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Anti-tyrosinase and anti-melanogenic potential of shallots (*Allium ascalonicum*) from various cultivation sites in Thailand

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ABSTRACT

Objectives: The present study aimed to investigate the anti-tyrosinase effect of shallots (*Allium ascalonicum*) in Thailand and to prepare an optimised shallot extract for use as a skin-lightening agent. **Materials and Methods:** Aqueous and ethanolic shallot extracts from 14 cultivation sites were investigated for anti-tyrosinase effect and cytotoxicity. Then the potential shallots were selected for the preparation of optimized shallot extract. **Results:** Shallots from Phayao exhibited low cytotoxicity and a high tyrosinase inhibitory effect compared with shallot from other cultivation sites. Therefore, Shallots from Phayao were selected to prepare optimized shallot extract, AA 20-4-40. Cytotoxicity and genotoxicity of the extract were not observed in human dermal fibroblast cells treated with the extract at 1–10 mg/ml. The extract also decreased both mushroom tyrosine activity and intracellular tyrosinase activity in B16F10 cells. In addition, the extract significantly decreased melanin content in cells. **Conclusion:** These results indicated that AA 20-4-40 has the potential to be used as a skin-lightening agent in cosmeceuticals.

Keywords: *Allium ascalonicum*, anti-melanogenesis, anti-tyrosinase, shallots, Thailand

INTRODUCTION

Cosmeceuticals made from natural compounds are now widely used. Many plants and herbs have been investigated for pharmacological effects on the skin. A skin-lightening effect is one popular property of cosmeceuticals. Melanin pigments are produced by melanocytes in the epidermis and hair follicles.[1] Melanogenesis is the cascade of the biochemical pathway to convert tyrosine to melanin. Initially, tyrosine is hydroxylated by tyrosinase to become l-3,4-dihydroxyphenylalanine (L-DOPA) which is rapidly oxidized to dopaquinone. Then, dopaquinone spontaneously undergoes by a free radical coupling pathway to form melanin. Tyrosinase hydroxylation is the rate-limiting step in melanin synthesis.[2,3]

Shallots (*Allium ascalonicum* L., family Amaryllidaceae) are an economically important crop in Thailand. They have been used in Asian foods and folk medicines since ancient times. The shallot bulb contains many compounds such as sulfur-containing compounds and high levels of flavone and polyphenolic derivatives.[4-6] These compounds in shallots also exhibit pharmacological effects, reported to include antimicrobial and antiviral,[7-9] anti-inflammatory,[10] and antioxidant effects.[6,10] These effects, especially anti-oxidation, are related to the high contents of flavone and polyphenolic derivatives.[11]

The major compounds of shallots are similar to those found in onions (*Allium cepa* L.), but shallots have higher amounts. Onions have been widely used in cosmeceuticals for several skin problems. Many studies have reported the pharmacological effects

of onions, such as antimicrobial,[12,13] immunosuppressant,[14] tyrosinase inhibition,[2] and anti-inflammatory effects.[15]

Our previous study indicated that shallot extracts contained a high flavonol glycosides content including quercetin-4′-glucoside, quercetin-3,4′-diglucoside, and quercetin, which contributed to the antioxidant activity.[16] In addition, many studies have reported the inhibitory effect on melanogenesis of quercetin and quercetin derivatives from plants.[2,17,18]

Shallot may, therefore, have effects like onions that can be used in cosmeceuticals. There have not been any reports about the anti-tyrosinase activity of shallots. Shallots are cultivated in various sites in Thailand. Different environmental conditions and cultivation factors can affect the production and accumulation of primary and secondary metabolites in plants.[19-21] Therefore, this study aimed to investigate the anti-tyrosinase effects of extracts of shallot planted in various cultivation sites in Thailand and the optimized shallot extracts. The toxicity of the optimized shallot extracts on human dermal fibroblast cells was also investigated.

MATERIALS AND METHODS

Reagents

Mushroom tyrosinase, L-DOPA, arbutin, kojic acid, Triton-X, cytochalasin B (Cyto-B), mitomycin C (MMC), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypsin-EDTA were purchased from Gibco® (Life Technologies, MD, USA).

Plant Materials

Shallots used in this experiment were collected from various sites in Thailand, including six provinces in the northeast region – Buri Ram (BR), Nakhon Phanom (NP), Nakhon Ratchasima (NR), Roi Et (RE), Si Sa Ket (SK), and Yasothon (YT); five provinces in the north region – Chiang Rai (CR), Phayao (PY), Phetchabun (PB), Nan (NN), and Uttaradit (UD); and three provinces in the central region – Kanchanaburi (KB), Nakhon Pathom (NP), and Ratchaburi (RB). The list of cultivation sites is shown in Table 1.

Preparation of Shallot Extracts

Dried shallots were mashed to reduce the particle size. Then, 5 g of dried shallots was extracted using 100 ml of either 95% ethanol or purified water on an orbital shaker for 6 h and macerated for 18 h. The supernatant was collected and evaporated on a water bath. The extract was freshly prepared by dissolving in buffer or medium.

Preparation of Optimized Shallot Extract

According to optimized conditions, optimized shallot extract AA 20-4-40 was prepared by the extraction of fresh shallots collected from Phayao, with 20% ethanol at 40°C for 4 h. The extract was freshly dissolved in buffer or medium before use in the experiments.

High-performance Liquid Chromatography (HPLC) Analysis

AA 20-4-40 was dissolved in 80% HPLC grade methanol, filtered through a 0.45 μm nylon filter, and then subjected to analysis using an HPLC (Agilent 1260) with a photodiode array. Poroshell C18 (2.1 mm ×150 mm, 4 μm) was used as a column. The C18 guard column was placed in a column oven set at 30°C. The injection volume was 10 μl, and the flow rate was 0.3 ml/min. The mobile phase consisted of a gradient of acetonitrile and water with 0.1% formic acid (pH 2.6), as shown in Table 2. UV spectra were recorded at 370 nm. Data were integrated using OpenLAB CDS EZChrom.

Cell Culture

Primary human dermal fibroblast cells (HDF, PCS 201 010™) were used for cytotoxicity and genotoxicity testing. B16 F10

Table 1: Cultivation sites of shallots in Thailand

No.	Code	Province	Global positioning system
Northeast region			
1.	BR	Buri Ram	14°59′42″N 103°6′12″E
2.	NP	Nakhon Phanom	17°05’45.7”N 104°44’48.0”E
3.	NR	Nakhon Ratchasima	15°13’7464”N 101°69’8463”E
4.	RE	Roi Et	16°3′12″N 103°39′12″E
5.	SK	Si Sa Ket	15°27’4874”N 104°28’2960”E
6.	YT	Yasothon	15°39′11″N 104°18′32″E
North region			
7.	CR	Chiang Rai	20°8′48″N 99°51′12″E
8.	PY	Phayao	19.1664° N, 99.9020° E
9.	PB	Phetchabun	16°25’9235”N 101°09’9296”E
10.	NN	Nan	18°35′54″N 100°44′24″E
11.	UD	Uttaradit	17.6100° N 99.9968° E
Central region			
12.	KB	Kanchanaburi	13.8649° N 99.5896° E
13.	NT	Nakhon Pathom	14.0284° N 100.1784° E
14.	RB	Ratchaburi	13.6120° N 99.6119° E

Table 2: Mobile phase gradient of the high-performance liquid chromatography analysis

Time (min)	Acetonitrile (%)	0.1% formic acid in water (%)
0	5	95
5	20	80
10	30	70
30	35	65
35	50	50
38	95	5
40	95	5
41	5	95
45	5	95

(CRL 6475™), a murine melanoma cell line from a C57BL/6J mouse, was used for melanin inhibition assay and tyrosinase activity assay. Both cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, MD, USA) in a humidified incubator at 37°C with 5% CO_2. Cells were subcultured at 80–90% confluence.

Cell Viability Assay

Cell viability after treatment with shallot extracts was determined by MTT assay. HDF cells were seeded at a density of 5×10^3 cells/well onto a 96-well plate overnight. After that, they were treated with various concentrations of shallot extracts for 24 h. Then, the medium was replaced with MTT solution (5.0 mg/ml in PBS) and incubated at 37°C for 4 h. MTT solution was removed and 100 µl DMSO was added to solubilize the formazan product. The intensity of the formazan product was measured at 570 nm using a microplate reader (Biorad, CA, USA). Cell viability was expressed as the percentage calculated from the optical density of treated cells relative to the controlled cells.

Nuclear Staining Assay

The mode of cell death was determined using Hoechst 33342 and PI costaining. HDF cells were seeded at a density of 5×10^4 cells/ml onto a 96-well plate and incubated overnight. Then, cells were treated with AA 20-4-40 for 24 h. After that, cells were incubated with 10 µg/ml of Hoechst 33342 and 5 µg/ml of PI for 30 min at 37°C. Apoptotic cells with condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus I × 51 with DP70, Olympus America Inc., Center Valley, PA, USA).

Cytokinesis-block Micronucleus Cytome Assay

The genotoxic effect of AA 20-4-40 was determined by cytokinesis-block micronucleus cytome assay (CBMN-Cyt assay) following the method of Fenech.[22] HDF cells were seeded at a density of 1×10^5 cells/ml onto a 35 mm dish. Then, cells were treated with AA 20-4-40 at various concentrations for 24 h. After that, cells were cultured in the presence of 4 µg/ml of Cyto-B, a cytokinesis blocker, for 24 h. After cells became binucleated (BN), they were fixed with 4% paraformaldehyde and then strained with Hoechst 33342 for 30 min at 37°C. Scoring was carried out at ×200 with a fluorescence microscope (Olympus I × 51 with DP70, Olympus America Inc.) using the criteria described by Fenech.[22] A total of 2000 BN cells were scored for the presence of micronuclei (MN, a biomarker of chromosome breakage or loss), nucleoplasmic bridges (NPB, a biomarker of asymmetrical chromosome rearrangement), and nuclear buds (NBud, a biomarker of gene amplification). MMC was used as a positive control. The nuclear division index (NDI) was also determined for measuring the proliferative status of viable cells. NDI was calculated from 500 cells according to the formula: NDI = (M1 + 2M2 + 3M3 + 4M4)/N, where M1, M2, M3, and M4 represent the number of cells with one, two, three, and four nuclei and N is the total number of viable cells scored.

Tyrosinase Inhibitory Assay

Mushroom tyrosinase inhibitory effects were determined following the method of Uchida *et al.*[23] Briefly, 20 µl of shallot extracts in phosphate buffer (50 mM, pH 6.8) was added in a 96-well plate. Then, 115 µl of phosphate buffer and L-DOPA, at the stock concentration of 4.57 mM, was added. L-DOPA was used as the substrate. The reaction was started by 25 µl of tyrosinase solution at the stock concentration of 403.05 unit/ml. Then, the reaction was incubated at 25°C for 10 min. The product as dopachrome was measured at 450 nm using a microplate reader. The percentage of tyrosinase inhibition effect was calculated from the optical density of treated cells relative to the controlled cells. Kojic acid (20 µM) was used as a positive control.

Cellular tyrosinase activity was further confirmed using B16-F10 cells. On treatment with AA 20-4-40, cellular tyrosinase activity assay was measured following the method of Ye *et al.*[24] with minor modification. Cells were seeded at a density of 5×10^4 cells/ml into a 96-well plate and allowed to adhere at 37°C for 24 h. Then, cells were treated with AA 20-4-40 at various concentrations for 48 h. Cells were washed with PBS and lysed in PBS containing 0.1% Triton X at –80°C for 30 min. After that, cells were placed at room temperature for 30 min. The substrate, 5.07 mM L-DOPA, was added at 100 µl/well. The reaction was incubated at 37°C for 1 h. The product, dopachrome, was measured at 450 nm using a microplate reader. The percentage of cellular tyrosinase activity was calculated from the optical density of treated cells relative to the controlled cells. Arbutin was used as a positive control.

Determination of Melanin Content in B16-F10 Melanoma Cells

Cellular melanin was measured as previously described by Arung *et al.*[2] with slight modification. Briefly, B16-F10 cells were seeded onto a 24-well plate (1×10^5 cells/ml) and cultured for 24 h. Cells were treated with various concentrations of AA 20-4-40 for 48 h. Then, the medium was removed and cells were washed twice with PBS. After that, 1 N NaOH was added at 1 ml/well to break the cell membrane and dissolve melanin in cells. Melanin content was measured at 450 nm using a microplate reader. Arbutin was used as a positive control. Cell viability was determined by MTT assay.

Statistical Analysis

Data were obtained from three independent experiments and presented as means ± standard error. Statistical analyses were performed using one-way ANOVA and *post hoc* test (Turkey's test) at a significance level of $P < 0.05$. SPSS 25.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses.

RESULTS

Screening of Cytotoxic Effects of Shallot Extracts from 14 Cultivation Sites in Thailand

To determine the cytotoxic effects of the shallot extracts, HDF was treated with either aqueous extracts or ethanolic extracts

of shallots from 14 cultivation areas in Thailand. Cells were treated with the extracts at concentration of 1 and 5 mg/ml for 24 h. Then, cell viability was determined by MTT assay. The treatment with aqueous extracts and ethanolic extracts of shallots at the concentration of 1 mg/ml resulted in cell survival of approximately 75% or more, except the ethanolic extract from Phetchabun (PB-E). At the concentration of 5 mg/ml, ethanolic extracts of shallots significantly decreased cell viability compared with the control group, except the extract from Phayao (PY-E). In addition, the viability of cells treated with aqueous extracts did not significantly decrease compared with the control group, except the extract from Nan (NN-W; Figure 1). The results showed that the extracts from Phayao (PY-W and PY-E) exhibited lower cytotoxic effects than the others.

Screening of Tyrosinase Inhibition Effects of Shallot Extracts from 14 Cultivation Sites in Thailand

All the aqueous extracts and ethanolic extracts of shallots from 14 cultivation sites in Thailand were assessed using the mushroom tyrosinase inhibitory assay. The percentage of inhibition of kojic acid (20 μM) was 41.85 ± 3.60%. Water shallot extracts, CR-W, NN-W, RB-W, KB-W, and PY-W at the concentration of 1 mg/ml exhibited approximately 10–15% tyrosinase inhibition. Ethanolic shallot extracts, BR-E, YT-E, CR-E-, NN-E, NP-E, RB-E, KB-E, and PY-E, showed approximately 10–15% tyrosinase inhibition, as shown in Figure 2. The tyrosinase inhibition of shallot from KB was highest but did not significantly different from the result of PY-W and PY-E. Shallot from Phayao was, therefore, selected for the preparation of the optimized shallot extract because of its lower cytotoxic effects and high tyrosinase inhibitory effects compared with shallot from other cultivation sites. The extraction of shallot from Phayao was then optimized using the full factorial design of the Unscrambler program with 36 conditions. Extraction with 20% ethanol for 4 h at 40°C yielded an optimized extract (AA 20-4-40) with a high content of quercetin and quercetin-4′-glucoside [Figure 3]. Therefore, AA 20-4-40 was further investigated in the next experiments.

Effects of AA 20-4-40 on HDF Cell Viability

To investigate the effect of AA 20-4-40 on normal human cells, the viability of HDF cells treated with AA 20-4-40 was

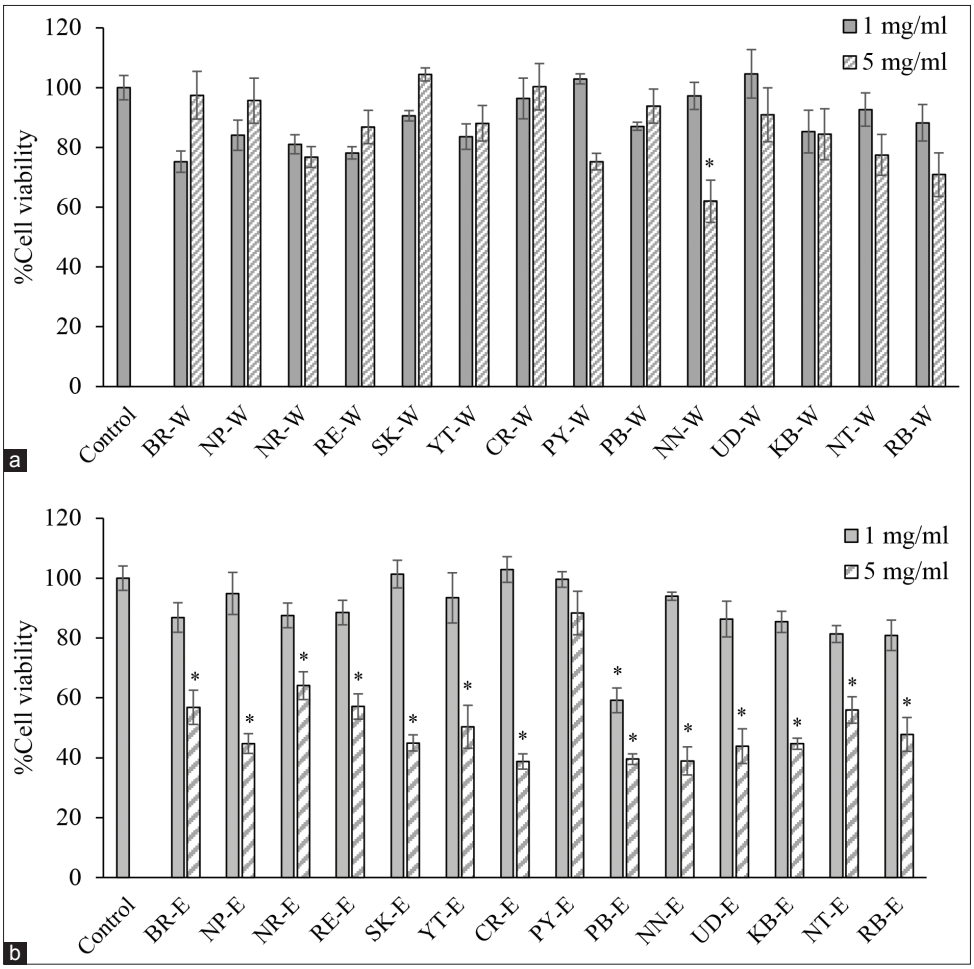

Figure 1: The percentage of HDF cell viability after treatment with aqueous extracts (a) and ethanolic extracts (b) of shallots from 14 cultivation sites in Thailand. Cells were treated with the extracts at the concentration of 1 and 5 mg/ml for 24 h. Then, cell viability was measured by MTT assay. Percentage of cell viability is represented as means of three independent triplicate samples ± standard error. *P <0.05 versus non-treated control

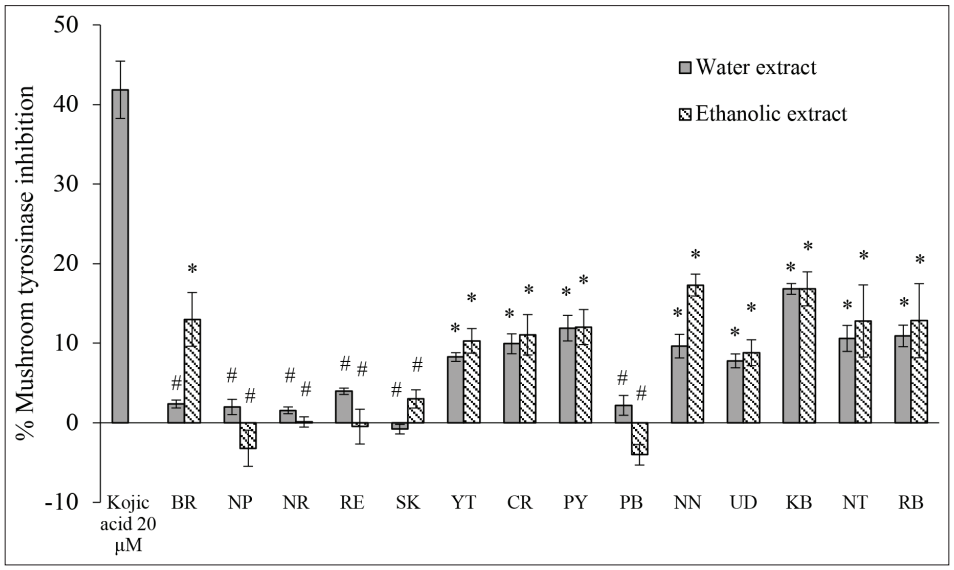

Figure 2: Anti-tyrosinase effects of shallot extracts from 14 cultivation sites in Thailand. Tyrosinase inhibitory effects of aqueous extracts and ethanolic extracts at the concentration of 1 mg/ml were determined using mushroom tyrosinase inhibitory assay. Kojic acid was used as the positive control. Percentage of tyrosinase inhibition was calculated relative to the control group. Values are means of three independent triplicate samples ± standard error. Means with different symbols (* and #) are significantly different ($P < 0.05$)

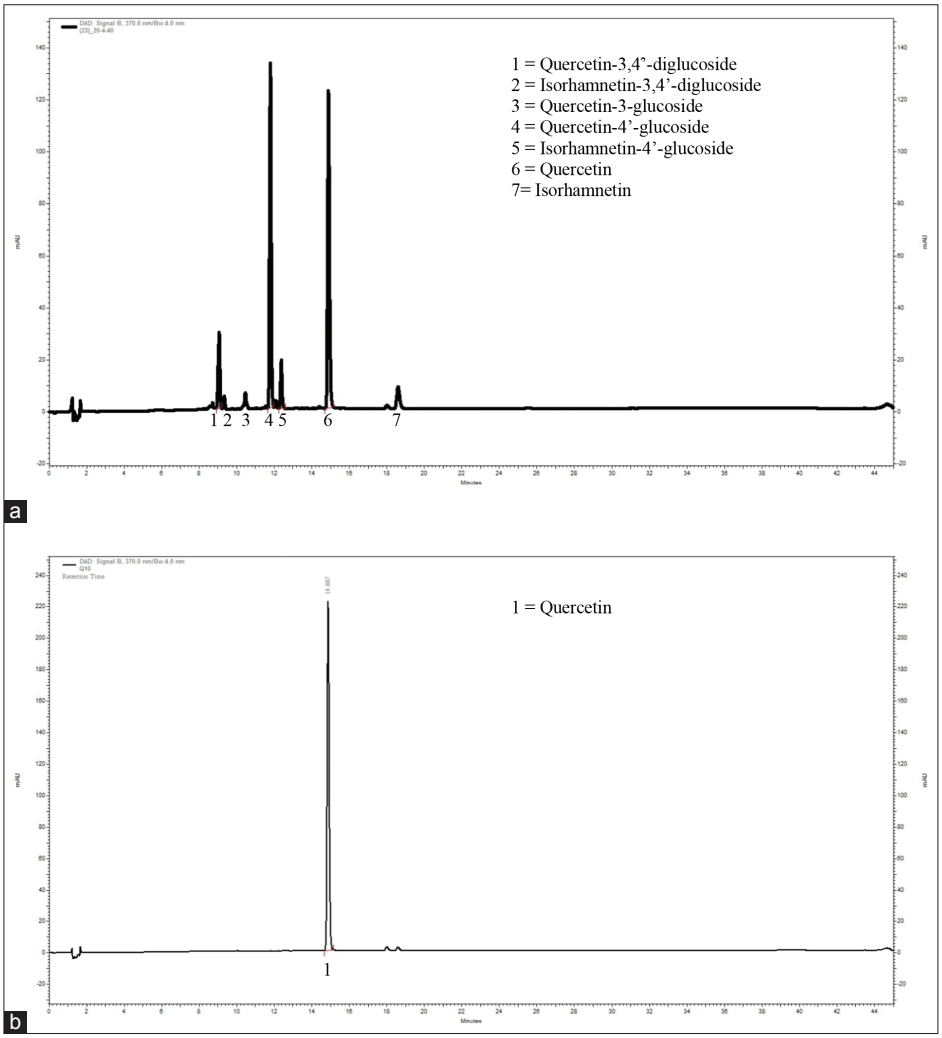

Figure 3: (a) High-performance liquid chromatography (HPLC) analysis of the optimized extract AA 20-4-40. (b) HPLC analysis of standard quercetin

determined using an MTT assay. Cells were exposed to AA 20-4-40 at the indicated concentrations for 24 h. The results showed that AA 20-4-40 significantly caused cytotoxic effects on HDF cells [Figure 4a]. Hoechst 33342/PI staining assay confirmed that apoptosis and necrosis were scarcely detectable in the AA 20-4-40-treated cells at the concentration of 10 mg/ml. Apoptotic and necrotic cells were detected among the cells treated with 20 mg/ml of AA 20-4-40 [Figure 4b]. Therefore, AA 20-4-40 at concentrations below 10 mg/ml was used in the following experiments.

Effects of AA 20-4-40 on DNA Damage

Genotoxicity of AA 20-4-40 was investigated using CBMN-Cyt assay. HDF cells were exposed to AA 20-4-40 for 24 h before cytokinesis was blocked using Cyto-B for 24 h. Then, nuclei were stained with Hoechst 33342. The results were reported as the number of BN cells with chromosomal damage or instability status (MN, NPBs, and NBUDs) per 1000 BN cells and NDI values to indicate the proliferation status of cells. Positive control (MMC, 5 μg/ml) significantly increased the frequency of BN cells with MN, NPBs, and NBUDs compared with the control group. The results showed that AA 20-4-40

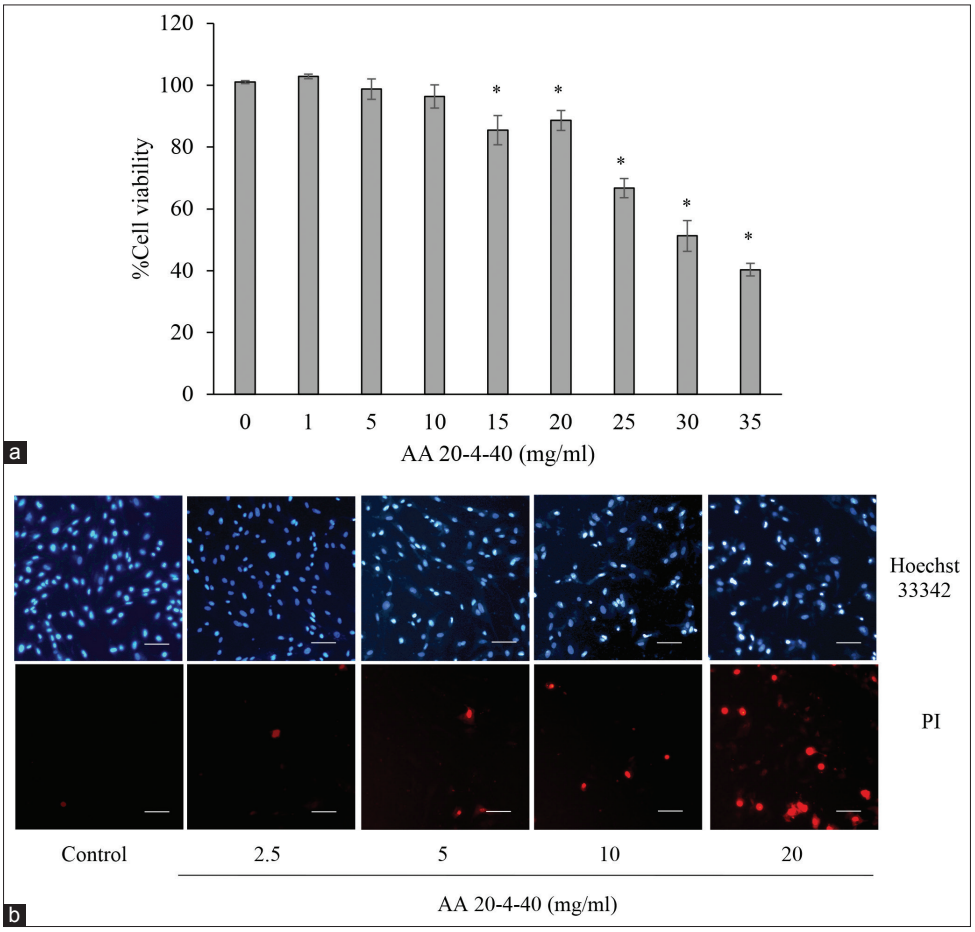

Figure 4: (a) Cell viability after treatment with AA 20-4-40 at various concentrations for 24 h was determined by MTT assay. Percentages of cell viability are means of three independent triplicate samples ± standard error. *P <0.05 versus non-treated control. (b) Nuclear morphology of the treated cells was detected by Hoechst 33342/PI costaining assay and visualized under a fluorescence microscope. Scale bar is 50 μm

Table 3: Frequencies of BN cells with MN, NPBs, and NBUDs (per 1000 BN cells) and NDI values in HDF cells treated with AA 20-4-40 at various concentrations for 24 h. *indicates significant difference (P<0.05) compared with control

Treatment	Concentration	Frequency of BN cells with MN, NPBs, and NBUDs (per 1000 BN cells) (mean±SE)	NDI (mean±SE)
Control		2.19±1.29	1.28±0.05
Mitomycin C	5 μg/ml	255.21±47.04*	1.22±0.02
AA 20-4-40	2.5 mg/ml	7.22±6.73	1.50±0.03*
	5 mg/ml	6.99±6.49	1.44±0.02*
	10 mg/ml	10.10±5.13	1.30±0.03

*P<0.05 compared with control. NDI: Nuclear division index

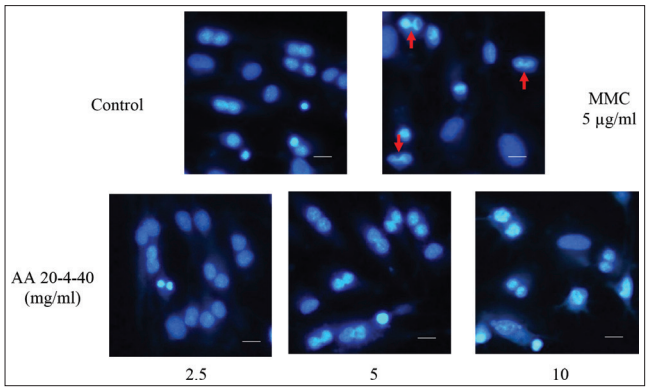

Figure 5: Genotoxicity of AA20-4-40 was determined using CBMN-Cyt assay. MMC was used as the positive control. Nuclear morphology of the treated cells was stained using Hoechst 33342 and visualized under a fluorescence microscope. Scale bar is 50 µm

did not cause genotoxic effects compared with the control group. The NDI values were also determined, to provide the proliferation status of viable cells. The results showed a significant increase in NDI values after treatment with AA 20-4-40 at 2.5 and 5 mg/ml [Table 3 and Figure 5].

Effects of AA 20-4-40 on Tyrosinase Activity

The anti-tyrosinase effects of AA 20-4-40 were determined using mushroom tyrosinase assay. The inhibition percentage of kojic acid, a positive control, was 48.75 ± 2.51%. AA 20-4-40 could inhibit mushroom tyrosinase activity in a concentration-dependent manner [Figure 6a]. The IC_{50} of AA 20-4-40 was 22.79 ± 3.49 mg/ml.

At the cellular level, B16F10 cells were exposed to AA 20-4-40 at various concentrations for 48 h. Arbutin, used as a positive control, significantly decreased intracellular tyrosinase activity. Intracellular tyrosinase activity was decreased significantly by AA 20-4-40 at concentrations of 2.5–10 mg/ml in a concentration-dependent manner [Figure 6b]. The IC_{50} of AA 20-4-40 was 12.40 ± 1.08 mg/ml.

Effects of AA 20-4-40 on Melanin Content and Cell Viability of B16F10 Cells

According to *in vitro* and cellular tyrosinase inhibitory activity of AA 20-4-40, which may affect cellular melanin content, the effect of AA 20-4-40 on melanin synthesis in melanocytes was next investigated using B16F10 cells. When cells were exposed to AA 20-4-40 at the indicated concentrations, the melanin content was significantly decreased in a concentration-dependent manner without affecting cell viability [Figure 6c].

DISCUSSION

Shallots are widely cultivated at various sites and consumed as food and folk medicines in Thailand. Shallots can grow in various environmental conditions, but several factors can affect the quality and quantity of particular constituents. Different cultivation sites can affect the pharmacological activity of plants because the production of primary and secondary metabolites can be affected. Studies have reported that environmental factors can influence the production of phenolic compounds such as tannins and flavonoids, which are responsible for many pharmacological activities.[20,21] For example, agro-climatic conditions are a significant factor that can influence the phytochemical composition of plants.[25] According to the previous findings, it was suggested that cold acclimation increases the production of flavonoids, anthocyanins, and mucilaginous substances.[26] In addition, a study reported that cultivation factors, such as time of lifting from the soil and field curing after lifting, have an effect on flavonoid content in onions.[19] Laksana *et al*. investigated the flavonoid glycoside content and anti-oxidation activities of shallot from various cultivation sites in Thailand. The results showed that shallots from different locations had varied relative quercetin and quercetin-4′-glucoside contents. The extract with the highest quercetin content showed the highest antioxidant activity.[16]

In this study, shallots from 14 cultivation sites in Thailand were collected and extracted with water and ethanol. The aqueous and ethanolic extracts of shallots from Phayao exhibited lower cytotoxic effects and higher tyrosinase inhibition effects than shallot extracts from the other cultivation sites. Therefore, shallots from Phayao were selected for the preparation of the optimized extract (AA 20-4-40) which had high quercetin and quercetin-4′-glucoside contents.

The cytotoxicity of AA 20-4-40 was investigated using HDF cells. The safe concentration of AA 20-4-40 is 1–10 mg/ml, whereas higher concentrations cause cell death, both apoptosis and necrosis. The genotoxicity of AA 20-4-40 was determined using CBMN-Cyt assay, which is the standard cytogenic test to investigate genotoxicity in both *in vivo* and *in vitro* studies. AA 20-4-40 at safe concentrations did not cause a genotoxic effect, although the previous studies have reported genotoxicity of quercetin in prokaryotic cells and eukaryotic cells.[27,28] Quercetin at high doses could induce micronucleus formation in the bone marrow of mice.[29] Our results were consistent with the many studies showing that quercetin does not induce DNA damage and micronucleus formation.[30,31] Many studies have indicated mostly negative results of quercetin in *in vivo* genotoxicity and carcinogenicity studies in mammals.[31-33] Further, AA 20-4-40 may increase the proliferation of HDF cells because of the increased NDI values after treatment. Considering the augmenting of fibroblast proliferation, AA 20-4-40 may accelerate the wound healing process. However, further investigation is necessary to determine the effect of AA 20-4-40 on fibroblast proliferation.

The effect of AA 20-4-40 on tyrosinase activity was examined using both enzyme assay and cell line. Although the inhibitory effect on melanogenesis was well correlated with the inhibitory effect on intracellular tyrosinase, the effects of AA 20-4-40 in cells (IC_{50} = 12.40 ± 1.08 mg/ml) were probably more potent than in enzyme assay (IC_{50} = 22.79 ± 3.49 mg/ml). It has been reported that the result of mushroom tyrosinase inhibition was not directly correlated with the results from the tyrosinase inhibition of cultured melanocytes.[34,35] A study showed that a parent compound has little depigmenting action, but its metabolite shows potent depigmenting activity in melanocytes.[36] Therefore, these results suggest that AA 20-4-40 may not directly inhibit tyrosinase activity, or there might be other mechanisms to decrease tyrosinase activity in cells.

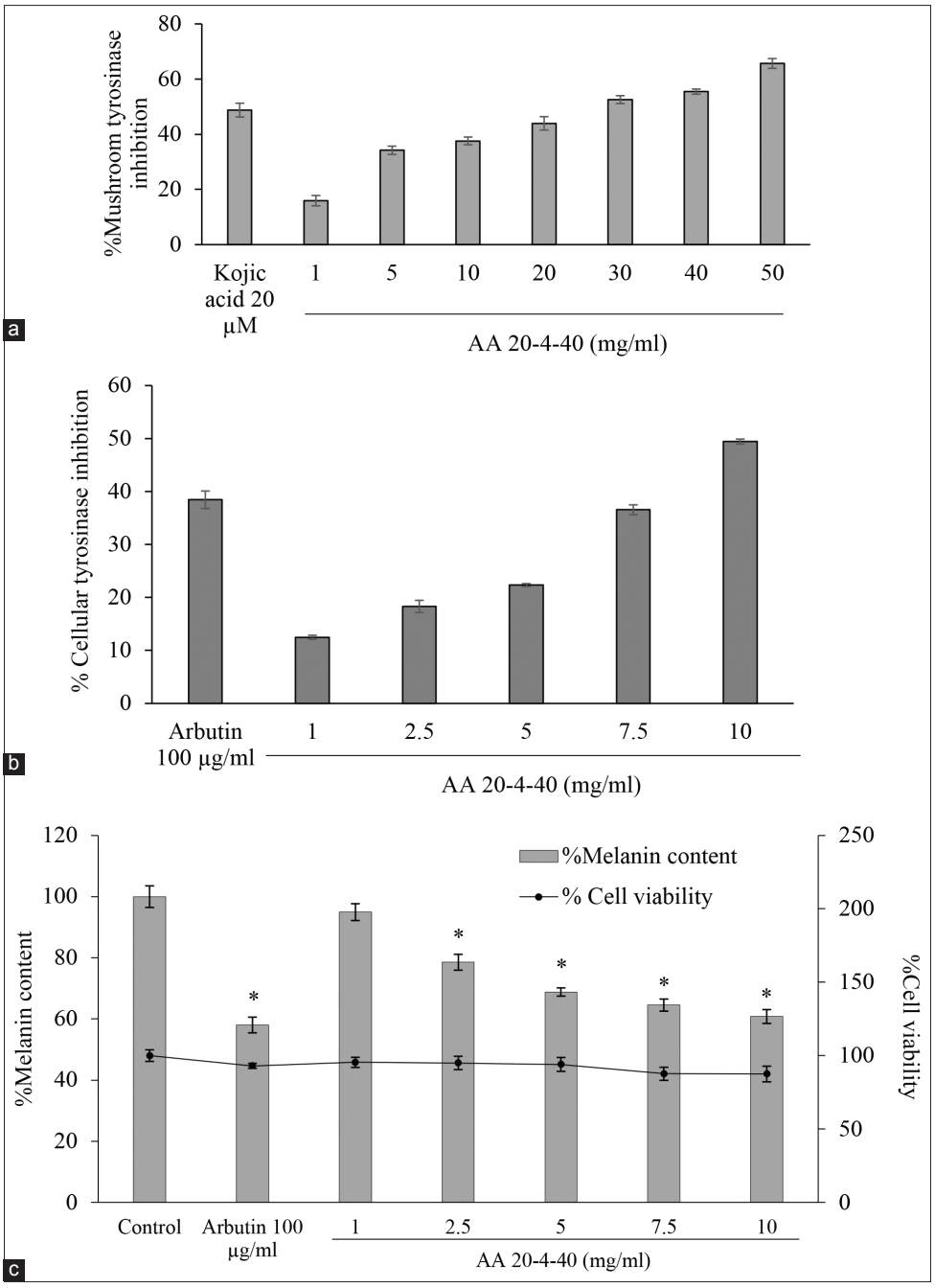

Figure 6: (a) Anti-tyrosinase effects of AA 20-4-40 by mushroom tyrosinase inhibitory assay. Kojic acid was used as the positive control. (b) Cellular tyrosinase activity was also measured. Percentage of tyrosinase inhibition was calculated relative to non-treated cells. Arbutin was used as the positive control. (c) The melanin inhibitory effect of AA 20-4-40 on B16F10 cells was investigated. After treatment with AA 20-4-40 (0–10 mg/ml) for 48 h, melanin content and cell viability were measured. Percentage of melanin content was calculated relative to the control group. Arbutin was used as the positive control. The data present means of three independent triplicate samples ± standard error. *P <0.05 versus non-treated control

AA-20-4-40 was found to decrease melanin content significantly in B16F10 mouse melanoma cells. The mechanism of AA-20-4-40 to inhibit melanin production could not be explained by cytotoxic effects because cells were treated with non-cytotoxic concentrations of the extract. The extract had an anti-melanogenesis effect due to the significant reduction in intracellular tyrosinase activity. The anti-melanogenesis properties of AA 20-4-40 may be related to the high content of quercetin and quercetin-4′-glucoside in the extract, although some studies have shown that quercetin could increase melanogenesis in human melanoma and melanocytes, as well as rodent melanoma cells.[35,37] Our results are consistent with the previous studies that showed the inhibitory effects of quercetin on melanin formation and intracellular tyrosinase activity in B16 melanoma cells.[2,18] Quercetin glycoside could inhibit melanogenesis in B16 melanoma cells with less potency than quercetin.[2,17]

To the best of our knowledge, this is the first study showing the anti-tyrosinase effects of shallots from various sites in Thailand and the anti-melanogenesis effects of optimized shallot extract. Shallot from Phayao was the most suitable for the preparation of the optimized extract because of less cytotoxicity and high tyrosinase inhibitory effects. The optimized shallot extract, AA 20-4-40, did not induce cytotoxic and genotoxic effects in primary human dermal fibroblasts. AA 20-4-40 exhibited a reduction in melanosomal tyrosinase activity and resulted in decreased intracellular melanin content.

CONCLUSION

Shallots from Phayao in Thailand had the potential to be developed as an optimized extract. AA 20-4-40 showed anti-tyrosinase activity in mushroom tyrosinase enzyme assay. In addition, the extract decreased intracellular tyrosinase activity and attenuated melanin content in melanoma cells. Therefore, AA 20-4-40 could be a beneficial ingredient in skin-lightening cosmeceuticals. However, the safety of the extract should be further investigated in *in vivo* study before use in humans.

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