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EVALUATION OF THE MELANIN SYNTHESIS-INHIBITORY POTENTIAL OF MORUS ALBA L. LEAF EXTRACT FOR THE DEVELOPMENT OF A NATURAL ANTI-HYPERPIGMENTATION FORMULATION

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ABSTRACT
Background: Although *Morus alba* (white mulberry) has long been used in traditional skin care, the scientific evidence for its leaf extract in anti-hyperpigmentation applications remains limited, particularly regarding its incorporation into topical formulations. This study investigated the melanin-synthesis-inhibitory, tyrosinase-inhibitory, and antioxidant activities of *M. alba* leaf extract and evaluated its potential in a stable topical gel formulation. **Methodology:** Melanin suppression was assessed in IBMX-stimulated B16F10 melanocytes using microscopic observation, pellet analysis, and quantitative melanin measurement. Tyrosinase inhibition was examined with a mushroom tyrosinase assay, while antioxidant capacity was evaluated via DPPH radical scavenging. A topical gel containing M. alba extract was developed and assessed for physicochemical properties, phenolic retention, bioactivity, and stability. **Result and Discussion:** The extract showed strong tyrosinase inhibition (IC_{50} = 5.70 ± 0.28 µg/mL) and antioxidant activity (IC_{50} = 16.22 ± 0.6 µg/mL), and significantly reduced melanin synthesis in B16F10 cells without cytotoxicity (≤ 200 µg/mL). The formulated gel maintained phenolic content, exhibited moderate tyrosinase inhibition, and demonstrated stable appearance, pH, spreadability, and bioactivity during storage. **Conclusion:** *Morus alba* leaf extract possesses potent anti-melanogenic and antioxidant properties and can be successfully incorporated into a stable topical gel, supporting its potential as a natural ingredient for anti-hyperpigmentation products. However, these findings are based on in vitro assays and preliminary formulation studies; further in vivo and clinical evaluations are needed to confirm its efficacy and safety in humans.

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INTRODUCTION

Hyperpigmentation is a common dermatological disorder characterized by overproduction and abnormal deposition of melanin in the skin, resulting in darkened patches or uneven skin tone. The process of melanogenesis is primarily regulated by tyrosinase, a key enzyme responsible for the hydroxylation of L-tyrosine to L-DOPA and its subsequent oxidation to dopaquinone, which eventually leads to melanin synthesis [1]. While melanin plays a crucial role in protecting the skin against ultraviolet (UV) radiation, its overproduction can lead to conditions such as melasma and post-inflammatory hyperpigmentation (PIH) [2].

Currently, synthetic brightening agents, including hydroquinone, arbutin, kojic acid, and azelaic acid, are widely used in cosmetic and dermatological products to inhibit melanogenesis [3]. However, these constituents have been reported to cause skin irritation; for example, long-term use of hydroquinone may lead to increased photosensitivity, contact dermatitis, skin thinning, or uneven depigmentation [4]. This highlights the need to research a safe and effective skin-lightening agent of natural origin [5].

Morus alba (Moraceae), commonly known as white mulberry, is a medicinal plant that has long been used in traditional medicine. Some of its known pharmacological effects include antioxidant, anti-inflammatory, and antibacterial activities [6]. *Morus alba* leaves have been reported to contain compounds such as flavonoids, phenolic acids, stilbenes, and alkaloids. Some of the isolated active compounds, including oxyresveratrol, mulberroside A, kuwanon G, and moracin C, are believed to play an essential role in the plant's ability to inhibit the tyrosinase enzyme [5]. Additionally, the reported antioxidant activity of mulberry leaves may be among the mechanisms contributing to the plant's skin-lightening effects [7]. To date, mulberry leaves have primarily been recognized for their ability to inhibit tyrosinase, but their practical application in topical skincare products remains limited. Furthermore, although most previous studies have focused on tyrosinase inhibition, other signaling pathways, such as the cAMP/PKA/CREB/MITF cascade, in relation to the skin-depigmenting effects of mulberry leaves, remain underexplored [8][9]. Conventionally, mulberry root has been the primary part used, with limited research on the leaves for their skin-lightening mechanisms [10]. In this study, leaves served as the raw material to evaluate their ability to inhibit melanin formation via the cAMP/PKA/CREB/MITF signaling pathway. Additionally, a gel formulation containing the leaf extract was evaluated for its in vitro skin-lightening effects.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Morus alba* (MA) were collected from Quang Nam Province, central Vietnam, and authenticated by Dr. Huynh Loi (Binh Duong University). A voucher specimen (MA-VN-0224) was deposited at the Pharmacognosy department, University of Health Sciences, Vietnam National University, HCM City, for future reference.

Chemical and media

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), DPBS, trypsin, penicillin, streptomycin, IBMX (3-Isobutyl-1-methylxanthine) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA), and B16F10 mouse melanoma cells was kindly provided as a gift by the Department of Biochemistry and Molecular Biology, School of Medicine, Kyung Hee University, Republic of Korea.

Folin–Ciocalteu reagent, mushroom tyrosinase, L-DOPA, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alpha-arbutin and phosphate buffer were obtained from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Sodium carboxymethyl cellulose (NaCMC) was obtained from Sigachi Industries (USP grade), while sodium benzoate was sourced from Annexe Chem (Indian Pharmacopoeia grade). Hydroxyethyl cellulose (HEC) was procured from Ashland, USA. Ethanol and isopropanol were supplied by Fisher, USA, and GHTECH, China, respectively. Propylene glycol was obtained from Shanghai Zhanyun Chemical Co., Ltd., and glycerin was purchased from Xilong, China.

Preparation of the extract

The leaves were thoroughly washed with distilled water and air-dried at room temperature in the shade for four days. The dried leaves were powdered using a mechanical grinder. The powdered leaves (50 g) were ultrasonicated in 500 mL of 90% ethanol (ELmaS60H, Germany). The extract was filtered using Whatman no. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (IKA, Germany). The concentrated extract was further dried in a desiccator until a constant weight was achieved, and then stored at -30°C for subsequent use.

Anti-tyrosinase assay for the ethanolic extract of *M. alba*

The tyrosinase inhibition assay was performed in a 96-well microplate format according to the method described by Masuda et al. [11], with slight modifications. The extracts were diluted in 50% (w/w) dimethyl sulfoxide. Each well was prepared with a reaction mixture containing 20 µL of the extract at varying concentrations, 140 µL of 50 mM phosphate buffer (pH 6.5), and 20 µL of tyrosinase solution (100 U/mL; Sigma, EC 1.14.18.1). Following a 10-minute re-incubation period, 20 µL of 0.85 mM L-DOPA (L-3,4-dihydroxyphenylalanine, Sigma) was introduced as the substrate. The reaction was then maintained at 25°C for 20 minutes, after which the absorbance was recorded at 475 nm using a microplate reader (Biotek, USA). Each sample was analyzed in parallel with a corresponding blank to account for background interference. Kojic acid (100 µg/mL) was employed as the positive control. The percentage of tyrosinase inhibition was calculated using the following equation:

$$\boldsymbol{Inhibition}\ (\%) = \left(\mathbf{1} - \frac{\boldsymbol{A_{sample}}}{\boldsymbol{A_{control}}}\right) \times \mathbf{100}$$

Where A_{sample} represents the absorbance of the test sample, and $A_{control}$ represents the absorbance of the blank control in the absence of the extract.

Cell-Based Melanin Inhibition Assay in B16F10 Cells

Cell cultures

B16F10 melanoma cells were routinely cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C.

Melanin contents asay

The melanin content assay was performed as described previously [8]. The cells were then seeded into a 24-well plate at a density of 0.05×10^6 cells per well and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, the culture medium was removed and replaced with fresh medium containing 50 µM IBMX to induce melanin production. Subsequently, various concentrations of the MA extract (25, 50, and 100 µg/mL) were added to the wells and incubated for an additional 48 hours. After 48 hours of incubation, the cells in each well were observed using a multidimensional image analysis system. The cells were detached from the 24-well plate and transferred to Eppendorf tubes. The Eppendorf tubes containing cell suspensions were centrifuged at 12,500 rpm for 20 minutes at 25°C using a refrigerated centrifuge. The supernatant was discarded, and the cell pellets resuspended in PBS and centrifuged again at 12,500 rpm for 15 minutes at 25°C. The PBS was removed, and the remaining cell pellets were visually examined for melanin content based on pigmentation intensity. 100 µL of 1N NaOH containing 10% DMSO was added to each tube, followed by incubation at 80°C for 90 minutes using a dry heating block. The resulting solutions were transferred to an ELISA plate, and intracellular melanin content was quantified by measuring absorbance at 490 nm with an ELISA reader and a multifunctional image analysis system. A 10% DMSO solution was used as the negative control (final DMSO concentration in the well was 0.5%). At the same time, alpha-arbutin (50 µg/mL) served as the positive control for melanin inhibition. The percentage of melanin content was calculated based on optical density using the following formula:

$$\boldsymbol{Melanin\ Inhibition}\ (\%) = \left(\mathbf{1} - \frac{\boldsymbol{A_{sample}}}{\boldsymbol{A_{control}}}\right) \times \mathbf{100}$$

MTT cell viability assay

Cell viability was determined using the MTT assay [12]. B16F10 cells in the logarithmic growth phase were seeded into 96-well plates (flat bottom; 100 µL/well) at a density of 7.500 cells/well and cultured for 24 hours under standard conditions (37°C, 5% CO_2). After 24 hours, the culture medium was replaced, and test samples were added. Cells were then incubated for an additional 48 hours. The MTT assay was performed by adding 100 µL of DMEM containing 10% MTT solution to each well. Cells were incubated for 30 minutes under light-protected conditions (37°C, 5% CO_2). The medium was removed, and a DMSO-PBS (2:1) solution was added. Cells were further incubated for 10 minutes under light-protected conditions. A 50 µL aliquot of the resulting solution was transferred to an ELISA plate. Absorbance was measured at 540 nm to determine cell viability.

Antioxidant assay

The antioxidant activity of *Morus alba* extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, following the method described by Gerasimova [13] with slight modifications. A 0.1 mM DPPH solution was prepared in methanol and stored in the dark. The assay was performed by mixing 100 µL of the extract solution at concentrations of 400, 200, 100, 50, 25, and 12.5 µg/mL with 100 µL of DPPH solution in a 96-well plate. The reaction was incubated at room temperature in the dark for 30 minutes, and the absorbance was measured at 517 nm using a microplate reader. Ascorbic acid was used as the positive control, and the percentage of DPPH radical scavenging activity was calculated using the following equation:

$$DPPH\ Scavenging\ (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

Where A_{sample} is the absorbance of the extract-treated sample, and $A_{control}$ is the absorbance of the control (DPPH solution without extract). Ascorbic acid, with an IC_{50} value of 4.47 ± 0.28 µg/mL, was used as a positive control.

Determination of total phenolic content (TPC)

The total phenolic content was assessed using the Folin-Ciocalteu assay as described by Singleton and Margraf et al. [14]. Briefly, 25 µL of appropriately diluted *M. alba* extract was pipetted into each well of a 96-well plate. Subsequently, 125 µL of Folin–Ciocalteu reagent (diluted 1:10 in distilled water) was added, and the mixture was incubated at room temperature for 5 minutes. Following incubation, 100 µL of 7.5% sodium carbonate (Na_2CO_3) solution was introduced into each well. The plate was then incubated in the dark at 37°C for 30 minutes to allow the colorimetric reaction to develop. After incubation, the absorbance was measured at 765 nm using a microplate reader. A standard calibration curve was prepared using gallic acid at concentrations ranging from 10 to 200 µg/mL under the same experimental conditions. The total phenolic content was expressed as mg of gallic acid equivalents per gram of extract (mg GAE/g extract) using a standard curve. All measurements were performed in triplicate, and appropriate blanks were included to account for background absorbance.

Gel formulation and preparation

The gel base was evaluated using different gelling agents, including sodium carboxymethyl cellulose (Na-CMC) and hydroxyethyl cellulose (HEC), at concentrations of 0.5%, 1%, and 2% (w/w)**.** Ethanol (96%) was incorporated into the formulations at 10% and 20% (w/w)**.** For Na-CMC-based formulations, the preparation process began by dispersing Na-CMC in a beaker, followed by the addition of an appropriate amount of distilled water. The mixture was allowed to hydrate thoroughly before ethanol was added. The formulation was then stirred thoroughly and homogenized using an IKA T25 Digital Ultra-Turrax homogenizer (Dispersion Tool T25 S25KV-18G) at 3600 rpm for 5 minutes to ensure uniform consistency. In the case of HEC-based formulations, the preparation process initially involved dispersing HEC in ethanol under continuous stirring. Distilled water was then added, and the mixture was allowed to hydrate fully. The formulation was subsequently stirred and homogenized at 3600 rpm for 5 minutes to achieve uniformity.

Physicochemical stability, heavy metal, and microbiological contamination assessment of MA gel

The selected gel formulation will be subjected to accelerated aging studies using a temperature and humidity test chamber (Taisite, CHI-80P, 80L) under controlled conditions of 40°C and 75% relative humidity (RH), as well as real-time stability testing at room temperature, for a duration of three months, in accordance with ICH guidelines [15]. The following parameters were assessed:

- pH: Measured using a digital pH meter (Hanna HI 2211, Italia) at room temperature.
- Viscosity: Determined using a viscometer (Daihan WVS2M, Korea, 1-2,000,000 cP).
- Homogeneity: Examined visually for phase separation, syneresis, or precipitation.
- Spreadability: The spreadability of the gel was assessed by measuring the diameter of the gel spread when 1 g of the formulation was placed between two glass plates (20 × 20 cm) and allowed to spread for 1 minute.

The concentrations of lead (Pb), arsenic (As), and mercury (Hg) in the MA gel were determined in accordance with the ACM THA 05 (Revision No.1, 2006) guideline, issued by the ASEAN Consultative Committee for Standards and Quality (ACCSQ) under the ASEAN Cosmetic Committee (ACC).

Microbiological evaluation was conducted following international standards, including ISO 21149:2017/Amd 1:2022 for aerobic mesophilic bacteria, ISO 16212:2017/Amd 1:2022 for total yeast and mold, ISO 22717:2015/Amd 1:2022 for ***Pseudomonas aeruginosa***, ISO 22718:2015/Amd 1:2022 for ***Staphylococcus aureus***, ISO 18416:2015/Amd 1:2022 for ***Candida albicans***, and ISO 21150:2015/Amd 1:2022 for ***Escherichia coli*.**

Statistical analysis

All experiments were performed in at least three independent replicates. Results are expressed as the mean of three replicates ± standard deviation (SD). Differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test to compare each treatment group with the control group.

A p-value of < 0.05 was considered statistically significant. Data processing and statistical analysis were performed using GraphPad Prism version 10.4.2 (trial version; GraphPad Software, USA).

RESULT AND DISCUSSION

Result

Tyrosinase inhibitory activity of *Morus alba* extract

The *Morus alba* extract exhibited significant tyrosinase inhibitory activity, with an IC_{50} value of 5.70 ± 0.28 µg/mL. As a benchmark, kojic acid, used as the positive control at 100 µg/mL, inhibited tyrosinase activity by 22.33% under the same assay conditions. These results highlight the strong inhibitory effect of *M. alba* extract compared to the standard control. Tyrosinase is a key enzyme in melanogenesis, catalyzing the conversion of tyrosine to melanin pigments. In clinical practice, first-line management of hyperpigmentation often involves tyrosinase inhibitors, underscoring the potential relevance of our findings. Our findings also indicate that the tyrosinase inhibitory activity of mulberry leaf extract is stronger than that of other plant extracts. For instance, a previous study on *Vitis vinifera* leaf extract reported an IC_{50} of 3.84 mg/mL [16], while an extract from Greyia flanaganii had an IC_{50} of 32.62 µg/mL [17]. Additionally, a crude procyanidin extract from green soybean seeds exhibited an IC_{50} of 6.85 mg/mL [18]. Furthermore, our results show greater tyrosinase inhibition than a 2020 study on mulberry leaves from Thailand [19], suggesting that regional variations in soil and climate may affect the plant's chemical composition & biological activity.

Melanin inhibition in B16F10 cells

MITF is a key regulator of melanogenic enzymes, including tyrosinase, TRP-1, and TRP-2, which collectively promote melanin production in melanocytes [20]. Treatment with MA extract resulted in a dose-dependent reduction in intracellular melanin accumulation, as evidenced by the progressive decrease in dark granules from 25 µg/mL to 400 µg/mL (Figure 1). Cells treated with higher concentrations (200 and 400 µg/mL) showed markedly reduced pigmentation, comparable to the positive control (arbutin 50 µg/mL), while cells in the 0.5% DMSO group exhibited dense melanin accumulation. Microscopic analysis (Figure 1) revealed distinct differences in intracellular melanin accumulation across treatment groups. In the negative control group (0.5% DMSO), B16F10 melanocytes exhibited dense, widespread, darkly pigmented granules throughout the cytoplasm, confirming that IBMX stimulation induced melanogenesis. Conversely, the positive control group treated with arbutin (50 µg/mL) showed a substantial reduction in pigmentation, with very few visible melanin granules, indicating strong inhibition of melanin synthesis. Treatment with MA extract demonstrated a dose-dependent inhibitory effect on melanin production. At 25 µg/mL, cells showed a modest decrease in melanin granules, indicating partial inhibition of melanogenesis; increasing the concentration to 50 µg/mL further reduced melanin content, with fewer dark granules. At 100 µg/mL, only sparse pigmentation remained, resembling the pattern observed in arbutin-treated cells. Higher concentrations of 200 and 400 µg/mL resulted in an almost complete absence of melanin granules, and cells appeared morphologically normal, suggesting potent anti-melanogenic activity without apparent cytotoxicity. These findings confirm that *Morus alba* extract suppresses melanin synthesis in a concentration-dependent manner, supporting its potential as a natural skin depigmenting agent.

Figure 2 presents a qualitative evaluation of melanin inhibition by MA extract in a dose-dependent manner.

- Panel A displays the color of the culture medium, representing secreted (extracellular) melanin. A visible decrease in pink coloration is observed from tube 1 (0.5% DMSO, negative control) to tube 7 (MA extract at 400 µg/mL), indicating reduced melanin secretion.
- Panel B shows the appearance of cell pellets after centrifugation. The gradual lightening of the pellet color with increasing MA concentrations reflects reduced intracellular melanin accumulation.
- Panel C illustrates the pellets dissolved in 1 N NaOH containing DMSO to quantify intracellular melanin. The solutions appear progressively lighter from tube 1 to tube 7, indicating a dose-dependent decrease in melanin content.

Tubes 1–7 correspond to the following treatments:

1. 0.5% DMSO (negative control)
2. Arbutin 50 µg/mL (positive control)

3–7. MA extracts at 25, 50, 100, 200, and 400 µg/mL, respectively. These results visually confirm that MA extract effectively inhibits melanogenesis both extracellularly and intracellularly in a concentration-dependent manner.

Visual evaluation of melanin content (Figure 2) supported the microscopic findings. In the negative control group (tube 1), the culture medium (A1) exhibited the darkest color, indicating substantial melanin secretion. In contrast, the positive control (tube 2, arbutin 50 µg/mL) showed a markedly lighter medium, consistent with reduced melanin synthesis and release. Tubes 3–7, treated with increasing concentrations of MA extract, showed a gradual lightening of the medium color, reflecting a concentration-dependent reduction in extracellular melanin.

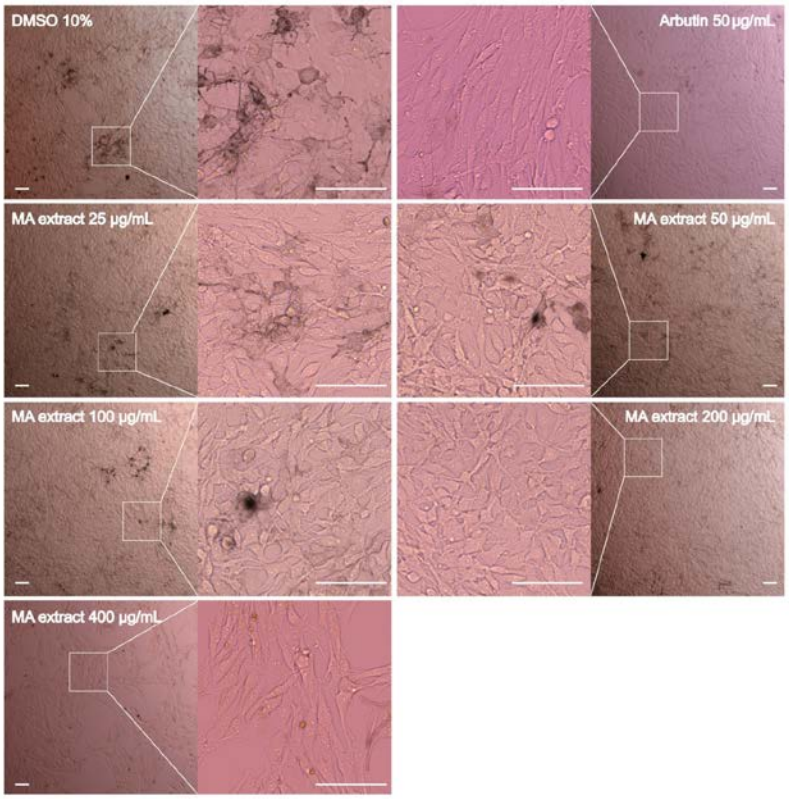

Figure 1: Microscopic evaluation of melanin content in B16F10 melanocytes treated with *Morus alba* (MA) extract (25–400 µg/mL) compared with controls. Cells were treated with 0.5% DMSO (negative control) or arbutin 50 µg/mL (positive control). Representative images show intracellular melanin accumulation at different magnifications

In panel B, cell pellets from the DMSO group appeared the darkest, consistent with significant intracellular melanin accumulation. Progressive lightening of pellet pigmentation was observed from tubes 3 to 7, with 200 and 400 µg/mL treatments (tubes 6 and 7) exhibiting pigmentation levels comparable to the arbutin group. Panel C further confirmed these trends. After dissolving cell pellets in 1 N NaOH containing DMSO, the solution from the DMSO control (tube 1) appeared darkest. In contrast, samples treated with increasing concentrations of MA extract displayed lighter coloration, indicating decreased intracellular melanin content. Collectively, these results provide strong evidence that *Morus alba* extract inhibits both extracellular and intracellular melanin synthesis in a dose-dependent manner, highlighting its potential as a promising skin-lightening agent.

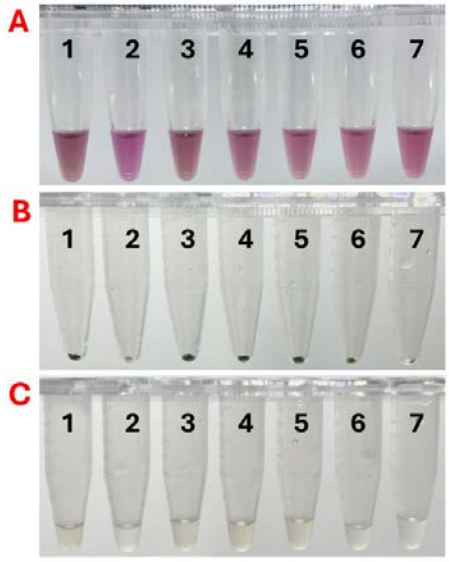

Figure 2: Visual assessment of melanin content in B16F10 melanocytes treated with Morus alba (MA) extract. (A) Extracellular melanin in culture medium, (B) Intracellular melanin in cell pellets, and (C) Total melanin after lysis. Representative images show a reduction in melanin content following MA extract treatment relative to controls.

Table 1: Melanin synthesis inhibition and cytotoxicity of *Morus alba* (MA) leaf extract at various concentrations in B16F10 melanocytes, compared with alpha-arbutin and DMSO (0.5%) control.

Sample	% Melanin content	% Viability cells
DMSO 0.5% - IBMX	100 ± 1.67	100
MA extract 25 µg/mL	90.83 ± 11.03	116.16 ± 12.37
MA extract 50 µg/mL	85 ± 9.27	116.21 ± 3.77
MA extract 100 µg/mL	81.11 ± 9.87*	115.79 ± 3.35
MA extract 200 µg/mL	66.39 ± 9.36***	94.33 ± 1.76
MA extract 400 µg/mL	55.83 ± 0.83***	74.90 ± 3.67**
Alpha-arbutin (50 µg/mL)	66.10 ± 3.57***	NA

Data are presented as mean ± SD (n = 3). *Statistical significance compared to the negative control (DMSO 0.5%) in melanin content: MA extract at 100 µg/mL (*p < 0.05); MA extract at 200 µg/mL (p < 0.001); MA extract at 400 µg/mL (***p < 0.001) and alpha-arbutin at 50 µg/mL (***p < 0.001). Statistical significance compared to the negative control (DMSO 0.5%) in cell viability: MA extract at 400 µg/mL (**p < 0.01)***

Table 1 summarizes the inhibitory effects of MA extract on melanin synthesis and its cytotoxicity in B16F10 melanocytes. The 0.5% DMSO group (stimulated with IBMX) served as the baseline (100% melanin content and 100% cell viability) for relative comparison. MA extract demonstrated a dose-dependent inhibition of melanin production. At 25 µg/mL, melanin content was reduced to 90.83 ± 11.03%, indicating mild inhibition. Increasing the concentration to 50 and 100 µg/mL further reduced melanin levels to 85.00 ± 9.27% and 81.11 ± 9.87% ($p < 0.05$), respectively. Notably, 200 and 400 µg/mL treatments resulted in a significant decrease, with melanin content reduced to 66.39 ± 9.36% and 55.83 ± 0.83%, respectively ($p < 0.001$), comparable to or exceeding the effect of alpha-arbutin (66.10 ± 3.57%; $p < 0.001$). Regarding cytotoxicity, the MA extract showed no detrimental effect on cell viability at 25–100 µg/mL, with values exceeding 115%, suggesting potential proliferative or antioxidant-enhancing effects. At 200 µg/mL, viability declined slightly to 94.33 ± 1.76%, while 400 µg/mL reduced viability to 74.90 ± 3.67% ($p < 0.01$), indicating mild cytotoxicity at higher concentrations. These findings support the conclusion that Morus alba extract effectively inhibits melanin synthesis while maintaining acceptable cell viability, particularly at concentrations up to 200 µg/mL, underscoring its potential as a safe and effective depigmenting agent.

Antioxidant activity of *Morus alba* extract

The antioxidant potential of *Morus alba* leaf extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay, yielding an IC_{50} value of 16.22 ± 0.6 µg/mL. This result indicates that *Morus alba* leaf extract exhibits potent antioxidant activity. Free radicals are believed to play a significant role in disrupting skin health, including conditions like melasma. Several studies also indicate that a high total polyphenol content is closely linked to a strong ability to neutralize free radicals. Therefore, total polyphenols can be considered a key factor in the treatment of hyperpigmentation [21], [22], [23].

Total polyphenol content in the extract

The total polyphenol content (TPC) of the MA leaf extract was found to be 32.60 ± 0.22 mg GAE/g extract, indicating a high concentration of phenolic compounds in the raw material. This is consistent with previous reports highlighting the richness of *Morus alba* leaves in bioactive polyphenols, such as chlorogenic acid, rutin, and quercetin derivatives, which contribute to antioxidant and anti-tyrosinase activities [24, 25].

Gel formulation

After evaluating gelling excipients, HEC was found to have better gelling properties and higher viscosity than Na-CMC. Both materials produced transparent gels. However, increasing the Na-CMC concentration led to a yellowish discoloration, whereas ethanol reduced the gel's viscosity. In contrast, HEC was not affected by these factors. Based on these findings, the final optimized formulation was selected using HEC at 2.0% (w/w) with 20% (w/w) ethanol. The gel composition is presented in Table 2.

Total polyphenol content and tyrosinase inhibitory activity of *Morus alba* gel

The TPC in the gel was quantified at 0.32 ± 0.04 mg GAE/g gel, indicating successful incorporation of the extract into the topical vehicle. Although this value represents a dilution from the original extract, it remains within a functional range for skin application, particularly in cosmetic products intended for antioxidant or skin-brightening effects [26]. The retention of

polyphenols in the gel formulation also demonstrates that the gel base is compatible with and capable of stabilizing polyphenolic compounds, a critical factor for maintaining biological efficacy during product storage and application.

The tyrosinase inhibitory activity of the MA gel was evaluated to assess its potential as a natural skin depigmenting agent. The results showed that the gel exhibited an IC_{50} of 0.76 ± 0.018 mg/mL, indicating moderate in vitro tyrosinase inhibition.

Table 2: MA (*Morus alba*) Gel composition

Ingredient	Concentration (%)	Function
***Morus alba* extract**	1.0	Active ingredient (anti-tyrosinase, antioxidant)
Hydroxyethyl cellulose (HEC)	2.0	Gelling agent
Sodium benzoate	0.25	Preservative
Ethanol	20.0	Solvent, penetration enhancer
Isopropanol	1.0	Solvent, antimicrobial
Propylene glycol	1.0	Humectant, penetration enhancer
Glycerin	1.0	Moisturizer, skin-conditioning agent
RO water	q.s. to 100%	Solvent

Gel characterization

Table 3 shows the results of the stability assessment of the *Morus alba* leaf extract gel over 3 months under two conditions: room temperature and accelerated aging conditions (40°C, 70% RH). Key parameters monitored include appearance, pH, viscosity, homogeneity, spreadability, and total polyphenol content (TPC).

Table 3: Stability monitoring

Condition	Assessment parameters	Month 0	Month 1	Month 2	Month 3
Room temperature	**Appearance**	Viscous gel, smooth, moss green color	Viscous gel, smooth, moss green color	Viscous gel, smooth, moss green color	Viscous gel, smooth, moss green color
	pH	5.71	5.68	5.64	5.62
	Viscosity (cP)	180,400	176,600	176,400	-
	Homogeneity	Uniform	Uniform	Uniform	Uniform
	Spreadability (g.cm/s)	55.46	56.86	56.30	56.86
	TPC (µg GAE/g gel) ± SD	322.47 ± 4.14	320.00 ± 0.95	317.80 ± 0.82	314.78 ± 1.90
Accelerated Aging (40°C, 70% RH)	**Appearance**	Viscous gel, smooth, moss green color	Viscous gel, smooth, moss green color	Viscous gel, smooth, moss green color	Viscous gel, smooth, moss green color
	pH	5.71	5.59	5.47	5.38
	Viscosity (cP)	180,400	174,000	173,600	-
	Homogeneity	Uniform	Uniform	Uniform	Uniform
	Spreadability (g.cm/s)	55.46	55.18	56.58	55.74
	TPC (GAE/g gel) ± SD	322.47 ± 4.14	317.53 ± 3.43	312.58 ± 1.71	310.66 ± 0.47

Under both storage conditions, the appearance, color, and homogeneity of the gel remained unchanged, maintaining a viscous, smooth, moss-green character and a uniform consistency throughout the 3 months. This visual stability suggests that the formulation is physically stable and does not undergo phase separation or discoloration, both common issues in herbal-based formulations. The pH of the gel showed a slight downward trend over time in both conditions, from 5.71 at Month 0 to 5.62 at Month 3 at room temperature, and to 5.38 under accelerated aging. Despite the decrease, pH values remained within the acceptable physiological range for topical application (4.5–6.5), indicating that the formulation is unlikely to cause skin irritation [27].

Viscosity declined modestly under both conditions, with a slightly sharper reduction under accelerated aging (from 180,400 cP to 174,000 cP at Month 1). Although viscosity measurements beyond Month 1 under accelerated conditions were unavailable, the initial reduction may indicate polymer relaxation or water loss due to the higher temperature. Nonetheless, the remaining viscosity values suggest the formulation maintained its semisolid gel consistency.

The spreadability of the gel remained relatively stable across all time points and conditions, with minor fluctuations ranging from 55.18 to 56.86 g·cm/s. These small changes are within acceptable formulation tolerances and do not significantly

impact the product's usability or application experience. The total polyphenol content (TPC) decreased gradually over time. At room temperature, TPC decreased from 322.48 ± 4.15 to 314.78 ± 1.90 µg GAE/g gel after 3 months, while under accelerated conditions, the decline was slightly more pronounced (to 310.66 ± 0.48 µg GAE/g gel). However, these changes represent only a 2.4–3.6% reduction, indicating good chemical stability of the active compounds over time. The results confirm that the gel matrix protects polyphenolic compounds from rapid degradation even under stress conditions.

Table 4: Heavy metal and microbial limits

No.	Test Parameter	Unit	Result
1	**Aerobic mesophilic bacteria**	CFU/g	< 10
2	**Total yeast, mold**	CFU/g	< 10
3	***Pseudomonas aeruginosa***	/ 0.1 gram	Not detected
4	***Staphylococcus aureus***	/ 0.1 gram	Not detected
5	***Candida albicans***	/ 0.1 gram	Not detected
6	***Escherichia coli***	/ 0.1 gram	Not detected
7	**Arsenic (As)**	mg/kg	Not detected
8	**Lead (Pb)**	mg/kg	Not detected
9	**Mercury (Hg)**	mg/kg	Not detected

As shown in Table 4, the MA gel met all safety criteria for microbial and heavy metal contamination. Aerobic mesophilic bacteria and total yeast/mold counts were <10 CFU/g, and no pathogenic microorganisms (*P. aeruginosa*, *S. aureus*, *C. albicans*, *E. coli*) were detected. Heavy metals (As, Pb, Hg) were not detected, confirming the product's microbiological and chemical safety for topical use. Furthermore, these results complied with the permissible limits established by international cosmetic regulations, including the ASEAN Cosmetic Directive, the European Union Regulation (EC No. 1223/2009), and US FDA guidance. This indicates that the formulation not only demonstrates promising biological efficacy but also meets essential international safety requirements for cosmetic applications in global markets.

Discussion

Although agents such as hydroquinone, kojic acid, and arbutin are commonly used to inhibit melanin synthesis, their long-term use raises safety concerns [28-30]. This has encouraged the search for safer, plant-derived alternatives. The *Morus alba* leaf extract revealed a concentration-dependent inhibitory effect on melanin production in B16F10 murine melanoma cells. At concentrations of 100 and 200 µg/mL, a marked reduction in melanin content was observed, indicating that the extract interferes with the melanogenesis pathway. This effect is consistent with the known depigmenting potential of *M. alba*, which has been attributed primarily to its rich polyphenolic content, including flavonoids and phenolic acids. However, cell viability declined at 400 µg/mL. This finding suggests a relatively narrow safety margin at higher concentrations and should be carefully considered when developing topical formulations. As this study was limited to in vitro assays, further in vivo and clinical evaluations are required to confirm the safe concentration range and establish a clear therapeutic window for cosmetic applications.

Melanin biosynthesis in melanocytes is primarily regulated by tyrosinase, a copper-containing enzyme that catalyzes the hydroxylation of L-tyrosine to L-DOPA and its subsequent oxidation to dopaquinone. By inhibiting tyrosinase activity, the extract likely disrupts the rate-limiting step of melanin production. The notable tyrosinase inhibitory activity observed in this study ($IC_{50} = 5.70 \pm 0.28$ µg/mL) supports this hypothesis. The extract's ability to reduce melanin synthesis without causing cell toxicity suggests that it selectively targets the melanin synthesis pathway. This is a crucial characteristic, as it demonstrates strong potential for application in developing skin-care products.

The results of this study align with previous reports, including Jeong et al. (2015), who reported that a 70% ethanol extract of mulberry leaves effectively inhibited α-MSH-induced melanogenesis in B16F10 cells [31]. These findings further solidify the biological effects of mulberry leaves [7]. Studies have also indicated correlations among total polyphenol content, antioxidant capacity, and tyrosinase inhibitory activity in plant extracts [30]. For example, Mapoung et al. (2021) analyzed 23 commercial cosmetic creams and reported that higher TPC content was associated with stronger antioxidant and tyrosinase-inhibitory activities [32]. Other studies on magnolia flower and bee pollen extracts have also shown a positive correlation among TPC content, antioxidant capacity, and tyrosinase inhibition [33],[34]. The polyphenol content may enhance the extract's antioxidant properties, thereby reducing the formation of reactive oxygen species (ROS) and attenuating oxidative stress–induced overactivation of melanogenic pathways. Although the extract demonstrated strong antioxidant activity and measurable TPC, only a single TPC value of the crude extract was

determined in this study. As such, statistical correlation analysis between TPC and biological activities was not possible. This represents a limitation of the current work, and future studies should investigate TPC across different fractions or concentrations to better elucidate its relationship with melanin inhibition and tyrosinase inhibition. Unlike most prior studies that limited their evaluation to crude extracts, this study successfully translated the bioactivity of *M. alba* into a topical gel formulation. The formulation retained phenolic content and inhibitory activity while demonstrating physicochemical stability & regulatory compliance, underscoring its novelty & practical relevance for cosmetic development.

Overall, this study on mulberry leaves provides new information about a plant commonly used in traditional medicine. Based on specific and reliable data, the results indicate that this medicinal plant can be used to develop a product beneficial for individuals with skin pigmentation disorders. In the future, further research, such as standardizing the extract and standardizing the gel production process, and conducting clinical trials, will be necessary.

CONCLUSION

In summary, the study demonstrates that mulberry leaf extract exhibits strong tyrosinase inhibitory activity (IC_{50} = 5.70 ± 0.28 µg/mL). In IBMX-induced B16F10 melanocytes, the Morus alba extract inhibited melanin production in a dose-dependent manner, both intracellularly and extracellularly, and demonstrated greater efficacy than arbutin at 400 µg/mL. In addition to its anti-melanogenic effects, the extract exhibited potent antioxidant activity (IC_{50} = 16.22 ± 0.6 µg/mL), which may contribute to its protective effects against oxidative stress-induced melanogenesis. These biological activities were further supported by its high total polyphenol content and the ability to retain and release polyphenols effectively when formulated into a topical gel. The optimized gel formulation, containing 1% MA extract and 2% hydroxyethyl cellulose, exhibited favorable physicochemical stability for three months under both room-temperature and accelerated conditions. The formulated gel retained measurable tyrosinase inhibitory activity (IC_{50} = 0.76 ± 0.018 mg/mL), suggesting that the extract's bioactivity is preserved within the vehicle.

Taken together, this study demonstrates that *Morus alba* leaf extract possesses significant anti-melanogenic and antioxidant activities and can be feasibly incorporated into a stable topical gel formulation. These findings contribute to the scientific understanding of M. alba as a potential source of natural depigmenting agents and highlight the importance of its formulation development. Nevertheless, further research, including standardized production processes and clinical evaluations, will be essential to confirm efficacy and safety for future cosmetic applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Nguyen Minh Nam was responsible for conceptualizing the study, conducting the investigation, and contributing to the manuscript review and editing. Bui Thi Phuong oversaw the project's administration and secured funding for the research. Nguyen Ngoc Dien was involved in data visualization and investigation activities. Nguyen Tan Tai conducted data curation and participated in the investigation. Tran Thi Huyen provided supervision and resources, drafted the original manuscript, and performed the formal data analysis.

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