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Urea-extracted sericin is potentially better than kojic acid in the inhibition of melanogenesis through increased reactive oxygen species generation

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A R T I C L E I N F O

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A B S T R A C T

Background: Hyperpigmentation is a skin disorder, which is caused by an excess production of melanin. The reduction in melanin content without causing undesirable effects is required for the treatment of hyperpigmentation. Sericin is increasingly used as a hyperpigmentation treatment because of its anti-tyrosinase activity. However, the various methods of sericin extraction have an effect on the composition and biological properties. The purpose of this study was to investigate the antioxidant and anti-melanogenic properties of sericin using different extraction methods including acid, base, heat, and urea extraction.
Methods: The chemical properties of extracted sericin were assessed in terms of amino acid components, thermal behavior, and UV–vis absorption. The inhibitory effects of sericin on melanogenesis were explored by determining the melanin content and cellular tyrosinase activity in B16F10 cells.
Results: Sericin from urea extraction provided different properties when compared with the other extraction methods. Our results indicate that urea-extracted sericin reduced the melanin content and cellular tyrosinase activity more effectively than the other extraction methods. Interestingly, the potential anti-melanogenic activity was more effective than kojic acid, a depigmenting agent used to treat hyperpigmentation. Moreover, treatment of urea-extracted sericin induced reactive oxygen species and subsequently activated antioxidant activity in B16F0 cells.
Conclusions: Our results present the potential inhibitory effect of urea-extracted sericin on melanogenesis. The therapeutic potential of urea-extracted sericin can be used in the treatment of hyperpigmentation and its complications.
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1. Introduction

Hyperpigmentation is a common disorder of the skin, particularly in people of Asian ethnicity, who have a greater predisposition to congenital and developed with acquired pigmentary skin disorders.[1] A common cause of hyperpigmentation is the increased production and/or redistribution of melanin in melanocytes, leading to pigment alteration.[2] This phenomenon can be associated with the occurrence of many primary disorders such as seborrheic dermatitis, atopic dermatitis, and tinea versicolor, or it can indicate the development of a life-threatening condition such as melanoma.[1] Moreover, hyperpigmentation can be caused by medication side effects or a phototoxic reaction.[3] Melanocytes are specialized skin cells that produce melanin in the basal epidermal layers, a process known as melanogenesis. Inhibition of melanogenesis is a

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List of abbreviations

TGA	Thermogravimetric analysis
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
DCFH-DA	2′-7′dichlorofluorescin diacetate
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
H_2O_2	Hydrogen peroxide

rational adjuvant approach to the therapy of hyperpigmentation disorders. Many therapeutic agents have been used in an attempt to attenuate melanin production in patients. The most widely prescribed therapies are chemical peels, laser treatments, and topical corticosteroids.[4] However, these applications also have undesirable effects.[3,5,6] Consequently, natural product-derived compounds present interesting possibilities for the treatment of hyperpigmentation, as they are perceived to reduce melanin production without inducing adverse side effects.

Silk sericin is a natural macromolecular protein produced in the middle silk gland (MSG) of the silkworm, *Bombyx mori* (*B. mori*). The biocompatible and biodegradable properties of sericin have driven growing interest in its biological applications. Sericin is responsible for numerous applications in biomedicine as it has powerful antioxidant, wound healing, antitumor, antimicrobial, and anti-inflammatory properties.[7–11] Furthermore, sericin is increasingly used as a hyperpigmentation treatment as it has antityrosinase activity.[12] Tyrosinase is the key regulator of melanin synthesis, and is a common target for reducing hyperpigmentation. A previous study demonstrated the antityrosinase activity of the sericin using urea extraction.[13] However, different silk varieties and extraction methods can lead to a variation in the composition of amino acids and secondary metabolites,[14] which could have an impact on tyrosinase inhibitory activities.[15] The extraction method of sericin and its mechanisms of action are important for providing a rationale for their efficacy.[16,17] However, the anti-melanogenic properties of sericin prepared by different extraction methods are yet to be explored in melanocytes.

The objectives of the present study were to investigate sericin obtained using different extraction methods regarding its inhibitory action against melanogenesis. The extraction methods significantly affected the suppression of melanin production in melanocytes. Furthermore, our results revealed a surprising effect of sericin extracted using urea in terms of inhibiting melanogenesis by ROS-dependent activation. Urea-extracted sericin could be used as a potential agent for the treatment of hyperpigmentation disorder and its complications.

2. Materials and methods

2.1. Extraction of silk sericin

2.1.1. Extraction of silk sericin by acid and alkali degradation

Silk sericin was extracted from cocoons using the modified protocol of Kurioka et al..[7] Briefly, the cocoons of mulberry (*B. mori*) silkworms were cut into small pieces and placed into a 1.25% citric acid (for acid degradation) or 0.5 M sodium carbonate (alkali degradation) solution (1 g of dry silk cocoon and 18 mL of citric acid solution) and boiled for 30 min. After that, insoluble fibers were removed by paper filtration. The obtained supernatant was filtered and immediately dialyzed in distilled water for 3 days using cellulose tubing [Cellusep T2; MWCO (molecular-mass cut-off) = 6000–8000; Sequin, TX, U.S.A.]. The protein solution was lyophilized using a Heto LL 3000 lyophilizer (HetoHolten A/S, Allerod, Denmark).

2.1.2. Extraction of silk sericin by high temperature under high pressure (autoclaving)

The cocoons of mulberry (*B. mori*) silkworms were cut into square pieces (approx. 5 mm^2). Cocoon pieces were extracted in 70% ethanol three times for 24 h at room temperature (25 °C). After drying, the residual cocoon shells (~97% from initial cocoon weight) were autoclaved in purified water (1 g of dry silk cocoon and 30 mL of water) at 120 °C and 15 lbf/in^2 (1 lbf/in^2 = 6.9 kPa) for 60 min (SS-320; Tomy Seiko, Tokyo, Japan). Centrifugation and filtration were performed to separate the silk fibroin along with other solid residues. After that, the protein solution was frozen and freeze-dried using a Heto LL 3000 lyophilizer (HetoHolten A/S, Allerod, Denmark).

2.1.3. Extraction of silk sericin by urea degradation

The silk sericin was isolated from the cocoons of mulberry (*B. mori*) silkwworms using urea solution as described by Aramwit et al..[13] Cocoon pieces (6 g) were soaked in 8 M urea (150 mL) for 30 min and then refluxed at 85 °C for 30 min. All insoluble residues were removed by centrifugation and filtration. The obtained protein solution was dialyzed in distilled water using cellulose tubing (Cellusep T2; MWCO = 6000–8000; Sequin) for 3 days. The protein solution was lyophilized using a Heto LL 3000 lyophilizer (HetoHolten A/S, Allerod, Denmark).

2.2. Characterization of silk sericin

2.2.1. . Ultraviolet–visible spectroscopy (UV–Vis)

UV–Vis spectra were analyzed using a spectrophotometer (Thermo, Varioskan Flash, England) in the wavelength range of 200–500 nm.

2.2.2. . Thermogravimetric analysis (TGA)

Thermogravimetric analysis was used to determine the thermal behavior. The composition of silk sericin obtained by different extraction methods was determined by thermogravimetric analysis (TGA) using a TGA Q50 instrument (TA Instrument, USA) with heating from 0 to 700 °C at a rate of 10 °C/min under nitrogen atmosphere.

2.2.3. . Analysis of amino acid composition

The amino acid compositions of silk sericin were measured using an amino acid analyzer (Hitachi L-8500A; Hitachi, Tokyo, Japan). The silk sericin was prepared for analysis by hydrolysis in methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole (Wako Pure Chemical Industries, Tokyo, Japan) at 100 °C for 24 h under vacuum. All experiments were carried out three times in triplicate.

2.3. . Cell culture

The B16F10 melanoma cell line (ATCC number CRL-6475) was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), and 1% antibiotic-antimycotic solution (Gibco, USA). Cells were grown and maintained at 37 °C with 5% CO_2 in a humidified incubator.

2.4. . Cytotoxic assay

The PrestoBlue™ reagent (Invitrogen, USA) was used to evaluate the influence of silk sericin on cell viability. Metabolically active

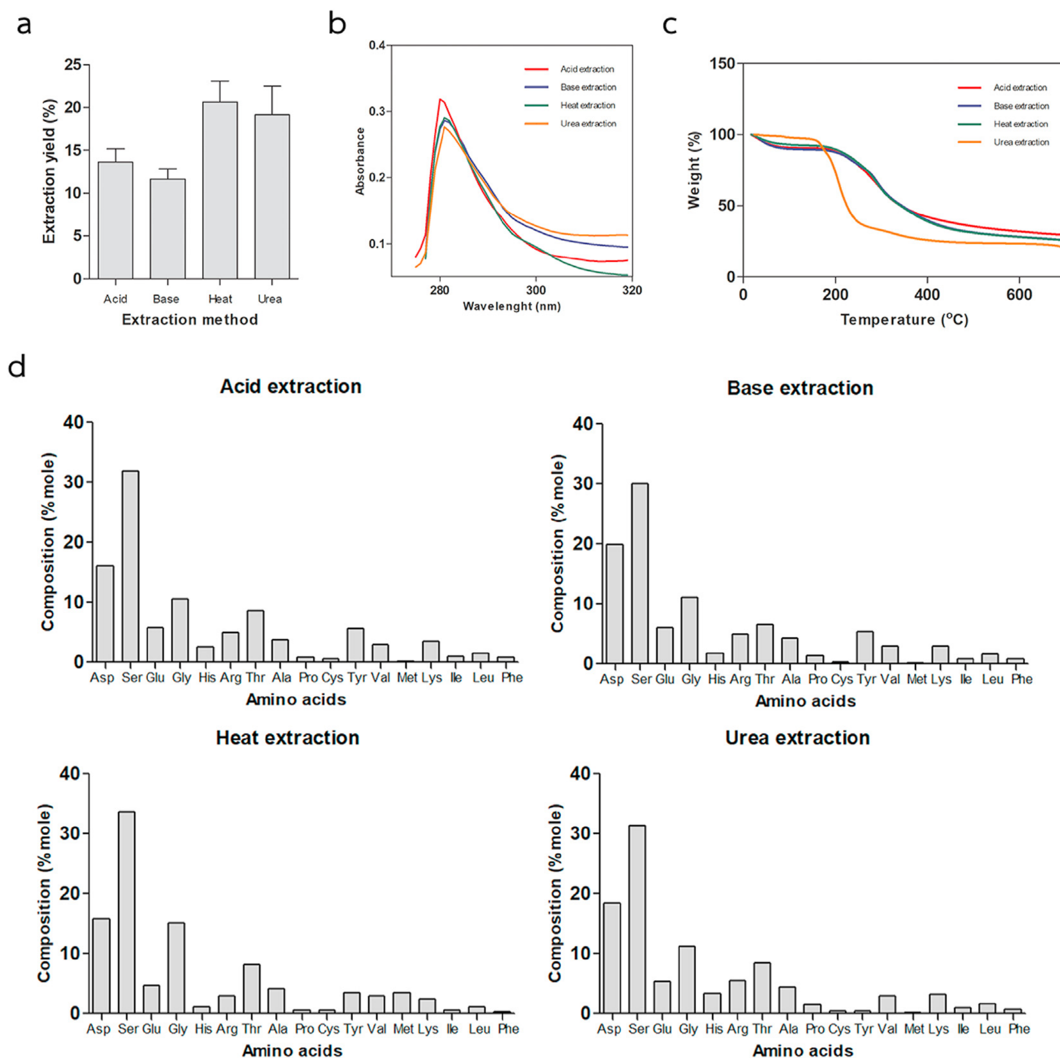

Fig. 1. Characteristics of extracted sericin from different extraction methods. The yield of sericin obtained from different extraction methods (a). The extracted sericin was analyzed by UV absorption spectrum (b), thermal behavior (c), and amino acid composition (d).

cells are capable of reducing the PrestoBlue reagent, with colorimetric changes used as an indicator to quantify the viability of cells. B16F10 cells were seeded in 96 well plates at a density of 5×10^3 per well in cell culture medium and incubated for 24 h to allow cell adherence. Post incubation, cells were pretreated with different concentrations (10, 30 and 50 μg/mL) of sericin from various extraction methods for 24, 48, and 72 h. Following incubation, 10 μl PrestoBlue solution was added to each well, and then plates were placed back into the incubator for a further 30 min incubation. Fluorescence was measured using a microplate reader at 560 nm excitation and 590 nm emission (Thermo, Varioskan Flash, England). Morphology was examined using a light microscope.

2.5. Determination of intracellular antioxidant activity

The intracellular antioxidant activity of sericin against H_2O_2 was evaluated using the 2′-7′dichlorofluorescin diacetate (DCFH-DA) assay (Invitrogen, USA). B16F10 cells were seeded into 96-black well plates at a density of 5×10^3 cells per well in cell culture medium and incubated for 24 h. Post incubation, the culture medium was replaced by fresh medium containing different concentrations (10, 30 and 50 μg/mL) of sericin and incubated for 48 h. Cells were washed with PBS and incubated with 0.1 μM of DCFH-DA at a volume of 100 μL/well for 30 min (min). After that, medium containing 2 mM H_2O_2 was added and incubated for 60 min. Post incubation, fluorescence was measured in a microplate reader at excitation and emission wavelengths of 485 and 528 nm (Thermo, Varioskan Flash, England).

2.6. Detection of intracellular reactive oxygen species (ROS) generation

The formation of intracellular ROS was measured by monitoring the changes in 2′,7′-dichlorofluorescein-diacetate (Invitrogen, USA) fluorescence. B16F10 cells were seeded into 96-black well plates at a density of 5×10^3 cells per well in cell culture medium and incubated for 24 h. Cells were washed with PBS and incubated with 0.1 μM of DCFH-DA at a volume of 100 μl/well for 30 min. After that, cells were washed with PBS again and treated with sericin for 48 h. Fluorescence was measured using a microplate reader at excitation and emission wavelengths of 485 and 528 nm (Thermo, Varioskan Flash, England).

2.7. Estimation of melanin content

Intracellular melanin was quantified in B16F10 cells cultured in

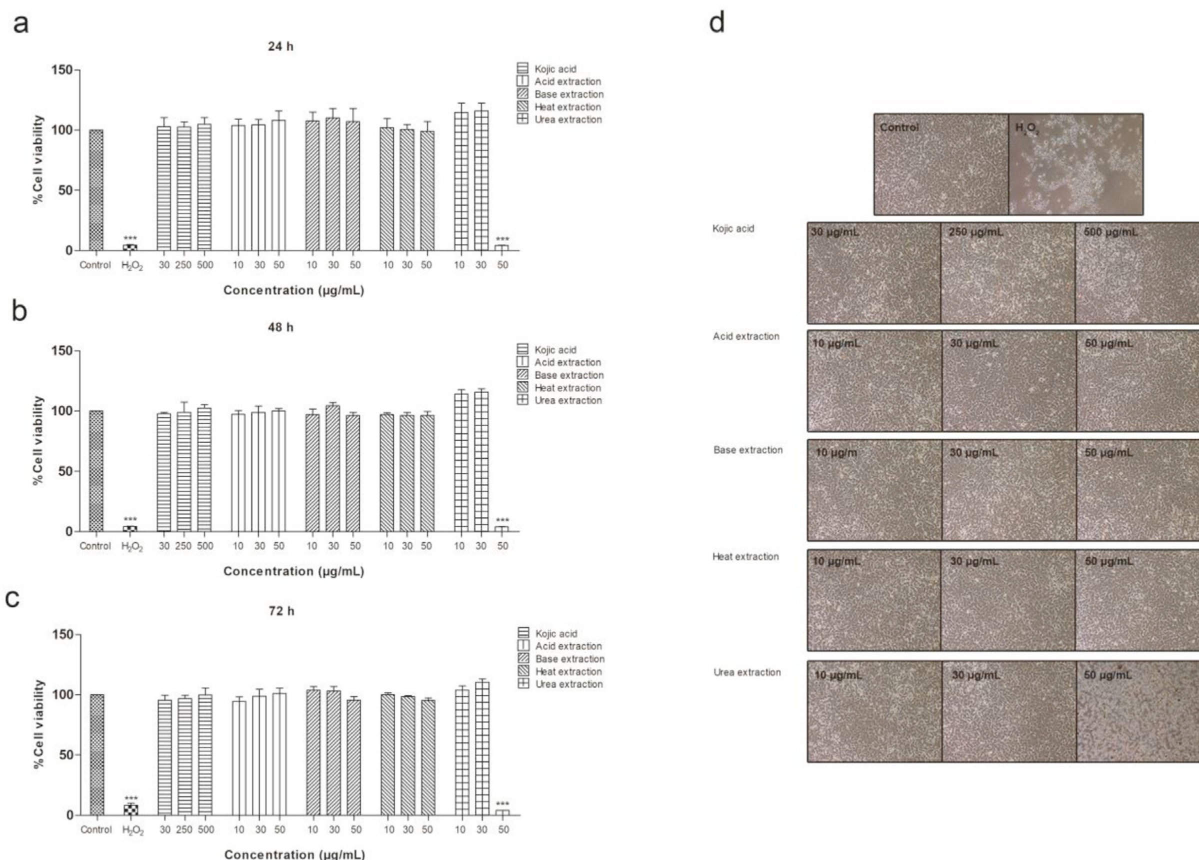

Fig. 2. Evaluation of cell viability after treatment of sericin from different extraction methods. Cell viability results of B16F10 cells treated with sericin for 24 (a), 48 (b), and 72 h (c) and qualitative visualization of the morphology (d). Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the control group.

DMEM with sericin. B16F10 cells were seeded into 12 well plates at a density of 1×10^5 cells per well in cell culture medium and incubated for 24 h to allow cell adherence. Post incubation, the media was replaced with fresh media containing sericin. The cells were collected after 48 h of incubation and the cell pellets were solubilized in 1 N NaOH for 1 h at 70 °C to dissolve melanin. 200 µl of cell lysates were placed in 96 well plates and absorbance was recorded at 475 nm using a microplate reader (Thermo, Varioskan Flash, England).

2.8. Cellular tyrosinase activity

B16F10 cells were seeded into 24 well plates at a density of 5×10^4 cells per well in cell culture medium and incubated for 24 h. After incubation, the medium was replaced with fresh media containing sericin and further incubated for 48 h. The cellular tyrosinase activity was measured using a commercial kit (Abcam, Cambridge, MA, USA). All the operating steps were conducted according to the manufacturer's instructions.

2.9. Real-time PCR

The total RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA concentrations and quality were measured by nanodrop spectrophotometer (Thermo Scientific, USA). One microgram of total RNA was used for cDNA synthesis with a First strand cDNA synthesis kit (Thermo scientific, USA) according to the manufacturer's instructions. qPCR was performed with Express SYBR GreenER qPCR Supermix Universal (Invitrogen). The reaction was conducted on a StepOnePlus Real-Time PCR System (ABI Applied Biosystems), in the following thermocycling conditions: 94 °C for 15 s, 60 °C for 30 s and 72 °C for 40 s, for 40 cycles. The CT (threshold cycle) values of the target genes were normalized with housekeeping gene and relative to the normalized calibrator. The primers used for real-time PCR were as follows: Nrf2 forward, 5′-AGCCCAGCA-CATCCAGTCA-3′ and reverse, 5′-TGCATGCAGTCATCAAAGTACAAAG-3'; HO-1 forward, 5′-AAGCCGAGAATGCTGAGTTCA-3′ and reverse, 5′-GCCGTGTAGATATGGTACAAGGA-3'; NQO1 forward, 5′-TTCTGTG GCTTCCAGGTCTT-3′ and reverse, 5′-TCCAGACGTTTCTTCCATCC-3'; GCLC forward, 5′-GGGGTGACGAGGTGGAGTA-3′ and reverse, 5′-GTTGGGGTTTGTCCTCTCCC-3′ and β-actin forward, 5′- TATTGG-CAACGAGCGGTTC-3′ and reverse, 5′- ATGCCACAGGATTCCATACCC-3' (Table S1).

2.10. Small interfering RNA

Briefly, B16F10 cells were seeded into 12 well plates at a density of 5×10^4 cells per well in cell culture medium and incubated for 24 h to allow cell adherence. Post incubation, B16F10 cells were transfected with Nrf2 small interfering RNA (siRNA) and siRNA negative control (Origene, USA) using siTran 2.0 siRNA transfection reagent (Origene, USA) for 18 h, according to the manufacturer's instructions. After incubation, the transfection medium was replaced with 1 mL of standard growth medium and the cells were maintained at 37 °C humidified incubator supplemented with 5% CO_2.

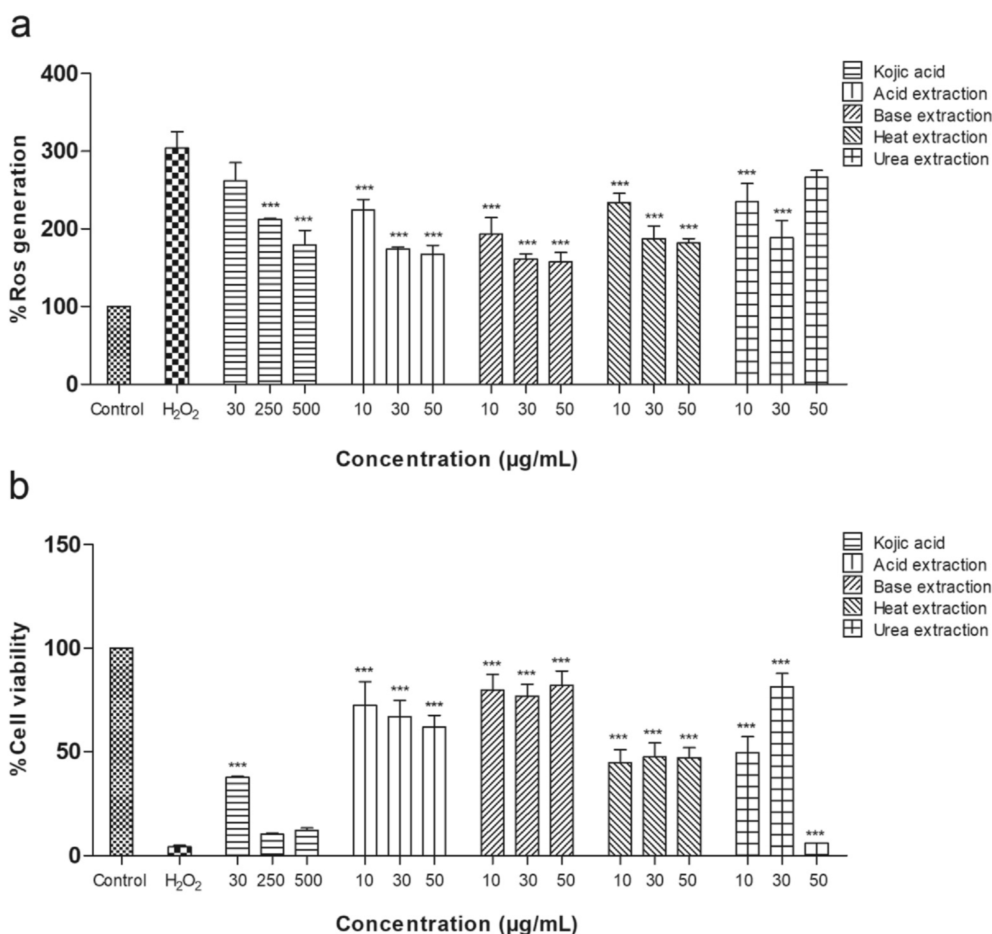

Fig. 3. Protective effect of sericin from different extraction methods against H_2O_2 induced oxidative damage and promotion of cellular viability. The measurement of ROS levels was observed after H_2O_2 exposure (a). The pre-treatment of sericin significantly increased the cell viability (post 24 h of H_2O_2 treatment) (b). Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the H_2O_2 group.

2.11. Statistical analyses

All experiments were performed in triplicate. The results were expressed as mean ± standard deviation for n = 3. Statistical analysis was performed using one-way ANOVA and Tukey post-test using GraphPad Prism 5.0 (Graph-Pad Software Inc., CA, USA). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Extraction yields

The extraction yields of sericin by different methods of extract were presented in Fig. 1a.

3.2. Characterization of sericin

3.2.1. UV–vis spectra of sericin

The percentage yield of the sericin was presented in Fig. 1a. The extracted sericin was characterized using UV–vis spectroscopy. The maximum absorption of the extracted sericin was at around 280 nm (Fig. 1b). This result indicated that the typical UV–vis profile of sericin with a maximum absorption peak at around range of 280–285 nm could be attributed to aromatic amino acids.

3.2.2. Thermal decomposition analysis

The thermogravimetric analysis of sericin from different extraction methods is shown in Fig. 1c. The TGA curve showed that the thermal transition occurred in two temperature ranges: <100 and 150–400 °C. The initial weight loss at below 100 °C was due to water evaporation. Weight loss occurred again at a temperature over 150 °C. There was no significant change in the decomposition pattern of the acid-, base-, and heat-extracted sericin. The thermal properties of urea-extracted sericin dramatically differed from the other extraction methods. The weight losses from the evaporation of the physically adsorbed moisture were observed at around 2%. The thermal degradation of urea-extracted sericin was observed at around 100–300 °C.

3.2.3. Amino acid composition of sericin

The amino acid composition of sericin is shown in Fig. 1d. The results show that sericin from different extraction methods contained a high serine content. It was also found that the contents of aspartic acid and glycine were greater than other amino acids, except for serine. Other amino acids were present in very small amounts.

3.3. Cytotoxicity of sericin

We first examined whether sericin has an influence on cell viability. After melanocytes were exposed to sericin for 12, 48 and

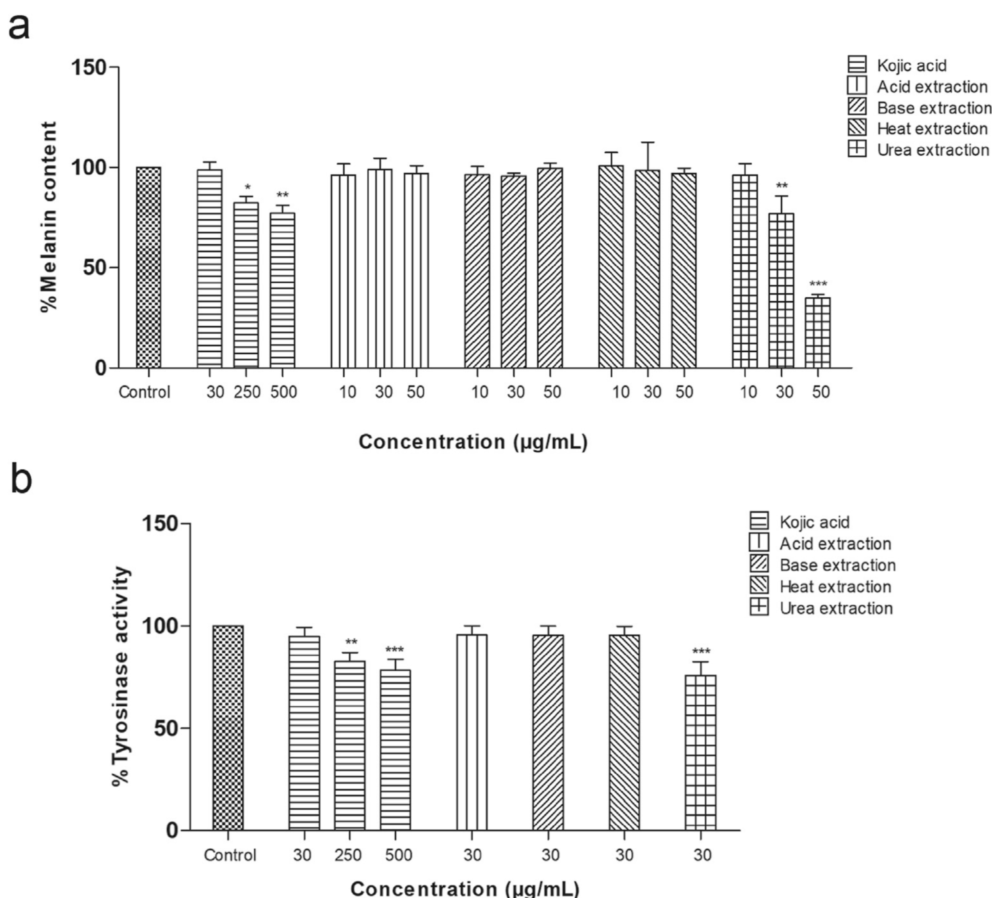

Fig. 4. Inhibitory effect of urea-extracted sericin on melanin production. Inhibitory effect of treatment with sericin from different extraction methods for 48 h on melanin production (a) and intracellular tyrosinase activity (b). Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the control group.

72 h, the Presto Blue assay was performed to determine the viability and morphology of the cells (Fig. 2a–d). Treatment with H_2O_2 led to intense damage to melanocytes; this was used as a positive control. The acid-, base-, and heat-extracted sericin were found to be non-toxic to cells at all incubation time points by comparing the treated and control groups. There were no noticeable morphological differences between the sericin-treated and control groups. However, cells treated with a high concentration (50 μg/mL) of urea-extracted sericin showed significantly lower ($p \leq 0.001$) viability., indicative of alterations in mitochondrial function.

3.4. *Antioxidant potential of sericin against H_2O_2-induced oxidative stress*

The effect of pre-treatment of cells with sericin for 48 h is shown in Fig. 3. Overall, a significant difference was observed in the ROS reduction of sericin untreated and pretreated cells in comparison with control. After 24 h of H_2O_2 exposure, it was observed that pre-treatment of acid-, base-, and heat-extracted sericin at 10, 30 and 50 μg/mL significantly increased cell viability (post 24 h of H_2O_2 treatment). Treatment with urea-extracted sericin at 30 μg/mL showed better protection when compared with 10 μg/mL of urea-extracted sericin. Pre-incubation with 50 ng/mL of urea-extracted sericin did not show significant protection against H_2O_2-induced oxidative stress since this was a toxic dose. Sericin from all extraction methods improved cell viability (post 24 h of H_2O_2 treatment) compared to the positive control (kojic acid).

3.5. *Inhibitory effect of urea-extracted sericin on melanogenesis*

The anti-melanogenic activity of sericin was assessed in terms of melanin content and *in vitro* tyrosinase inhibitory activity. We first measured the reduction in melanin levels induced by sericin obtained using different extraction methods. As shown in Fig. 4a, 30 μg/mL of urea-extracted sericin exerted a significant inhibitory effect on the melanin content in B16F10 cells. Interestingly, the inhibitory effect of urea-extracted sericin was higher than that of kojic acid at the same concentration. Next, to determine the effect of sericin on melanogenesis, the tyrosinase activity was quantified. As shown in Fig. 4b, treatment with urea-extracted sericin decreased the tyrosinase activity in sericin-treated cells, indicating that the decrease in melanin content might be due to the inhibition of tyrosinase activity. Conversely, the treatment of sericin obtained by acid, base, and heat extraction had no significant anti-melanogenic property on B16F10 cells. These findings clearly showed that urea-extracted sericin exerts its anti-melanogenic effect through the inhibition of tyrosinase activity and subsequently reduces melanin formation in B16F10 cells without inducing cytotoxicity.

3.6. *ROS-dependent melanogenesis inhibition by urea-extracted sericin*

We further investigated the role of free radicals in the anti-melanogenic properties of urea-extracted sericin. As demonstrated in Fig. 5, treatment with sericin obtained from acid, base,

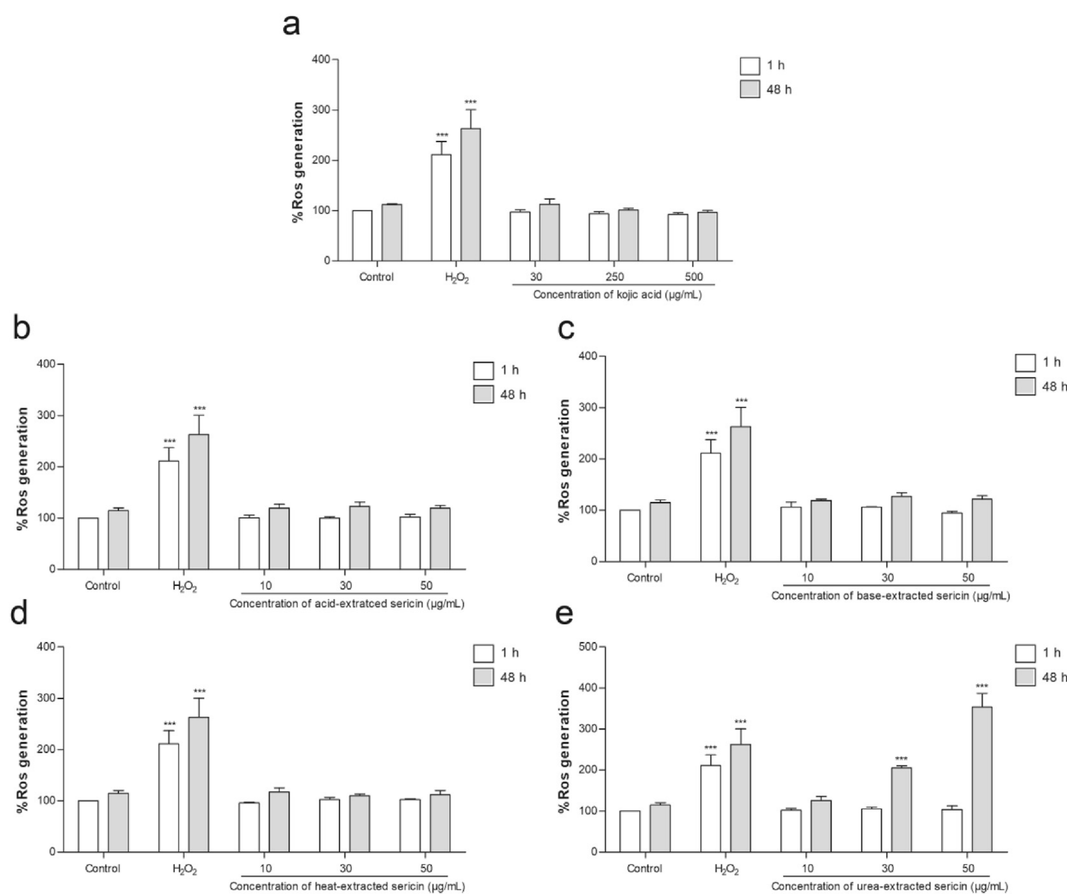

Fig. 5. Activation of ROS levels by urea-extracted sericin. Measurement of ROS levels was observed after the treatment of kojic acid (a) and sericin from the acid (b), base (c), heat (d), and urea extraction (e). Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the control group.

and heat extraction did not induce ROS production in treated cells. ROS generation induced by sericin obtained from acid, base, and heat extraction was similar to that of the positive control. However, treatment with urea-extracted sericin significantly induced free radical production, which also reduced melanogenesis in B16F0 cells (Fig. 4). Taken together, these findings demonstrate that the inhibitory effects of urea-extracted sericin on melanogenesis in B16F10 cells might be mediated through the induction of ROS generation and the inhibition of tyrosinase activity in B16F10 cells.

3.7. Urea-extracted sericin inhibits melanogenesis by up-regulating of antioxidant related genes

We assumed that the response of antioxidants may be due to the activation of Nrf2 signaling for the inhibition of melanogenesis. To elucidate the possible mechanism, we examined the expression levels of Nrf2 in B16F10 cells after urea-extracted sericin treatment. The result indicated that treatment with urea-extracted sericin significantly induced the antioxidant related genes. As shown in Fig. 6, treatment with urea-extracted sericin at 30 µg/mL showed markedly induced Nrf2 expression. To further examine tThe effect of Nrf2 on its downstream target genes, HO-1, NQO1 and GCLC mRNA levels were measured following treatment of urea-extracted sericin. The results indicated a signifcant increase in the expression of HO-1, NQO1, and GCLC. Conversely, the treatment with kojic acid and urea-extracted sericin at 10 µg/mL did not change in the expression of Nrf2, HO-1 and GCLC.

3.8. Transfection of Nrf2 siRNA decreases Nrf2 expression and downstream

The results identified that transfection with Nrf2 siRNA was able to inhibit the expression levels of Nrf2, NQO1, and GCLC (Fig. 7a, c-d). However, the results showed that the expression of HO-1 did not change in urea-extracted sericin + Nrf2 siRNA group (Fig. 7b). Moreover, melanin content significantly increased in urea-extracted sericin + Nrf2 siRNA group (Fig. 7e). These results suggest that melanogenesis is modulated by Nrf2 signaling for the inhibition of melanogenesis in B16F10 cells following treatment of urea-extracted sericin.

4. Discussion

In this study, we aimed to investigate the anti-melanogenic potential of sericin from various extraction methods including acid and alkali-degradation, high temperature under high pressure, and urea degradation, and found that urea-extracted sericin possesses potent anti-melanogenic and anti-oxidant activities. This is the first study to demonstrate the potential inhibitory effect of urea-extracted sericin on melanogenesis possibly through ROS production in B16F10 cells. Interestingly, the anti-melanogenic activity of urea-extracted sericin is more potent than kojic acid at the same concentration.

Sericin, a globular protein produced by silkworms, is extracted from silk by detaching it from the fibroin part; this protein has

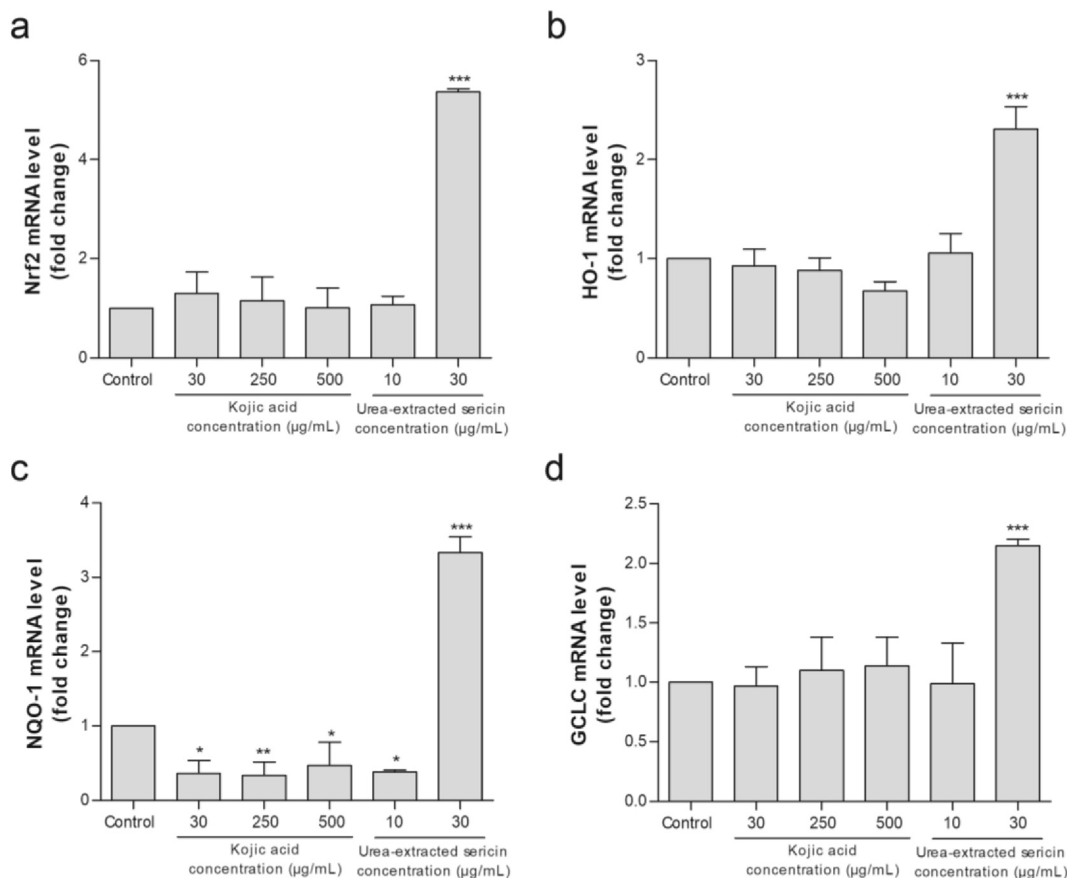

Fig. 6. Urea-extracted sericin up-regulates the antioxidant related genes in B16F10 cells. Cells were treated with urea-extracted sericin for 48 h and the mRNA expression showed the effects of urea-extracted sericin on the activation of Nrf2, HO-1, NQO1, and GCLC. Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the control group.

several useful applications in various fields. The biological functions of sericin vary based the extraction method, resulting in secondary metabolites and variations in the amino acid composition.[7,12] The present study investigated the chemical properties of sericin extracted using different extraction methods. The thermal properties of sericin were evaluated by TGA. Acid-, base-, and heat-extracted sericin exhibited similar TGA curves. Similarly, the major thermal degradation of sericin (*B. mori*) was observed at 200–400 °C. The decomposition temperatures were attributed to the breakdown of side chain groups of amino acid residues and the cleavage of peptide bonds in sericin.[18] However, urea-extracted sericin showed less thermal stability and variable degradation profiles. These results suggest that this extraction method produces a different composition and results in changes to the structure of a protein and thermal behavior. After that, the UV–vis absorption of sericin showed that the maximal absorption wavelength was at around 280 nm. The maximal absorption wavelength indicates the absorption peaks of peptides and amino acids in sericin.[19] The amounts of amino acids differed in each type of extracted sericin, but serine was the most abundant in all samples. Serine has a high content of the hydroxyl group, which contributes to the functions of sericin.[20]

Cytotoxicity assays were performed to evaluate the potential cytotoxic effect of sericin from various extraction methods before further testing. A previous study showed that treatment with sericin is biocompatible up to a concentration of 400 μg/mL.[7] In our study, urea-extracted sericin at 50 μg/mL was the only treatment that presented cellular toxicity in B16F10 cells. In comparison with the previous study, urea extraction method severely damaged cells at concentrations higher than 100 μg/mL. However, the toxicity effect of urea-extracted sericin was not observed at low concentrations.[21]

Reactive oxygen species or ROS is oxygen-containing radicals and generated during mitochondrial oxidative metabolism for regulation of cellular homeostasis. The production of several ROS plays an important role in melanin production.[22–25] Therefore, the effect of different extractions of sericin was explored by evaluating intracellular antioxidant behavior. The suppression of intracellular ROS generation was observed by pre-treatment with sericin from various extraction methods. After H_2O_2 treatment, the efficacy of the antioxidant potential of heat-extracted sericin was less than sericin obtained from acid, base, and urea extraction. A previous study showed lower antioxidant activity in sericin extracted by the autoclaving method compared to base extraction.[7] Pre-treatment with sericin led to a better protective effect against H_2O_2-induced oxidative damage than kojic acid at the same concentration. Previously, ascorbic acid and arbutin did not promote significant cell recovery after 24 h of H_2O_2 exposure.[7] Protection against oxidative stress is the potential mechanism of preventing and modulating melanogenesis in hyperpigmentation disorders.[26,27] In addition, maintenance of ROS levels at basal levels contributes to the inhibition of melanin synthesis.[14] These data indicate that sericin extracted using different extraction methods suppressed ROS generation and increased cell viability after H_2O_2-induced oxidative stress. Sericin can plays a role in the regulation of melanogenesis by attenuating oxidative stress-induced melanogenesis.

Treatment of hyperpigmentation disorders can be achieved by regulating melanin production and the distribution in melanocytes.[28] Previous research has demonstrated that urea-extracted sericin has anti-melanogenic effects by the inhibition of tyrosinase activity using *in vitro* models of allergy induction.[13] However, the anti-melanogenic properties of sericin obtained with a number of extraction methods have not been well-documented. The results show that urea-extracted sericin inhibited the formation of melanin more than a standard inhibitor kojic acid at 30 μg/mL. In accordance with tyrosinase activity, the inhibitory action of urea-extracted sericin was more potent than that of kojic acid. Tyrosinase is a rate-limiting enzyme in the melanin synthesis pathway. Tyrosinase inhibitory activity prevents the biosynthesis of melanin in melanocytes.[29] Although all types of extracted sericin are considered to be potent antioxidants, the only urea-extracted sericin exerted the inhibitory effects on melanin production in B16F10 cells. Therefore, it is thought that the extraction method affected the composition of extracted sericin, resulting in an alteration of its biological functions in melanogenesis. Based on these results, urea-extracted sericin effectively suppressed cellular tyrosinase activity and reduced the melanin content in B16F10 cells.

The existence of reactive oxygen species is a key regulator of many intracellular pathways.[30–32] We further investigated the reduction in melanogenesis via reactive oxygen species. Remarkably, the addition of urea-extracted sericin significantly increased intracellular ROS levels in B16F0 cells. On the other hand, treatment with kojic acid did not affect change ROS levels in the treated cells for anti-melanogenesis effect. was significantly reduced in melanin contents and tyrosinase activity. We suggest that increased ROS levels play a crucial role in the inhibitory effect of melanin production and tyrosinase activity by urea-extracted sericin. Thus, our result is in contrast to those of earlier studies showing that the induction of intracellular peroxide production contributes to melanogenesis in B16F0 cells.[33,34] We suggest that the antioxidant system responds to increased ROS levels by sustaining redox homeostasis,[35] leading to suppression of melanogenesis. Nrf2 is a master regulator of antioxidant and cytoprotective genes that protects against oxidative damage by binding to the antioxidant response element (ARE) and upregulate several antioxidant genes including HO-1, NQO1, and GCLC.[36] The role of urea-extracted sericin-induced Nrf2 activation in B16F10 cells was determined. To explore the role of Nrf2 in B16F10 cells following treatment with urea-extracted sericin, Nrf2 siRNA was utilized to inhibit Nrf2 expression and its downstream target genes. Our study also suggested that the action of Nrf2-ARE pathway plays an important role in the inhibition of melanogenesis, which was consistent with

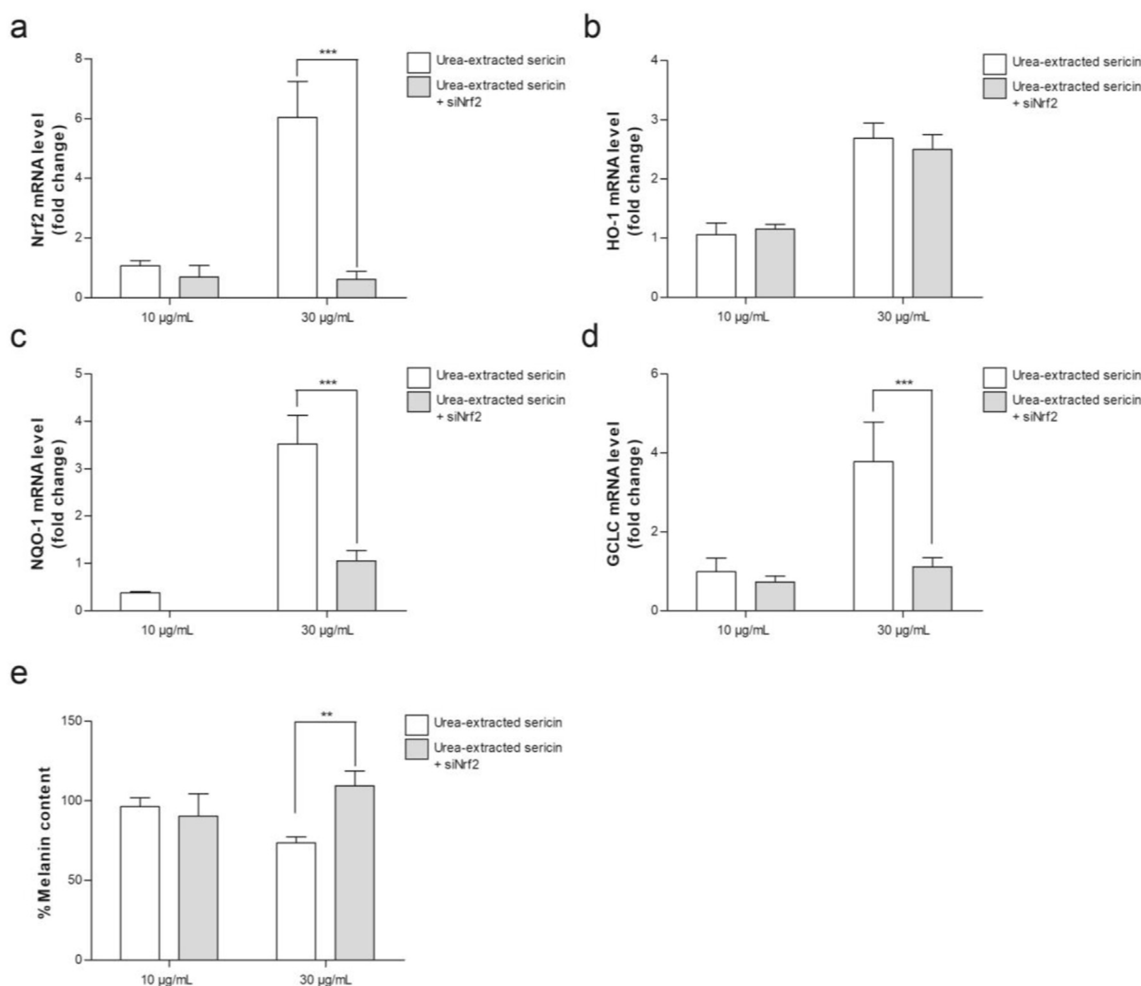

Fig. 7. Effect of Nrf2 knockdown on melanogenesis in B16–F10 cells. Cells were treated with urea-extracted sericin at 30 μg/mL for 48 h after transfection of Nrf2 siRNA. The expression of Nrf2, NQO1, and GCLC were reduced in the urea-extracted sericin + Nrf2 siRNA group. The changes in melanin content was increased to the pattern of Nrf2 expression. Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the urea-extracted sericin group without transfection of Nrf2 siRNA.

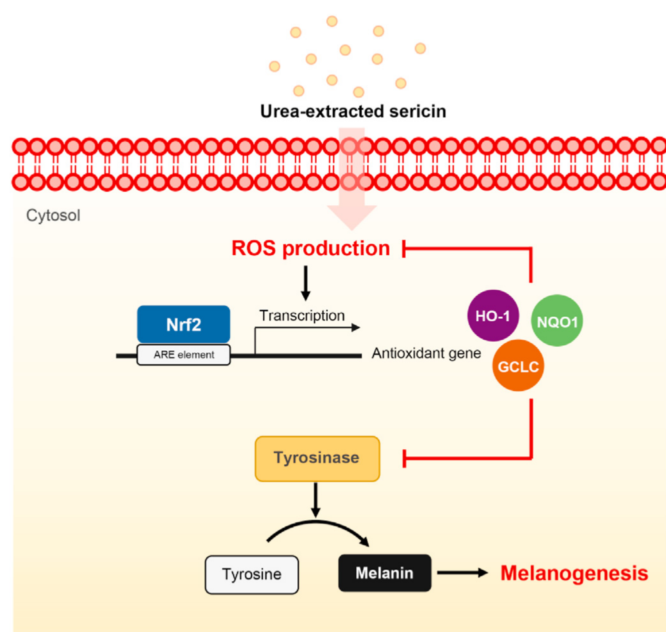

Fig. 8. A possible mechanism of urea-extracted sericin for the inhibition of melanogenesis and their role in the activation of ROS levels. The urea-extracted sericin induces generation of reactive oxygen species (ROS), resulting in activation of a Nrf2-ARE signaling pathway, which further induces the expression of HO-1, NQO1, and GCLC. The action of Nrf2-ARE signaling pathway subsequently inhibits melanin synthesis in B16F10 cells.

previous reports.[37,38] Nrf2 knockdown cells exhibit the reduction of anti-oxidative properties in melanocytes.[39] A previous report suggests that oxidative stress may lead to hypopigmentation by the downregulation of melanogenic enzymes.[40] However, treatment with urea-extracted sericin did not show cytotoxic effects at the effective concentration of anti-melanogenic activity. We propose that urea-extracted sericin inhibited melanogenesis and suppressed ROS-induced damage in melanocytes. Nevertheless, further study is required to fully understand these mechanisms. The possible mechanisms responsible for urea-extracted sericin-reduced melanogenesis in B16F0 cells are illustrated in Fig. 8.

5. Conclusion

From the above data, we concluded that low concentration of urea-extracted sericin (at 30 μg/mL) downregulates melanin production via the suppression tyrosinase activity, indicating its anti-melanogenesis efficacy. Additionally, the inhibitory effects of urea-extracted sericin in melanogenesis was also involved in the activation of intracellular ROS production. Urea-extracted sericin mediated beneficial effects via the activation of the Nrf2 pathway, which induces the antioxidant related genes HO-1, NQO1 and GCLC. Therefore, urea-extracted sericin exerts potential therapeutic effects in hyperpigmentation disorders and its complications.

Declaration of competing interest

Authors declare no conflict of interest involved in this study.

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Appendix A. Supplementary data

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