiple oxidation steps that generate reactive oxygen species (ROS). Extracts rich in antioxidants, including flavonoids and phenolic compounds, are often associated with anti-melanogenic effects [22, 23, 37]. However, our study did not reveal a clear correlation between antioxidant capacity and tyrosinase inhibition, with only R. rugosa, R. chinensis, and P. lactiflora showing substantial total antioxidant capacity (TAC). Further chemical profiling of these extracts is needed to elucidate the compounds responsible for their antioxidant and anti-melanogenic activities.

Historically, the development of skin depigmentation agents has relied heavily on abTYR due to challenges in expressing and purifying hsTYR. It was often assumed that inhibitors identified using abTYR would also be effective against human tyrosinase. Advances in recombinant protein expression and structural analysis now enable the production of hsTYR and determination of its 3D structure. These studies have revealed notable differences in amino acid sequence and active site conformation between human and mushroom tyrosinases [39, 40]. Consequently, many compounds effective against abTYR show minimal inhibition of hsTYR [41]. Our results mirror this discrepancy: only three of the ten TCM extracts exhibited comparable activity against both enzymes, while six were selectively active against one or the other. These findings emphasize that reliance on abTYR alone is insufficient for predicting human tyrosinase inhibition [38, 42, 43]. Comprehensive evaluation using multiple assays—including human tyrosinase, mammalian cells, or cell-free extracts—is essential when developing depigmentation agents intended for human use.

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