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Ittipon Siridechakorn

Chulalongkorn University

Junsuda Pimpa

Chulalongkorn University

Siwattra Choodej

King Mongkut's University of Technology Thonburi

Khanitha Pudhom (✉ khanitha.p@chula.ac.th)

Chulalongkorn University

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Synergistic Impact of Arbutin and Kaempferol-7-*O*- α -L-rhamnopyranoside from *Nephelium lappaceum* L. on Whitening Efficacy and Stability of Cosmetics Formulations

Ittipon Siridechakorn¹, Junsuda Pimpa¹, Siwattra Choodej², Khanitha Pudhom^{1,*}

¹Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand.

²Department of Chemistry, Faculty of Science, King Mongkut's University of Technology Thonburi, 10140, Thailand.

*Corresponding author: Khanitha Pudhom

Email address: khanitha.p@chula.ac.th

ABSTRACT

Four flavonoid glycosides, namely quercetin-3-*O*-rhamnoside (**1**), kaempferol-3-*O*- β -D-glucopyranosyl (**2**), kaempferol-7-*O*- α -L-rhamnopyranoside (**3**), and kaempferol-3-*O*- β -D-glucopyranosyl-7-*O*- α -L-rhamnopyranoside (**4**), from *Nephelium lappaceum* L. seeds were evaluated for their efficacy against melanin inhibition in B16F10 melanoma cells and tyrosinase inhibition. Among them, kaempferol-7-*O*- α -L-rhamnopyranoside (**3**) displayed the highest potency in both activities without any significant cytotoxicity. The combination of compound **3** and arbutin in specific proportions demonstrated a synergistic effect ($CI < 1$) in inhibiting melanin production in B16F10 cells and tyrosinase inhibition. Additionally, a cosmetic formulation containing compound **3** and arbutin as active ingredients exhibited favorable stability under accelerated storage conditions. Quantitative analysis indicated that compound **3** and arbutin levels in the formulation were above 90% after one month of storage.

Introduction

Melanin, a pigment responsible for shielding the skin against ultraviolet (UV) damage and regulating skin pigmentation, plays a pivotal role in maintaining skin integrity. However, excessive melanin production and accumulation lead to various skin pigmentation disorders, including melasma, freckles, and dark spots. Hyperpigmentation profoundly affects an individual's physical appearance and self-assurance in social interactions. Presently, the treatment of hyperpigmentation entails the utilization of whitening agents, chemical peels, and laser therapy.¹ Among these modalities, combination therapy emerges as the optimal treatment approach for hyperpigmentation, as it harnesses synergistic effects while mitigating the risk of unintended consequences.

Polyphenolic compounds, characterized by an aromatic ring containing at least one hydroxyl group, are prominent secondary metabolites found in nature. These compounds can be categorized based on their chemical structures, including phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids.²⁻⁴ Notably, kempferol-7-O- α -L-rhamnopyranoside, a prominent constituent found in various plant species, including *Nephelium lappaceum* L., has been documented for its ability to inhibit nitric oxide (NO) production, along with its antioxidant and anti-melanogenic activities.⁵⁻⁷

Synergy is a phenomenon wherein the combined effect of two or more substances surpasses the cumulative impact of their individual effects. In addition to its impact on biological activity, synergy can also influence the physicochemical properties of compounds or mixtures, including solubility and absorption efficiency.⁸ Given the aim of enhancing the efficacy of bioactive phenolics derived from Thai fruit wastes, investigating their synergistic effects in conjunction with commercially available whitening agents holds significant relevance.

Thailand is renowned for its agricultural prominence, characterized by the cultivation of a diverse array of crops. The country's thriving fruit processing industry generates a substantial proportion of byproducts, encompassing peels, seeds, and unused flesh, during various stages of processing. According to the Food and Agricultural Organization (FAO) of the United States, a significant quantity of agricultural residues, accounting for up to 50%, is annually available as byproducts, while processing activities result in waste production ranging from 25% to 30%.⁹ In this context, *N. lappaceum* L., an economically significant crop in Thailand, holds considerable potential as a source of agro-industrial waste for utilization in the cosmetic industry. This approach not only aids in waste reduction but also facilitates value addition. Presently, there is a growing preference for natural products within the cosmetic industry to circumvent the potential side effects associated with synthetic chemicals.

Hence, the present study aims to investigate the inhibitory activity of phenolic compounds from *N. lappaceum* L. on melanin production, while also exploring the potential synergistic effects when combined with melanin-inhibiting whitening agents and/or tyrosinase inhibitors. Additionally, the chemical stability of both these substances will be examined within cosmetic formulations.

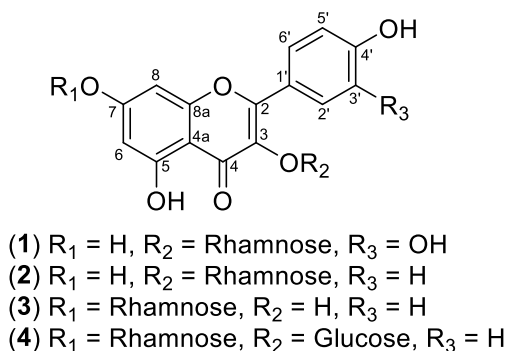


Figure 1. Structures of compounds **1-4** isolated from *N. lappaceum* L. seed extract.

Results and discussion

Anti-melanin activity and cytotoxicity of compounds 1-4. To assess the effectiveness of phytochemicals isolated from the *N. lappaceum* L. seed extract in inhibiting melanin production, a B16F10 cell-based anti-melanogenic assay was conducted on four specifically chosen compounds (**1-4**) (Figure 1). As a positive control, arbutin, a widely recognized tyrosinase inhibitor frequently employed as a cosmetic whitening agent, was utilized.¹⁰ Among them, compound **3** exhibited the most potent anti-melanogenic activity in a concentration-dependent manner, while no significant toxicity was observed (Figure 2C). It provided an IC₅₀ value of $23.0 \pm 1.3 \mu\text{M}$, indicating compound **3** was about 35-fold more potent than positive control arbutin (IC₅₀ = $807.6 \pm 2.9 \mu\text{M}$) (Table 1). The remaining three compounds **1-2** and **4** could also suppress melanin production in B16F10 cells in a dose-dependent manner, with IC₅₀ values of 134.8 ± 2.1 , 190.5 ± 2.2 , and $>200 \mu\text{M}$, respectively (Figures 2A, 2B and 2D). From these data, some structure-activity relationships could be seen. The position of the rhamnose moiety might play an important role in melanin inhibition and cytotoxicity. Attachment of rhamnose at the C-7 position to kaempferol core skeleton as in **3** exerted a remarkably potent melanin reduction, whereas the C-3 existence of rhamnose in **2** caused much decrease in anti-melanogenic activity but increase in cellular toxicity. In addition, it was implied that the presence of a glucosyl moiety at C-3 position as in **4** made the compound loss of both melanin suppression and toxicity. Surprisingly, Tang et al. previously reported that kaempferol itself did induce melanin production and melanocyte growth.¹¹

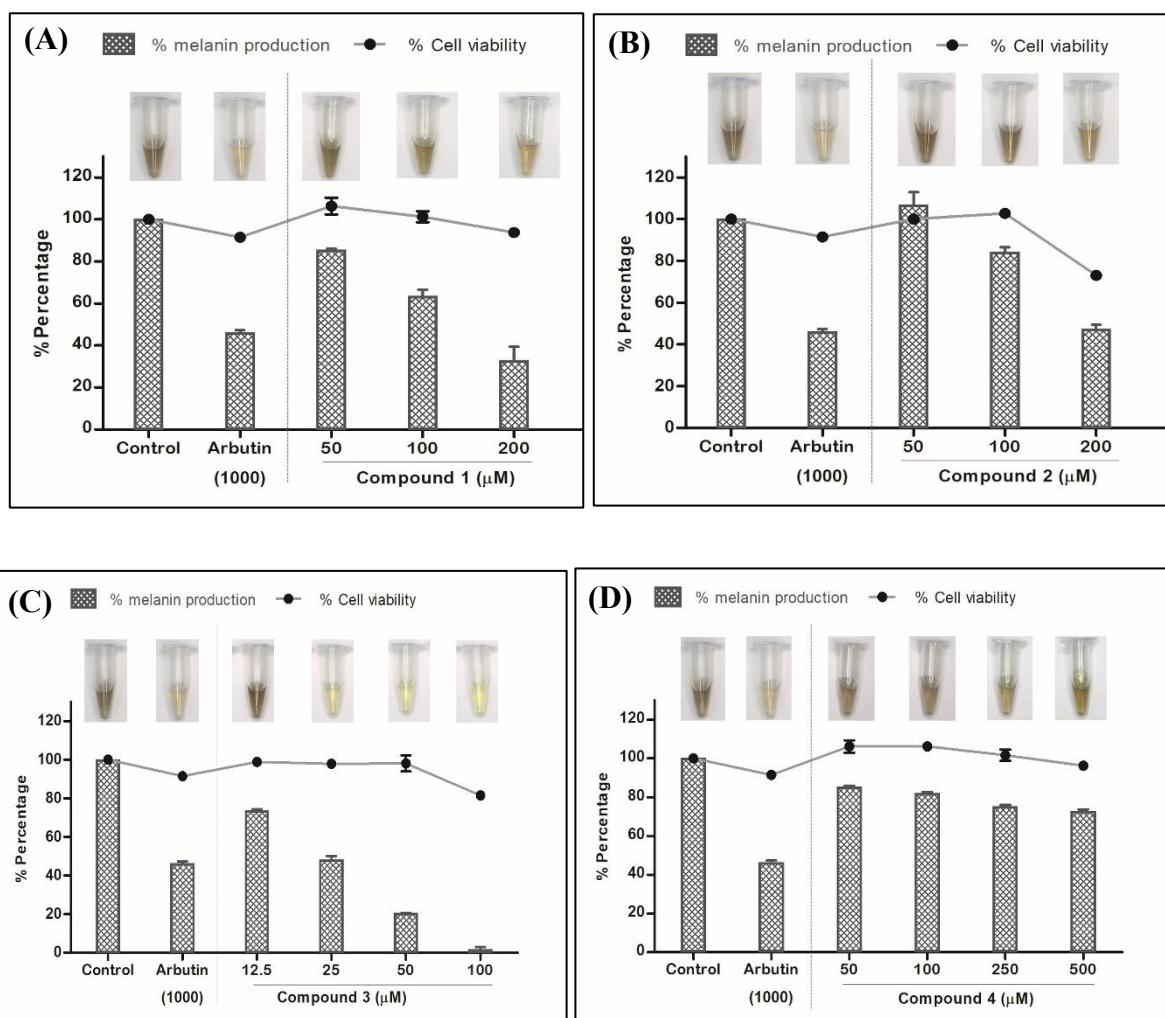


Figure 2. %Melanin content and %cell viability of compounds 1-4 (A-D)

Compounds	IC ₅₀ (μM)	Cytotoxicity (μM)
1	134.8 $\pm$ 2.1	>200
2	190.5 $\pm$ 2.2	200
3	23.0 $\pm$ 1.3	>1000
4	>200	>1000
Arbutin	807.6 $\pm$ 2.9	>1000

Data are expressed as mean $\pm$ SD (n = 3)

Table 1. Anti-melanogenic activity of compounds 1-4 and arbutin on B16F10 melanoma cells.

Anti-tyrosinase activity of compound 3. Tyrosinase plays a pivotal role as an essential enzyme, exhibiting multifarious catalytic functionalities within the melanogenesis process.¹² Hence, inhibiting tyrosinase represents a crucial strategy in addressing disorders associated with pigmentation. In this study, the inhibitory activity of the compound on tyrosinase was evaluated through the employment of a mushroom tyrosinase assay. Based on the anti-melanin activity, compound **3** was chosen for subsequent investigation owing to its pronounced melanin inhibition activity, in addition to the absence of discernible cytotoxicity even after 72 hours of treatment with concentrations as high as 1000 μM . Most notably, its anti-melanogenic activity exhibited a 35-fold superiority to that of arbutin. Compound **3** exhibited strong anti-tyrosinase activity, with an IC_{50} value of $25.3 \pm 0.1 \mu\text{M}$, comparable to kojic acid (positive control) and much potent than arbutin (active ingredient in commercial skincare products) (Table 2). This heightened activity might be attributed to the hydroxyl groups present in kaempferol, which facilitate binding to the enzymatic site of tyrosinase. Consequently, this binding impedes the catalytic oxidation of L-DOPA to L-DOPA quinone via catecholate activity.¹³

Sample	$\text{IC}_{50} (\mu\text{M})$
Compound 3	25.3 ± 0.1
Arbutin*	1166 ± 0.04
Kojic acid**	39.0 ± 0.02

*Active ingredient in commercial skincare products

**Positive control

Table 2. Anti-tyrosinase activity of compound **3**, arbutin and kojic acid.

Synergistic Impact of arbutin and kaempferol-7-*O*- α -L-rhamnopyranoside (3). Recent research findings have revealed the application of arbutin, a hydroquinone compound, and kojic acid as potent tyrosinase inhibitors within the realm of cosmetic formulations. Nevertheless, the usage of hydroquinone has been associated with undesirable consequences, including irritative

responses, dermatitis, and the impairment of melanin. Furthermore, arbutin derivatives undergo catalysis, leading to the production of benzene metabolites that may possess inherent hematotoxicity.^{14,15} Furthermore, when kojic acid is utilized at concentrations exceeding 1%, it has the potential to elicit skin irritations, contact dermatitis, and increased vulnerability to sunburn, particularly in individuals possessing sensitive skin conditions.¹⁶ The synergistic effect is a phenomenon that arises when two or more substances collaborate to yield an aggregated impact surpassing the cumulative effect of their individual properties. Moreover, the synergistic effects may extend to influence the physicochemical characteristics of compounds or mixtures, such as solubility.⁸ Arbutin is a natural product which has gained widespread employment as a depigmentation agent within the cosmetic industry. In current study, compound **3** was selected to be synergistically combined with arbutin to investigate their collective impact on suppressing tyrosinase activity and inhibiting melanin production in B16F10 cells. Various combinations of compound **3** and arbutin were prepared, utilizing ratios of 1:5, 1:10, 1:20, and 1:40 to reveal a notable reduction in melanin content within B16F10 cells (Figure 3). Treatment with compound **3** and arbutin mixtures resulted in reductions of 39.4%, 63.1%, 68.0%, and 71.5% in melanin content, respectively. These sample mixtures were further subjected to evaluation of their anti-tyrosinase activity, which manifested as a reduction in inhibitory percentages of 23.5%, 27.3%, 37.0%, and 49.6%, respectively (Table 3). The calculation of combination index (CI) values revealed that the mixtures comprising compound **3** and arbutin exhibited synergistic effects, as evidenced by CI values below 1.0 (Table 3). Notably, treatment with a 1:40 mixture of compound **3** and arbutin demonstrated the most potent inhibitory effects on melanin production in B16F10 cells, resulting in a remarkable reduction of up to 75.6%. These findings indicated that the combined mixture of compounds exhibits enhanced efficacy as melanin inhibitors compared to

their individual extracts. Additionally, Table 3 provides a summary of the percentage inhibition of tyrosinase activity and the corresponding CI values of the mixtures. The results indicated that the mixtures of compound **3** and arbutin also displayed a synergistic effect, as indicated by CI values below 1.0.

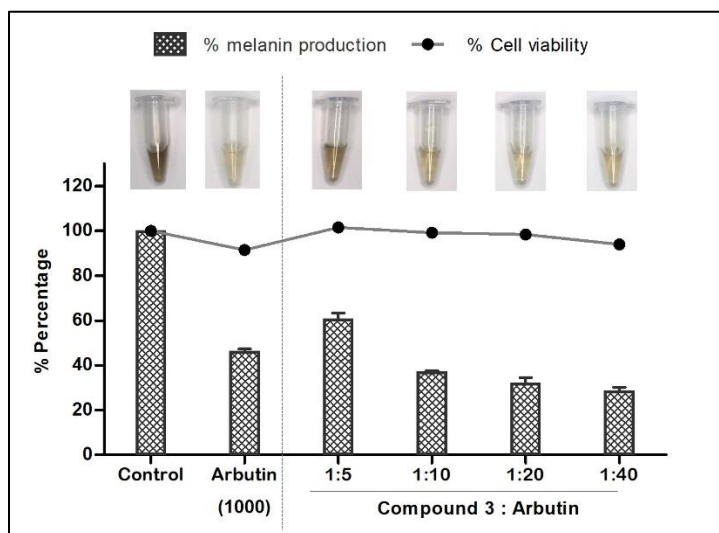


Figure 3. %Melanin content and %cell viability of the mixtures of compound **3** and arbutin

Concentration (μ M)		Ratio	Anti-melanin activity		Anti-tyrosinase activity	
Compound 3	Arbutin		% Inhibition	CI value	% Inhibition	CI value
12.5	62.5	1:5	39.4 $\pm$ 2.2	0.78	23.5 $\pm$ 3.4	0.92
12.5	125	1:10	63.1 $\pm$ 0.5		27.3 $\pm$ 4.3	
12.5	250	1:20	68.0 $\pm$ 2.0		37.0 $\pm$ 2.6	
12.5	500	1:40	71.5 $\pm$ 1.3		49.6 $\pm$ 1.6	

Table 3. Anti-melanin and anti-tyrosinase activities of the mixtures of compound **3** and arbutin.

Stability of Cosmetics Formulations. *Physical stability.* The physical attributes of formulation **F1** were examined, revealing no discernible alterations in color, odor, or texture. Moreover, **F1** demonstrated homogeneity, maintaining its uniformity without any indications of phase separation during centrifugation (Table 4). Additionally, over the duration of the accelerated storage period, **F1** displayed consistent pH values, with no significant deviations detected. In

contrast, it was observed that formulation **F2** experienced a color change after two weeks of the study (Figure 4). The observed phenomenon can be attributed to the utilization of triethanolamine as a pH-adjusting base in formulation **F2**, aimed at achieving an optimal pH range for the product. Subsequently, alkaline hydrolysis¹⁷ took place specifically with compound **3**, leading to a discernible alteration in the product color right from the initiation of the formulation process. Furthermore, during the storage period under accelerated conditions, the color change became increasingly conspicuous and evident.

Formulation F1



Formulation F2

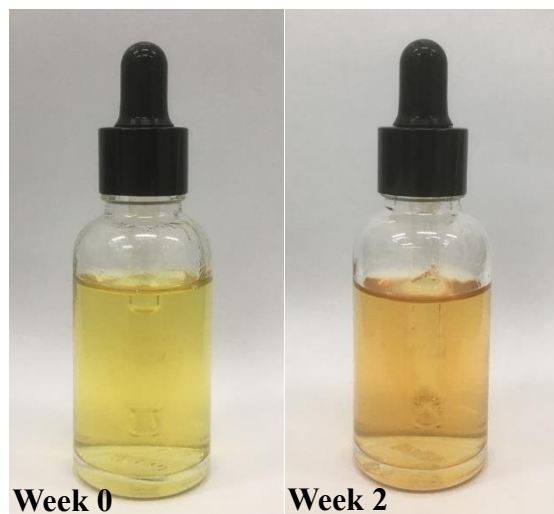


Figure 4. Color of the formulation **F1** and **F2** in the accelerated storage period.

Organoleptic test		Formulation F1	Formulation F2
Color change		Not Change	Change
Odor change		Not Change	Not Change
Phase separation		Not Change	Not Change
pH	Weeks		
	0	5.88 ± 0.01	6.85 ± 0.00
	1	5.88 ± 0.01	7.41 ± 0.07
	2	5.86 ± 0.01	7.50 ± 0.01
	3	5.85 ± 0.02	7.44 ± 0.05
	4	5.84 ± 0.01	7.44 ± 0.05

Table 4. Physical stability test of Formulation **F1** and **F2**.

Chemical stability. Formulation **F1** was employed for assessing the chemical stability, as it exhibited consistent physical stability under accelerated conditions without any observable changes. Performing the HPLC analysis using a single peak chromatogram of standard compounds, compound **3** and arbutin to generate the calibration curve (Figure 5). Calibration curves of compound **3** and arbutin were performed at different concentrations (compound **3**: 3.90, 7.81, 15.62, 31.25, 62.50 µg/mL, arbutin: 15.62, 125, 250, 500, 1000 µg/mL) (Figure 6). The amount of each compound **3** and arbutin was calculated based on a linear equation: $Y=99040X+352623$, $R^2=0.9964$ and $Y=10118X+566831$, $R^2=0.9992$, respectively (Figure 6). Each calibration point was conducted in triplicate. The formulation **F1** contained residual quantities of compound **3** and arbutin, with percentages of 96.00% and 97.44%, respectively (Table 5). Through the calculation of the remaining percentage of both active ingredients in the facial serum, it was established that the quantities of these active ingredients in the formulation remained above 90%¹⁸ after one month. Additionally, by employing the experimental results and the Q10 method for estimation, it was deduced that the serum possessed a maximum predicted shelf life of 5.2 months, during which no discernible alterations in its chemical and physical properties were observed.^{19,20}

Calculation for predicting the shelf life of a formulation using the Q10 method.

$$t_{30} = \frac{t_{30}(T_1)}{Q_{10}^{\left(\frac{\Delta t}{10}\right)}} = \frac{1}{3^{\left(\frac{-15}{10}\right)}} = 5.2 \text{ months}$$

Where, $T_{90}(T_1)$ = Shelf life at 45°C (T_1)

$T_{90}(T_2)$ = Estimated shelf life at 30°C (T_2)

ΔT = Temperature difference between T_2 and T_1 (30-45 = -15)

Q_{10} = Ratio of the rate of reaction with a temperature difference of 10°C

(3 is constant)

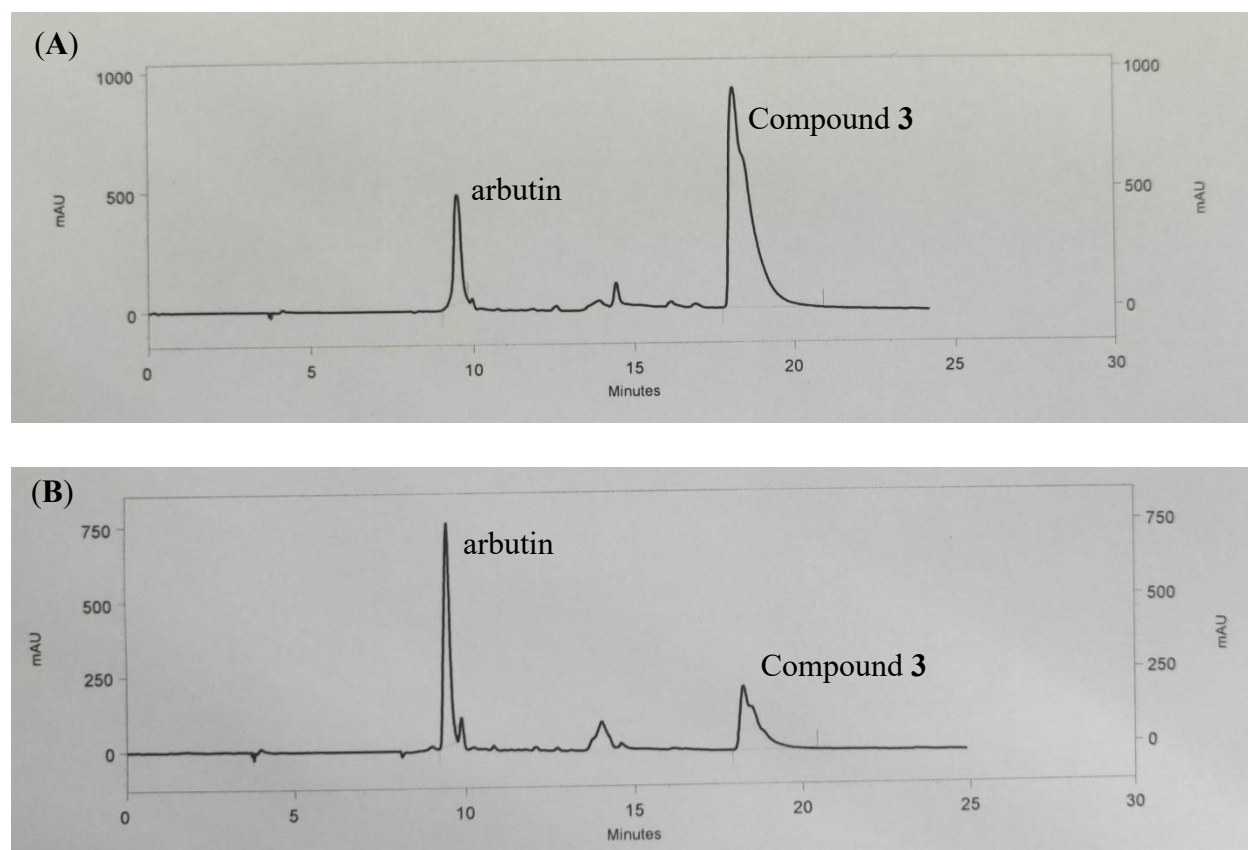


Figure 5. HPLC chromatogram of standard compound **3** and arbutin (A), and formulation **F1**

(B)

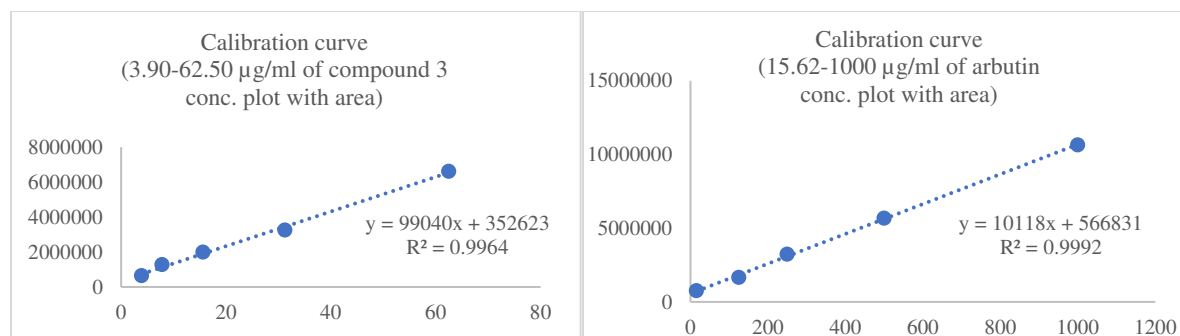


Figure 6. Calibration curve of compound **3** and arbutin

Compounds	LOD ¹ (%w/w)	LOQ ² (%w/w)	Formulation F1 (%w/w)		%Remaining
			Day 1	Day 30	
Compound 3	0.0016	0.0048	0.0050 ± 0.0005	0.0048 ± 0.0003	96.00%
arbutin	0.0124	0.0375	0.1991 ± 0.0004	0.1940 ± 0.0057	97.44%

¹LOD = Limit of detection, ²LOQ = Limit of quantification

Table 5. Compound **3** and arbutin contents in formulation **F1**

Conclusion

Four selected phytochemicals extracted from *N. lappaceum* L. seeds were assessed for their anti-melanogenic activity in B16F10 melanoma cells. Among them, kaempferol-7-*O*- α -L-rhamnopyranoside (**3**) exhibited the most potent activity without significant cytotoxicity. Moreover, it displayed remarkable anti-tyrosinase activity comparable to that of kojic acid, making it a promising candidate as an active ingredient in skin whitening products. Our study also demonstrated a synergistic compatibility effect when combining compound **3** and arbutin in specific proportions, assessed using the CI value to evaluate synergism. The results revealed a synergistic effect ($CI < 1$) between compound **3** and arbutin in inhibiting melanin production in B16F10 cells and tyrosinase inhibition using L-tyrosine as a substrate. Consequently, we selected compound **3** and arbutin as active ingredients for inclusion in cosmetic formulations. Furthermore, formulation **F1** demonstrated excellent stability under all accelerated storage

conditions, and the quantitative analysis confirmed that the levels of compound **3** and arbutin in the formulation remained above 90% after one month of storage. To determine the shelf life of the formulation, we utilized the Q10 method, which estimates a shelf life of approximately 5.2 months from the date of manufacture.

Materials and method

Materials. Quercetin-3-*O*-rhamnoside (**1**), kaempferol-3-*O*- β -D-glucopyranosyl (**2**), kaempferol-7-*O*- α -L-rhamnopyranoside (**3**), and kaempferol-3-*O*- β -D-glucopyranosyl-7-*O*- α -L-rhamnopyranoside (**4**) were isolated from the air-dried powdered seeds of *N. lappaceum* L. with several chromatographic techniques as our previous report.²¹ The *N. lappaceum* (Sapindaceae) seeds were gathered in Amphoe Laem Sing, Chanthaburi Province, Thailand. All methods were carried out in accordance with relevant guidelines. Arbutin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), kojic acid and Tween 20 were purchased from Tokyo Chemical Industry, Japan. L-Tyrosine and mushroom tyrosinase were purchased from Sigma-Aldrich (USA). Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, trypan blue solution, trypsin 0.25 (1x) solution were purchased from Hyclone (Austria).

Methods.

Cell Culture. Murine melanoma B16F10 cells (obtained from DS Pharma Biomedical, Japan) were cultivated in Dulbecco's Modified Eagle Medium (DMEM), a liquid medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated at a temperature of 37 °C within a controlled humidified environment containing 5% CO₂. To facilitate subculture, the cells were detached from the culture flask using trypsin solution. Subsequently, the cells were subjected to staining with trypan blue solution and quantified using a hemocytometer for further analysis.

Anti-melanogenic assay. Murine melanoma B16F10 cells were gently rinsed with phosphate-buffered saline (PBS) and detached from the cell tissue culture dish using trypsin. The cells were subsequently seeded in 24-well culture plates at a density of 5×10^4 cells per well and allowed to incubate for 24 hours before being subjected to treatment with the experimental samples. Arbutin was used as a positive control. Following the treatment, the cells were further incubated for an additional 72 hours, and the absorbance was measured at 510 nm using a microplate reader. Melanin production was expressed as a percentage relative to the control cells treated with DMSO. All experiments were conducted in triplicate. The IC_{50} value was calculated. The percentage of total melanin content was determined using the following equation:

$$\% \text{ Total melanin content} = \frac{\text{Abs}(\text{treated cells}) - \text{Abs}(\text{blank of treated cells})}{\text{Abs}(\text{control}) - \text{Abs}(\text{blank of control})} \times 100$$

Cytotoxicity assay. Cytotoxicity was assessed through the utilization of the MTT assay. The cell culture was initiated in 24-well plates, with a seeding density of 5×10^4 cells per well. After treatment with samples for 72 hours, 10 μ L of MTT solution (5 mg/mL in PBS) were added to each well and incubated for additional 3 hours. Then, supernatant was removed and DMSO (250 μ L) was added to dissolve the formed formazan crystals. The absorbance was measured at 570 nm using a microplate reader. Cells treated solely with DMSO served as the control. All experiments were conducted in triplicate. The percentage of cell viability was determined using the following calculation:

$$\% \text{ Cell viability} = \frac{\text{Abs}(\text{treated cells}) - \text{Abs}(\text{blank of treated cells})}{\text{Abs}(\text{control}) - \text{Abs}(\text{blank of control})} \times 100$$

Anti-tyrosinase assay. The assessment of tyrosinase inhibitory activity was conducted following the method outlined by Saewan et al., with slight modifications.²² L-tyrosine served as a substrate

and kojic acid was utilized as a positive control. The compounds (50 μ L) were added to the 96-well plate, followed by tyrosinase solution (50 μ L, 250 units/mL in phosphate buffer, pH 6.8). After the first incubation at 35°C for 10 min, 50 μ L of substrates (2.5 mM *L*-tyrosine) were added, followed by second incubation at 35°C for 20 min. The absorbance was measured at 492 nm using a microplate reader. All experiments were replicated in triplicate. The percentage inhibition of tyrosinase was determined using the following equation:

$$\% \text{ Tyrosinase inhibition} = \frac{\text{Abs}(\text{control}) - \text{Abs}(\text{sample})}{\text{Abs}(\text{control}) - \text{Abs}(\text{sample of control})} \times 100$$

Cosmetic formulation and accelerated stability test. The development of cosmetic formulations has resulted in the creation of two distinct formulations, outlined as follows. Formulation **F1** consists of the following ingredients: compound **3** (0.005% w/w), glycerin (3.00% w/w), propylene glycol (3.00% w/w), xanthan gum (0.5% w/w), ActiveProtectMOX (Sodium Diethylenetriamine Pentamethylene Phosphonate, Disodium EDTA, Sodium Metabisulfite, Sodium Glucaptate) (0.60% w/w), arbutin (0.20% w/w), phenoxyethanol (0.5% w/w), and water (92.195% w/w). Formulation **F2** consists of the following ingredients: compound **3** (0.005% w/w), glycerin (2.00% w/w), propylene glycol (1.00% w/w), hydroxyethyl cellulose (0.5% w/w), Acrylates/C10-30 Alkyl Acrylate Crosspolymer (0.10% w/w), triethanolamine (0.05% w/w), phenoxyethanol (0.5% w/w) and water (95.845% w/w). An accelerated stability test was performed by placing the formulation in a 45°C chamber for 30 days. The physical and chemical stability of the test products were assessed through organoleptic and HPLC analyses.

*Determination of kaempferol-7-O- α -L-rhamnopyranoside (**3**) and arbutin in cosmetic formulation by HPLC.* The stock solution was prepared by dissolving 1 mg of the standard compounds in 1 mL of methanol, resulting in a concentration of the stock solutions at 1000 ppm. Subsequently,

serial dilutions were conducted using methanol to establish a standard curve. The concentrations varied from 3.9 to 62.5 ppm for kaempferol 7-*O*- α -L-rhamnopyranoside (**3**) and from 15.6 to 1000 ppm for arbutin. For the analysis of the sample cosmetic formulations, precisely 200 mg was weighed and placed into a centrifuge tube, then dissolved in 1 mL of methanol. The resulting mixture was vortexed for 1 minute, followed by sonication at 25 °C for 15 minutes and subsequent freezing at -80 °C for 10 minutes. The mixture was then subjected to centrifugation at 3,000 rpm for 5 minutes. The sample solution was filtered through a nylon syringe filter with a pore size of 0.45 μ m, and 20 μ L of the filtrate was injected into a reverse-phase Vertical C18 column (150 mm x 4.6 mm, 5 μ m) equipped with a Thermo Scientific UV6000LP detector and Thermo Scientific P200 pump. The ChemQuest version 5.0 software controlled the system. The mobile phase, consisting of water (A) and acetonitrile (B), followed a linear gradient program of 0-100% B over 30 minutes, with a flow rate of 0.6 mL/min. The UV detector was set at 280 nm, and the injection volume was 20 μ L.

Statistical Analysis. The 50% inhibition concentration (IC₅₀) value is determined with GraphPad prism software, version 5. Data were presented as mean $\pm$ standard deviation (SD) from three independent experiments. Values were evaluated by one-way analysis of variance, followed by Dunnett's multiple tests multiple comparison test (GraphPad prism, version 5). The difference was considered significant when the p-value was < 0.05. The values of combination index (CI) were calculated by following equation:

$$CI = \frac{IC_{50amix}}{IC_{50a}} + \frac{IC_{50bmix}}{IC_{50b}}$$

where IC_{50a} and IC_{50b} are the IC₅₀ value of compound a and compound b, and IC_{50amix} and IC_{50bmix} are the concentration of compound a and compound b in the mixture that cause 50% inhibition.

The CI value < 1 indicates synergistic, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism.¹⁴

Data availability

All data and materials are the result of research and are available from the corresponding author upon reasonable request and are appropriately cited in the manuscript.

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Author contributions

Conceptualization, I.S. (Ittipon Siridechakorn), K.P. (Khanitha Pudhom), and S.C. (Siwattra Choodej); methodology and experimental design, I.S., S.C., and K.P.; Validation, I.S., J.P. (Junsuda Pimpa), S.C., and K.P.; investigation, J.P., and S.C.; writing-original draft preparation, I.S., S.C., and K.P.; writing-review and editing, I.S., S.C., and K.P.; visualization, I.S. and S.C.; project administration, K.P.; funding acquisition, K.P. All authors have read and agree to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to K.P.