

Urea-Extracted Sericin is Potentially Better Than Kojic Acid in the Inhibition of Melanogenesis Through Increased ROS Generation

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Research article

Keywords: sericin, melanogenesis, sericin extraction, tyrosinase activity, ROS

Posted Date: November 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-95384/v1>

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Abstract

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 antityrosinase 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.

Conclusions: Our results present the potential inhibitory effect of urea-extracted sericin on melanogenesis. Our results support the therapeutic potential of urea-extracted sericin in the treatment of hyperpigmentation and its complications.

Background

Hyperpigmentation is a common disorder of the skin, particularly in people of Asian ethnicity, who have a greater predisposition to congenital and developed 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, known as melanogenesis. Inhibition of melanogenesis is a rational adjuvant approach to the therapy of hyperpigmentation disorders. A variety of 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 are 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*. 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 urea extraction of sericin (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). 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.

Methods

Extraction of silk sericin

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, Allerød, Denmark).

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 mm²). 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/in² (1 lbf/in² = 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).

Extraction of silk sericin by urea degradation

The silk sericin was isolated from the cocoons of mulberry (*B. mori*) silk 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).

Characterization of silk sericin

UV-vis absorption spectroscopy

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

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was used to determine the thermal behavior. The composition silk sericin with 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.

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.

Cell culture

The B16F10 melanoma cell line (ATCC number CRL-6475) were 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% CO₂ in a humidified incubator.

Cytotoxic assay

The PrestoBlue™ reagent (Invitrogen, USA) was used to evaluate the influence of silk sericin on cell viability. Metabolically active 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 1×10^4 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 $\mu\text{g/ml}$) 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.

Determination of intracellular antioxidant activity

The intracellular antioxidant activity of sericin against H_2O_2 was evaluated using the 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, fresh media was replaced with different concentrations (10, 30 and 50 $\mu\text{g/ml}$) 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 minutes. 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).

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 minutes. 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).

Estimation of melanin content

Intracellular melanin was quantified in B16F10 cells cultured in DMEM with silk 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).

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 media 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 conducted according to the manufacturer's instructions.

Statistical analyses

All experiments were carried out three times in triplicate. The results are expressed as mean \pm 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 $P < 0.05$.

Results

Characterization of sericin

UV-vis spectra of sericin

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

Thermal decomposition analysis

The thermogravimetric analysis of sericin from different extraction methods is shown in **Figure 1c**. The TGA curve showed that the thermal transition occurred in two temperature ranges: <100 and $150-400^{\circ}\text{C}$. 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 $100-300^{\circ}\text{C}$.

Amino acid composition of sericin

The amino acid composition of sericin is shown in **Figure 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 a very small amounts.

Cytotoxicity of sericin

We first examined whether sericin has an influence on cell viability. After melanocytes were exposed to sericin for 12, 48 and 72 h, the Presto Blue assay was performed to determine the viability and

morphology of the cells (**Figure 2A-D**). Treatment with H₂O₂ 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.

Antioxidant potential of sericin against H₂O₂-induced oxidative stress

The effect of pre-treatment of cells with sericin for 48 h is shown in **Figure 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 H₂O₂ exposure, it was observed that pre-treatment of acid-, base-, and heat-extracted sericin at 10, 30 and 50 ng/ml significantly increased cell viability (post 24 h of H₂O₂ 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 H₂O₂-induced oxidative stress since this was a toxic dose. Sericin from all extraction methods improved cell viability (post 24 h of H₂O₂ treatment) compared to the positive control (kojic acid).

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 **Figure 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 **Figure 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.

***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 **Figure 5**, treatment with sericin obtained from acid, base, 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 (**Figure 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 pigmented melanoma cells.

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 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 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 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 (17). 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 (18). 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 (19).

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 (20).

The production of several reactive oxygen species plays an important role in melanin production (21). 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 H₂O₂ 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 H₂O₂-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 H₂O₂ exposure (7). Protection against oxidative stress is the potential mechanism of preventing and modulating melanogenesis in hyperpigmentation disorders (22, 23). 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 H₂O₂-induced oxidative stress. Sericin can play 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 (24). 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 (25). Although all types of extracted sericin are considered to be potent antioxidants, the only urea-extracted sericin exerted an inhibitory effect 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 (26). 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. Treatment with kojic acid was significantly reduced in melanin contents and tyrosinase activity. Nevertheless, treatment with kojic acid did not affect ROS levels in cells. 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 contrast to those of earlier studies showing that the induction of intracellular peroxide production contributes to melanogenesis in B16F0 cells (27, 28). We suggest that the antioxidant system responds to increased ROS levels by sustaining redox homeostasis (29), leading to suppression of melanogenesis. A previous report suggests that oxidative stress may lead to hypopigmentation by the downregulation of melanogenic enzymes (30). However, treatment with urea-extracted sericin did not show cytotoxic effects at the effective concentration of anti-melanogenic activity. Mitochondrial dynamics are involved in melanogenesis regulation. The activation of the ROS-ERK pathway by mitochondrial fission contributes to the proteasomal degradation of microphthalmia-associated transcription factor (MITF), which

subsequently downregulates melanogenesis (31). Thus, 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. 6. More information is required about the biological responses to sericin in the regulation of melanogenesis.

Conclusions

Our results show that a high level of ROS generation by sericin obtained from urea extraction was significantly associated with reducing melanin production and tyrosinase activity. Urea-extracted sericin thus has a potential therapeutic effect in hyperpigmentation disorders.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by National Research Council of Thailand and Second Century Fund (C2F), Chulalongkorn University.

Authors' contributions

Sarocho cherdchom conducted, analyzed, interpreted the data and wrote the manuscript.

Amornpun Sereemasapun were involved in planning and supervised the work.

Pornanong Aramwit supervised the research project and contributed to the final version of the manuscript.

All authors read and approved the final manuscript.

Acknowledgements

This research project is supported by National Research Council of Thailand and Second Century Fund (C2F), Chulalongkorn University.

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Figures

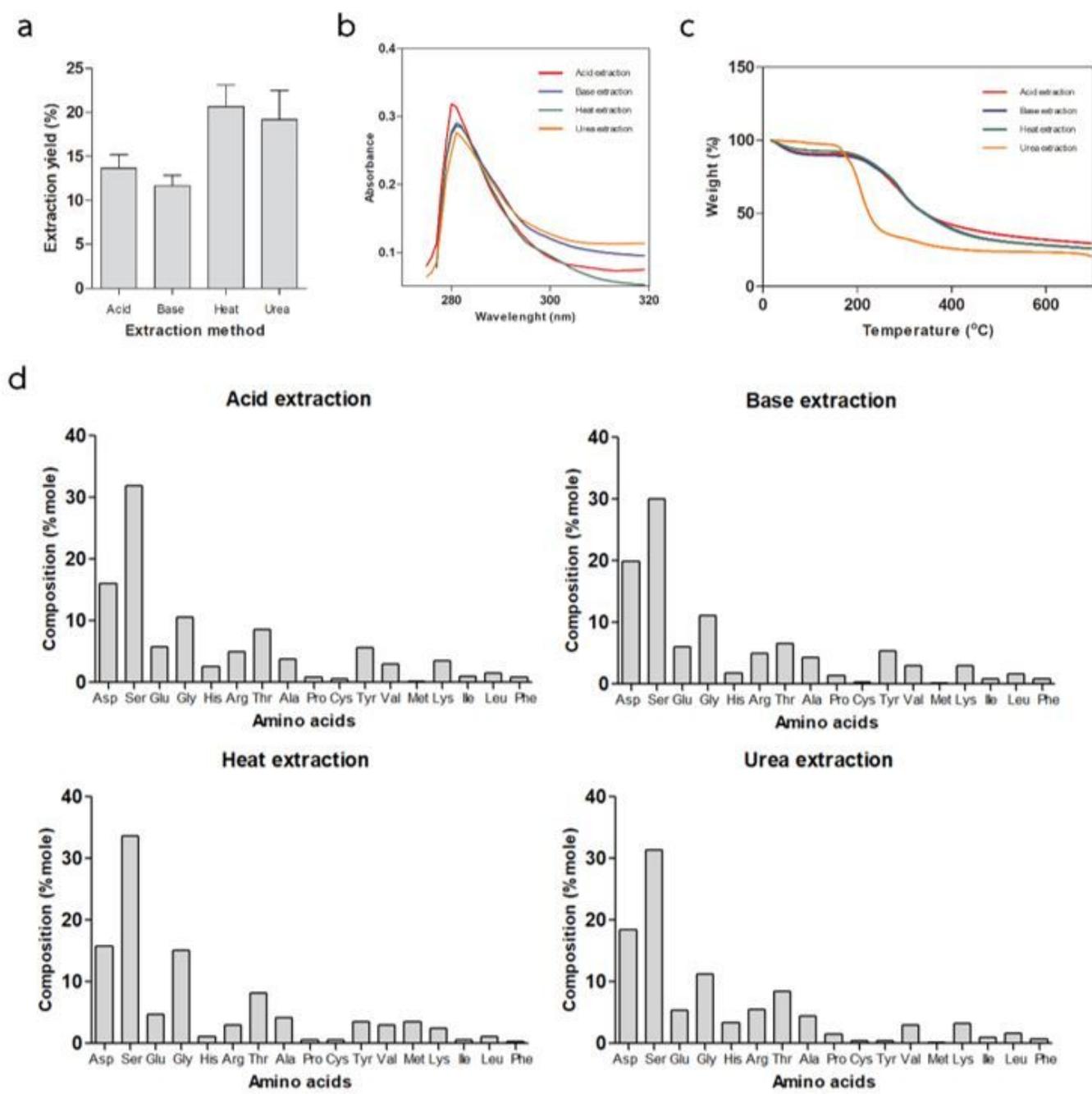


Figure 1

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).

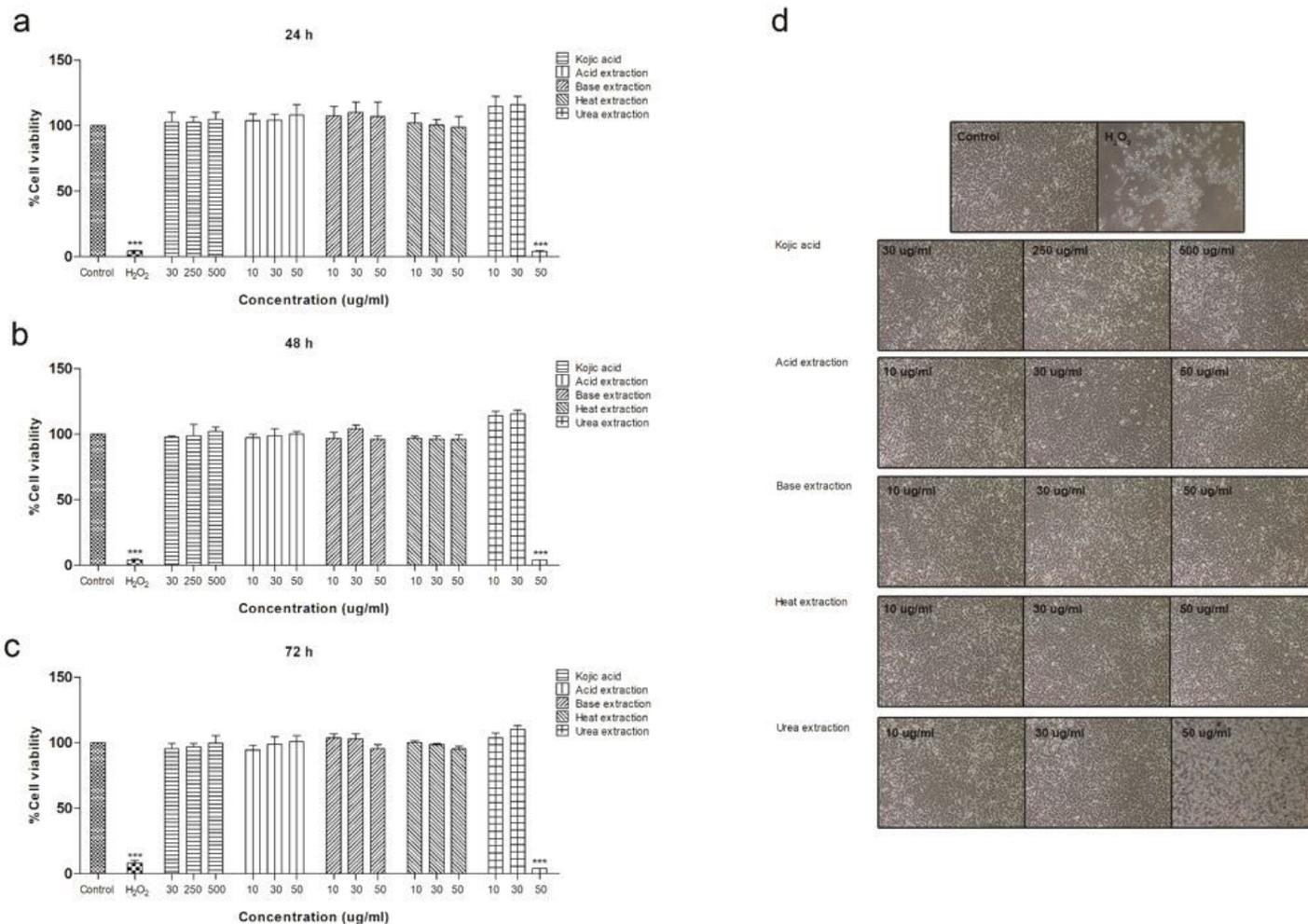


Figure 2

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 \pm standard deviation of three determinations. Statistically significant differences compared with the control group.

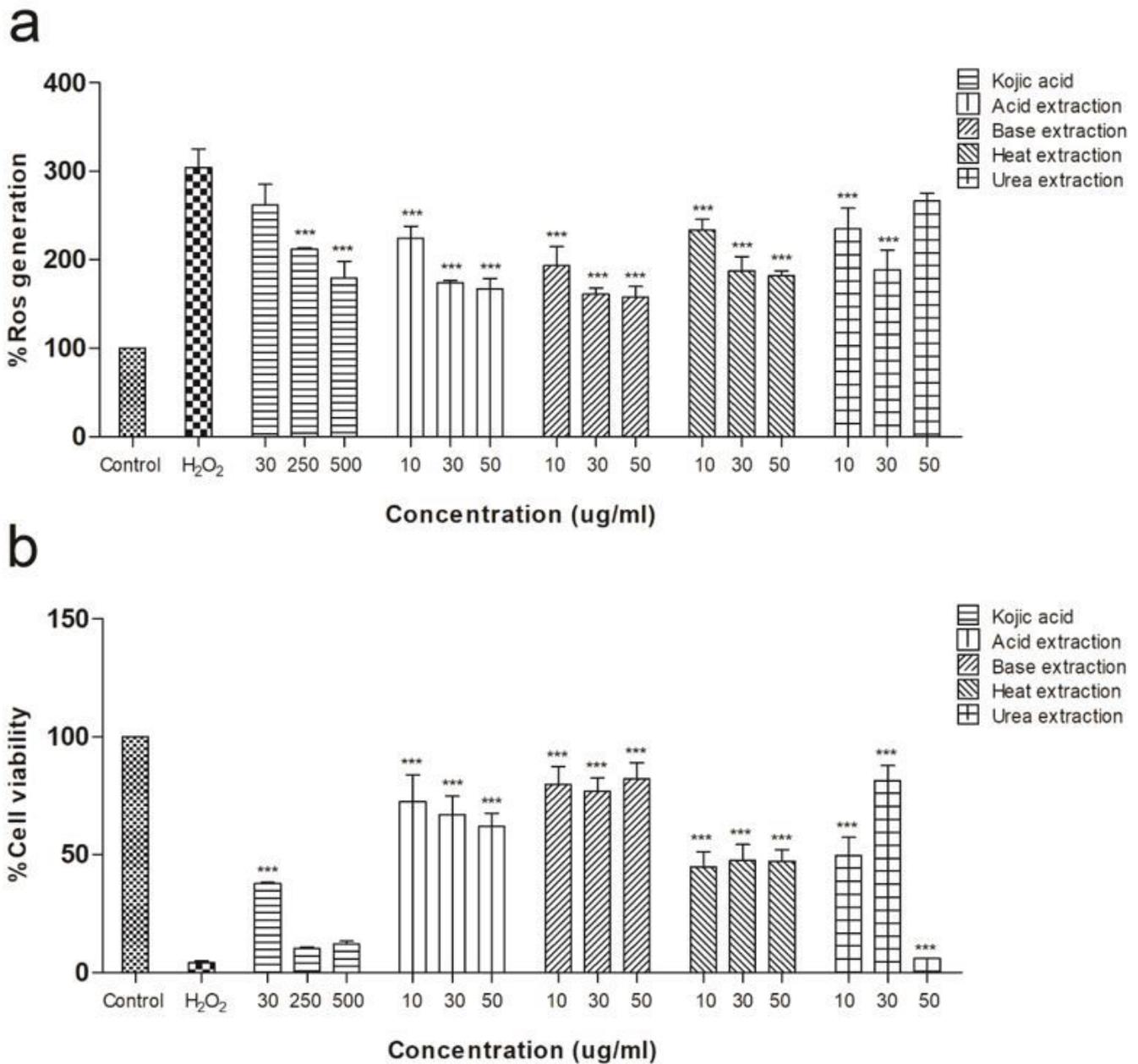


Figure 3

Protective effect of sericin from different extraction methods against H₂O₂ induced oxidative damage and promotion of cellular viability. The measurement of ROS levels was observed after H₂O₂ exposure (a). The pre-treatment of sericin significantly increased the cell viability (post 24 h of H₂O₂ treatment) (b). Values are mean ± standard deviation of three determinations. Statistically significant differences compared with the H₂O₂ group.

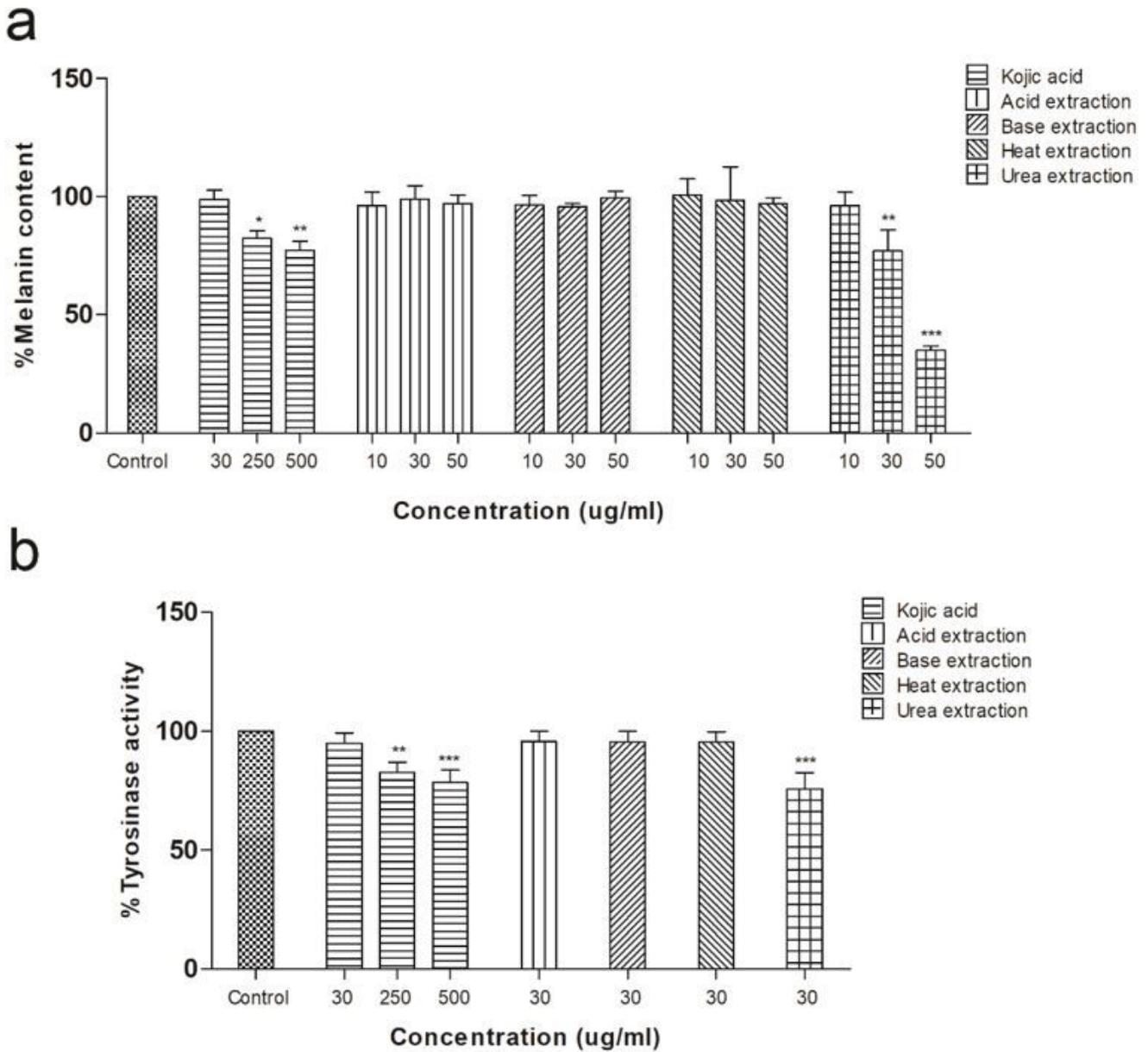


Figure 4

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) intracellular tyrosinase activity (b). Values are mean \pm standard deviation of three determinations. Statistically significant differences compared with the control group.

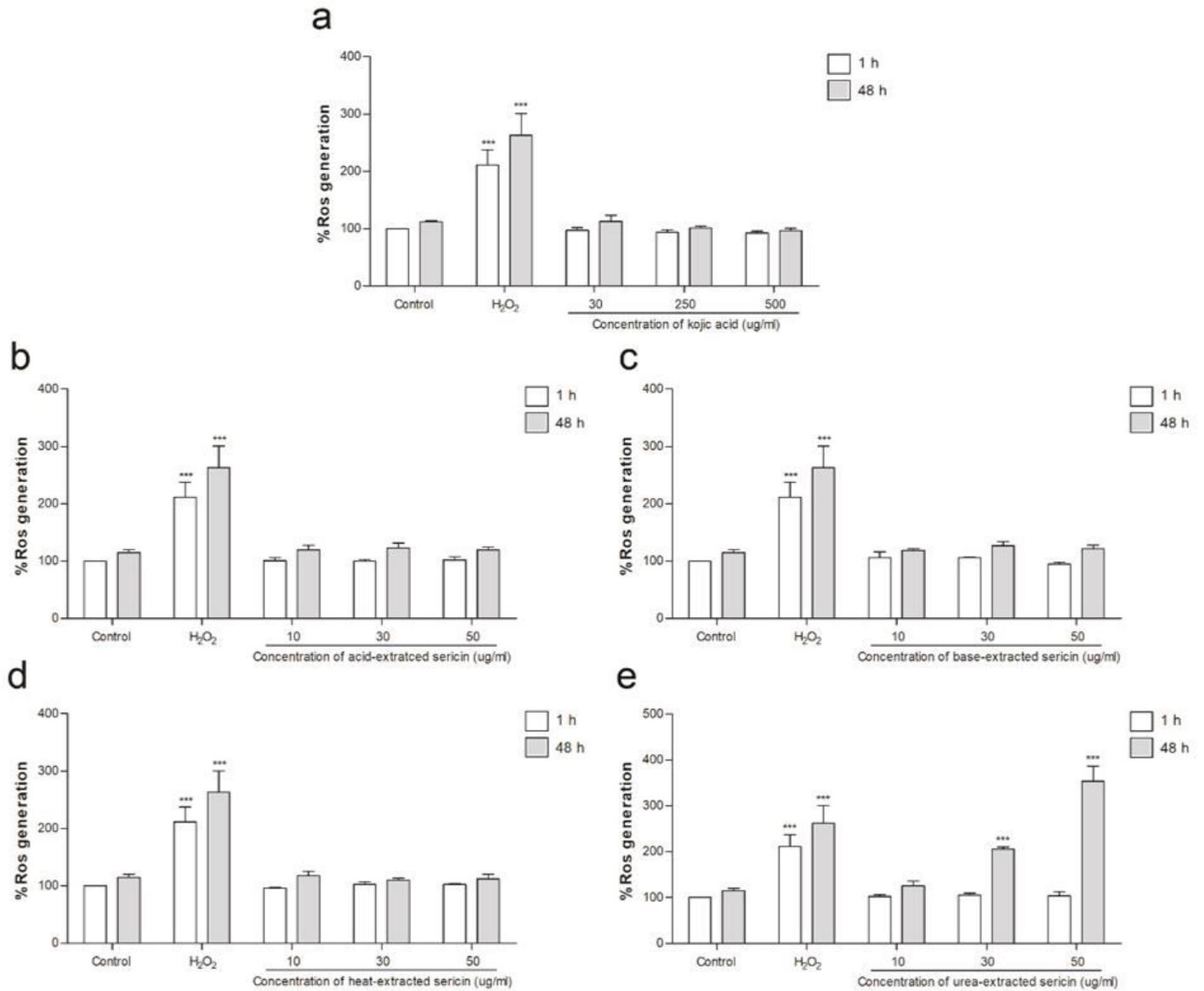


Figure 5

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 \pm standard deviation of three determinations. Statistically significant differences compared with the control group.

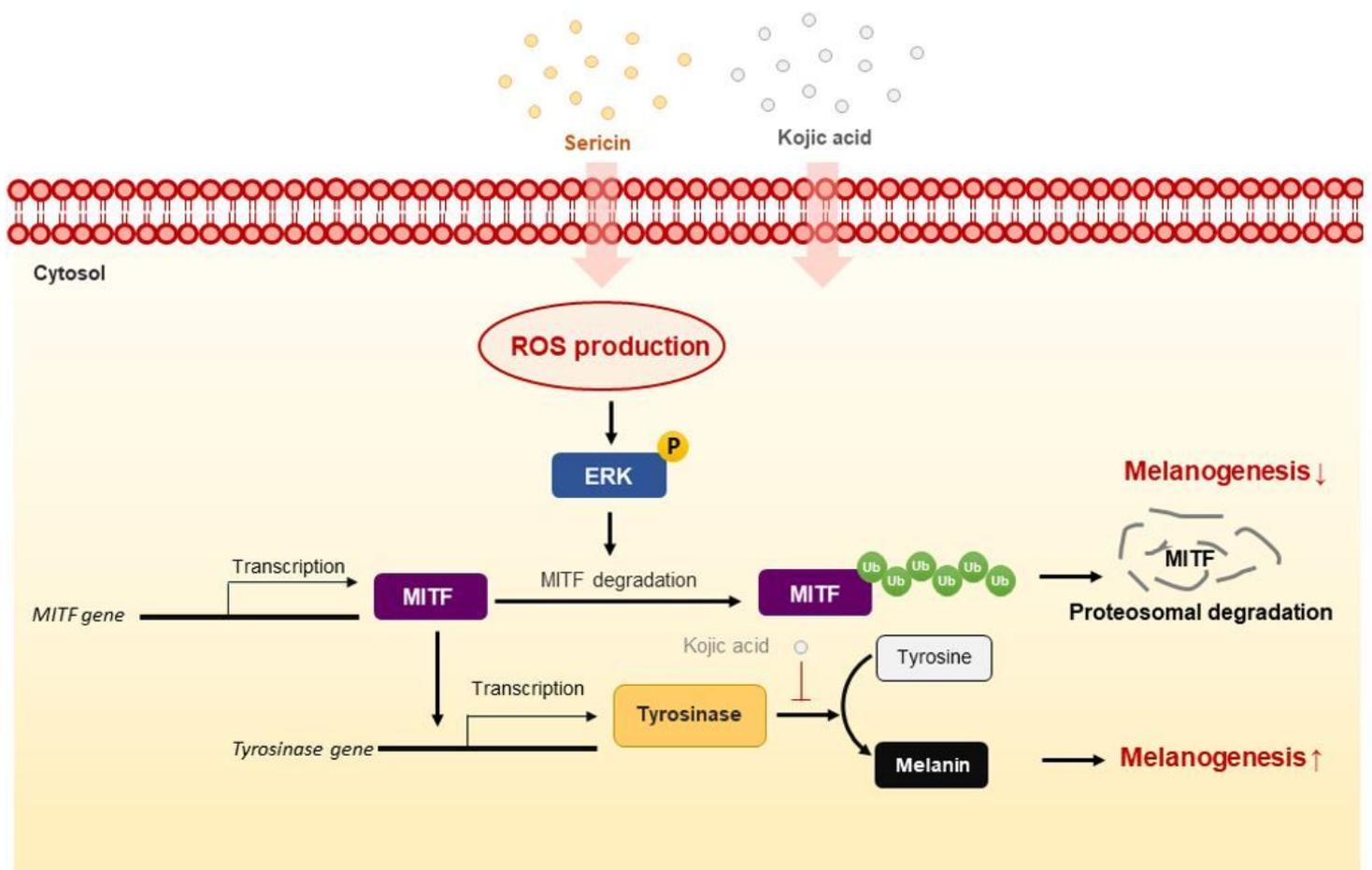


Figure 6

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 ROS-ERK signaling pathway, which further induces ubiquitination and proteasomal degradation of microphthalmia-associated transcription factor (MITF). The degradation of MITF subsequently downregulates melanogenesis in B16F10 cells.