Pharmacogn J. 2021; 13(4): 1030-1035
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

Azadirachta indica (Neem) Water Leaf Extract Inhibits Melanin Production and Tyrosinase Activity in B16F10 Melanoma Cells

Thanitsara Songtavisin[1,*], Benjamart Pratoomthai[2], Warachin Gangnonngiw[3], Jarinyaporn Naowaboot[4]

ABSTRACT

Introduction: Abnormal melanin production can cause pigmentary disorder such as hyperpigmentation. Finding a potent medicinal plants that can prevent pigment disorder. Objective: This study was to investigate the potential of leaf extract from *Azadirachta indica* var *siamensis valeton* to inhibit melanin formation or melanogenesis. **Materials and Methods:** *A.indica* leaf extract was tested for phenolic and flavonoid content assay. Tests using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were administered to determine the toxicity of *A.indica* leaf extract in B16F10 melanoma cells. Melanin content and tyrosinase activity assays were used to determine the potential for the inhibition of melanogenesis. Data was analyzed using SPSS. **Results:** It was found that *A.indica* leaf extract per gram has a total phenolic content of 28.73 ± 0.30 mg gallic acid equivalents and flavonoid of 12.48 ± 0.00 mg rutin equivalent. In addition, we found that these herbal extracts decreased the melanin content and intracellular tyrosinase activity in B16F10 melanoma cells without any toxicity. This study suggested that the melanin content and intracellular tyrosinase activity in B16F10 melanoma cells were decreased without any cytotoxicity by the *A.indica* leaf water extract. **Conclusion:** The findings demonstrated that *A.indica* leaf water extract inhibits melanin production through reducing tyrosinase activity. These results could be useful as a therapeutic treatment for skin hyperpigmentation disorders as well as an effective ingredient in whitening cosmetics.

Key words: *Azadirachta indica,* Tyrosinase, Melanin, Melanogenesis, Hyperpigmentation.

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History

- Submission Date: 17-04-2021;
- Review completed: 28-05-2021;
- Accepted Date: 07-06-2021.

DOI : 10.5530/pj.2021.13.133

Article Available online

http://www.phcogj.com/v13/i4

INTRODUCTION

Azadirachta *indica var siamensis valeton* (neem) belongs to the Meliaceae family which has therapeutic implications in disease prevention and treatment. Among phamacological properties of the neem tree includes antioxidant activities[1]. Leaf and bark extracts of *A. indica* have been studied for their antioxidant activities and results of the investigation clearly indicated that they have strong antioxidant potential[2-3]. As our skin is gets older, wrinkles are noticed. This is a result of use and degeneration of the body which is caused by an important component of the skin that loses strength and reduced flexibility which results in lack of moisture and dullness. In addition, there are abnormalities of the skin caused by the formation of too much melanin pigment, which causes the skin to become hyperpigmentation, referred to as melasma, freckles, age spot. While abnormal melanin production can also cause pigmentary disorder as hypopigmentation causes vitiligo, albinism and abnormal hair[4]. Therefore, when the color our skin changes to darker or lighter it is a result of changes in enzymes that stimulate melanin pigment synthesis.

Melanin is a component that causes skin color changes by synthesizing from the melanocyte cells in the epidermis cell layer that is supposed to protect against sunlight that damages the skin. The process of creating melanin is called melanogenesis which is controlled by the most active enzymes in melanin pigment synthesis, including tyrosinase, tyrosinase protein-1 (TRP-1), tyrosinase protein-2 (TRP-2)[5-6]. As mentioned above, tyrosinase plays an important role in melanin pigment synthesis. This is possible using the following processes: 1) the breakdown of tyrosine into a substance L-3, 4-dihydroxyphenylalanine (L-DOPA); 2) then transform the L-DOPA into dopaquinone using tyrosinase, which stimulates the synthesis of melanin to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP-1 that acts against oxidative stress[7] and changes from DHICA to indole. -5,6-quinone-2-carboxylic acid are both enzymes that stimulate the production of melanin.

Previous study showed that microphthalmia-associated transcription factor (MITF) was an important factor in regulating gene expression in various enzymes that stimulates the production of pigment including tyrosinase enzymes and TRP-1, TRP-2 [8]. There are also reports that MITF expression is controlled via 3 mechanisms: cAMP, SCF/cKit (via MAPKs and Or Akt) and the Wnt signaling mechanism, which affects the extracellular signal regulated kinase (ERK) that is a signal transduction protein subsequent melanogenesis[9,10,11].

There are also reports from in vitro studies of B16 melanoma cells. The extracts found in the neem leaves inhibited the process of melanogenesis using ethanol extraction method[12] but there is still no clear evidence of what point the inhibition occurs. There are many types of neem leaf substances that have biological and beneficial effects in pharmaceuticals and medicine such as antibacterial, anticancer, antioxidant, and skin ulcer[1]. Neem root extract has also been reported to inhibit melanin pigment production by inhibiting the expression of MITF, TRP-1 and TRP-2[13].

Cite this article: Songtavisin T, Pratoomthai B, Gangnonngiw W, Naowaboot J. *Azadirachta indica* (Neem) Water Leaf Extract Inhibits Melanin Production and Tyrosinase Activity in B16F10 Melanoma Cells. Pharmacogn J. 2021;13(4): 1030-1035.

There is currently more research being conducted in the area of anti-aging medicine as people are more committed to looking after their own health and enjoying the benefits of modern medicine that helps to improve their quality of life which includes the rejuvenation of youth and maintenance of beauty. The focus of society is that anti-aging medicine be scientifically accepted and results in good skin health care. However, the demand for using medical equipment and cosmetics that are scientifically accepted results in expensive products in the areas of production and distribution. Presently most cosmetics contain synthetic substances that may affect the health of consumers in the long run. Therefore, there are studies to find ways to reduce the risk of health and increase the use of such cosmetics. By using herbal extracts such as the neem extract as an alternative to synthetics in cosmetics results in less side effects in cosmetics and drugs. Researchers are interested in preliminary studies on the function of skin cells, including melanocyte, by inserting the extract from neem leaves into cells cultured in the laboratory and comparing the results of various chemical reactions in these cells with the control group that did not receive the extract. Information obtained from this research can be used as a basis for further development into human research studies. And the benefits from this study will create knowledge about the effects of extracts to the work of skin cells and will benefit the development of other extracts for use in the pharmaceutical and cosmetic industry in Thailand in the future. This project was aimed to study the effect of *A. indica* (neem) leaf water extract on melanogenesis inhibition. This was done to detect the composition of phenolic, flavonoid and then to test the toxicity of extracts by MTT assay in melanocyte cells. From there it was used to test the properties of neem leaf water extract to inhibit melanin pigment production and tyrosinase in melanocyte cells. The results provide scientific evidence for further study of Azadirachta *indica var siamensis valeton* in the application of therapeutic treatment for skin hyperpigmentation disorders.

MATERIALS AND METHODS

Chemicals and reagents

L-3,4-dihydroxyphenylalanine (L-DOPA), dimethyl sulphoxide (DMSO), synthetic melanin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), α-melanocyte-stimulating hormone (α-MSH) and kojic acid were from Sigma-Aldrich (USA). Primary antibodies specific for tyrosinase and MITF were from Santa Cruz Biotechnology (USA). Trypsin, penicillin, streptomycin, and fetal bovine serum (FBS) were from Gibco-BRL (USA). All other chemicals were from Sigma-Aldrich, unless otherwise noted.

Azadirachta indica var *siamensis valeton* leaf extracted

Plant material

The *A.incida* leaves was collected in Burirum Province, Northeast part of Thailand, between January and February 2018. Its botanical identity was determined and authenticated by a taxonomist. A voucher herbarium specimen (SKP 095 13 03 01) was given by the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkla Province, Thailand.

Extraction

Collected plant material (leaves) was cleaned thoroughly with water, cut into small pieces, boiled in distilled water (1 g of plant material in 10 mL of water) for 1 h and then filtered. Filtrates were concentrated using a rotary vacuum evaporator and subsequently lyophilized. Water extract yield was 4.0 %. The extract was stored in a freezer at –80 °C until it was further used.

Determination of Total Flavonoids Content[14]

The amount of total flavonoids were analyzed using aluminum chloride colorimetric method. Sample 100 μL was mixed with 30 μL of 10% aluminum chloride solution. The mixture was allowed to stand at room temperature (28 ± 2∘ C) for 10 min with intermittent shaking. Add 400 μL of DI water then mixed with 30 μL of 5% $NaNO_2$. The absorbance of the mixture was measured at 510 nm against a blank sample (methanol) without aluminum chloride using a UV-2550 spectrophotometer (Shimadzu, Japan). The total flavonoids content was determined using a standard curve of rutin (20- 100 μg/mL). The content was calculated as mean ± SD and expressed as milligrams of rutin equivalents (RE) in 1 g of the extract and dried powder.

Determination of Total Phenolic Compounds Content

The amount of total phenolics in extracts was determined according to the Folin-Ciocalteu procedure[15] Samples (200 µL, two replicates) were introduced into test tubes; 1.0 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured UV-2550 spectrophotometer (Shimadzu, Japan). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material.

Cell culture

B16F10 mouse melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (ATCC, USA) with 10% FBS and 1% penicillin/ streptomycin (10,000 U per100 mg mL), at 37 °C in an incutated with 5% CO_2. Cells were trypsinized, sub-cultured, and treated with *A.indica* leaf extract or kojic acids with various concentrations of tested samples for 72 h. Control cells were cultured with complete DMEM media without *A.indica* leaf extract.

Cell viability assay

Cytotoxicity of *A.incida* leaf extract on B16F10 melanoma cells was measured by using MTT assay[16] B16F10 melanoma cells (2.5×10^3 cells/well) were cultured into a 96 well plate. After 24 h, cells were treated with *A.incida* leaf extract (5, 10, 50, and 100 µg mL) or kojic acid (100 µg mL) for 72 h. After treatment, culture medium was removed and MTT solution (0.5 mg mL in PBS) was added and the plate incubated for 3 h at 37 °C. Separate supernatant out and DMSO was added to dissolve the formazan product. Negative control cells were treated in the same manner without the tested compound. After incubation at 37 °C for 15 min, optical intensity was measured at 490 nm using a microplate reader (TECAN, Switzerland).

Measurement of cellular melanin content

Total melanin content was measured according to Tsuboi *et al.*[17] with some modifications. Briefly, B16F10 melanoma cells (2.5×10^4 cells/well) were seeded into a 6-well plate and incubated for 24 h before being treated with various concentrations *A.indica* leaf extract (5, 10, 50, and 100 ug/mL) or kojic acid (100 ug/mL) in the presence of 100 nM of α -MSH for 72 h. After treatment, cell pellets were dissolved in 1 mL of 1 N NaOH at 100 °C for 30 min. The OD of the supernatant was measured at 405 nm using a microplate reader (TECAN, Mannedorf, Switzerland) and the melanin content was expressed as a percentage of the control value. Cells not stimulated with α-MSH were used as a negative control group.

Effect of *A.indica* leaf extract on tyrosinase activity in B16F10 melanoma cells

Measurement of mushroom tyrosinase activity

The effect of *A.indica* leaf extract on mushroom tyrosinase activity was determined spectrophotometrically as described previously[18] with a

slight modification. Briefly, 20 µL of mushroom tyrosinase (200 units/ml) and 20 µL of the sample were placed into a 96-well microplate, and then 160 µL of 5 mM L-DOPA in 0.1 M sodium phosphate buffer (PBS) pH 6.8 was added per well and incubated at 37 °C for 30 min. Tyrosinase activity was determined using a microplate reader (TECAN, Switzerland) at the absorbance of 475 nm, and results were expressed as a percentage of the control value (without *A.indica* leaf extract).

Measurement of intracellular tyrosinase activity

Intracellular tyrosinase activity was determined using the method described previously[19] with some modifications. Briefly, B16F10 melanoma cells (2.5x 10^4 cells/well) were seeded into a six-well plate and incubated for 24 h before being treated without or with various concentrations of *A.indica* leaf extract (5, 10, 50, and 100 ug/mL) or kojic acid (100 µg/mL) in the presence of 100 nM of α-MSH for 72 h. After treatment, cells were washed with cold PBS and lysed with PBS containing 1% Triton-X and then frozen at -80 ^{0}C for 30 min. After thawing, the lysates were centrifuged and the protein concentration was determined by using the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA). Then 100 µg protein lysates (adjusted to 100 µl with PBS) and 100 µL of 5 mM L-DOPA were placed into a 96-well plate. After incubation at 37 ^{0}C for 1 h, the absorbance was measured at 475 nm using a microplate reader (TECAN, Mannedorf, Switzerland).

Measurements of cell-free tyrosinase activity

Cell-free tyrosinase activity was determined according to the method described by Kim *et al.*[20]. with a slight modification. Briefly, cell lysate of α-MSH-induced B16F10 melanoma (without *A.indica* treatment) was prepared as described above. The lysate containing tyrosinase (50 µL of 100 µg crude lysate) was incubated with 50 µL of SG (5, 10, 50 and 100 µg/ml) or kojic acid (100 µg/mL), and then 100 µL of 5 mM L-DOPA was added to microplate wells. After incubation at 37 °C for 1 h, the absorbance was measured at 475 nm using a microplate reader (TECAN), and results were expressed as a percentage of the control value.

STATISTICAL ANALYSIS

All data were carried out triplicate and the results were presented as mean ± standard error of the mean (SEM). The values were compared using the one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparisons. P values less than 0.05 were considered to be statistically significant by utilizing the SPSS statistical analysis software (SPSS Inc., Chicago, IL, USA)

RESULTS

Phenolic and flavonoid compound

Previous studies reported the effects of phenolic compounds and flavonoids found in beans and other cereals to have an important role in inhibiting melanin pigment production. Similarly, in this study, it was found that the amount of these compounds was found to be high in *A.indica* leaf when compared to the standard values (Table. 1 at the end of the manuscript). Therefore, it is possible that with the right amount of substance Neem leaves can also be effective in inhibiting melanin production.

A.indica leaf extract exhibits no toxicity effect on B16F10 melanoma cells

B16F10 melanoma cells treated with various concentrations of *A.indica* leaf extract (5, 10, 50 and 100 µg/ml) or kojic acid (100 µg/mL) for 72 h in the absence or presence of α-MSH revealed no significant effect on cell viability (Figure 1).

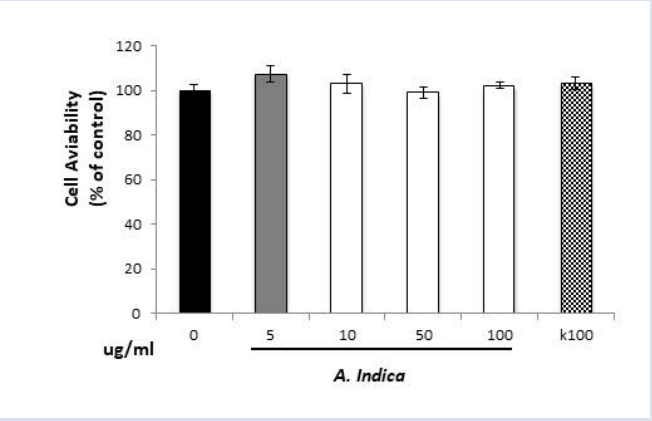

Figure 1: Effect of *A.indica* leaf extract and kojic acid on cell viability in B16F10 melanoma cells. Baseline cell viability in control wells not exposed to *A.indica* leaf extract or kojic acid was set at 100%. Data revealed changes correlating with the percentage of control. Each column represents the mean ± SD of three independent experiments.

Table 1: A.indica leaf extracted has a total flavonoid content of 12.48 ± 0.00 mg rutin equivalent per gram of extract and phenolic acid 28.97 ± 0.27 mg gallic acid equivalent per gram of extract.

A.indica leaf extracted	Flavonoid (mg/g)	Phenolic acid (mg/g)
	12.48 ± 0.00	28.97 ± 0.27

The toxicity test of *A.indica* leaf extract was performed by MTT assay by using extracted solvents at different concentrations of 5, 10, 50, 100 ug/mL and using kojic acid which is a commercial whitening agent as a positive control group. Extracts with the highest concentration of 100 ug/mL had no affect on cell death. Therefore, the *A.indica* leaf extract, which has a concentration of ≤100 ug/mL can be used for the investigation of an effect of melanogenesis in the B16F10 melanoma cells. The result of this experiment found that *A.indica* leaf extract at indicated concentrations (up to 100 ug/mL) with 72 hours incubation is useful for further studies on melanogenesis. Cytotoxicity showed no significant effects after *A.indica* leaf extract treatment (Figure 1).

The effects of *A.indica* leaf extract on melanin content in B16F10 cells

Intracellular melanin content was examined after cells were incubated with *A.indica* leaf extract. The results from the spontaneous melanogenesis assay showed a significant decrease in melanin levels in cells in a dose-dependent manner (Figure 2a). Cells treated with increasing concentrations of *A.indica* leaf extract (5, 10, 20, 50, and 100 µg mL) showed levels of melanin content decreasing to 5, 9, 11, 16 and 69 % of control, respectively. Kojic acid decrease the melanin level in cells by 13% of control (100 µg mL). Moreover, when cells were incubated with *A.indica* leaf extract in the presence of α-MSH, melanin content was also significantly reduced in a dose-dependent manner, compare to cells incubated with α-MSH alone. Melanin content of B16F10 melanoma cells treated with α-MSH become greater to 86% of control (cells without α-MSH treatment) (Figure 2b).

Effect of *A.indica* leaf extract on tyrosinase activity in B16F10 melanoma cells

This study was conducted to observe the effect of *A.indica* leaf extract and determine whether to directly inhibit it to the tyrosinase enzyme. The experiment was carried out using the mushroom tyrosinase activity method. The results showed that *A.indica* leaf extracts cannot be directly inhibited (Figure 3a), while kojic acid bleach can be directly

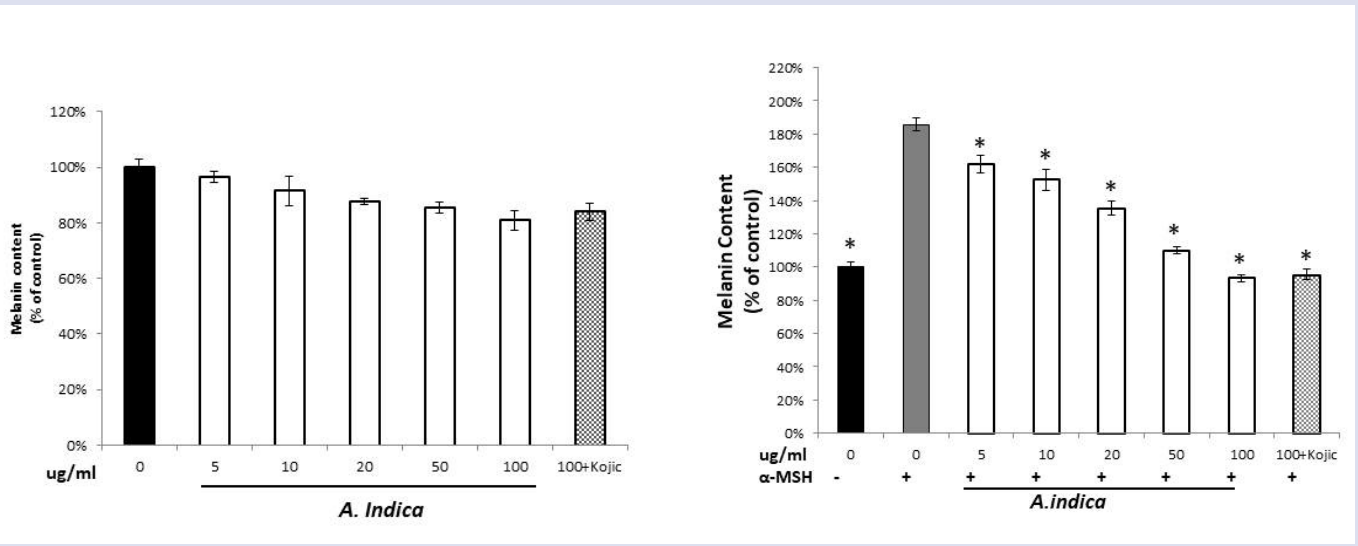

Figure 2: The effect of *A.indica* leaf on melanin in cells B16F10 produced a different level of concentration than cells where α-MSH were not administered (a). A comparison study was done with cells receiving α-MSH 100 nM (b) within 72h to check their melanin content using kojic acid for the positive control group. The baseline melanin content of this group (0) of which kojic acid, α-MSH or *A.indica* leaf were not administered to was determined. *indicates value significantly different from the α -MSH treatment group ($P < 0.05$).

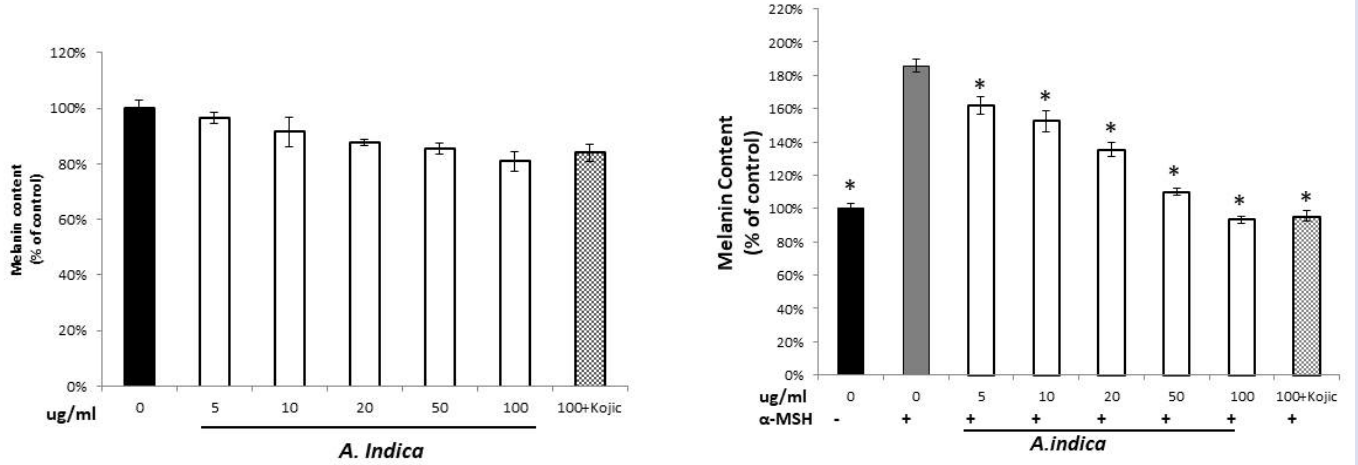

Figure 3: a and b The effects of *A.indica* leaf extract on direct mushroom tyrosinase activity compared with kojic acid, which is the positive control group (a) and compared with the effect of the extract on the cellular tyrosinase activity when stimulated by adding α-MSH (b). The baseline tyrosinase activity in the control group does not include the addition of *A.indica*, kojic acid or α-MSH (100% of control). Each column represents the mean±SD of three independent experiments. # indicates value significantly different from the α-MSH treatment group ($P<0.05$).

inhibited. From the graph (Figure 3b), the addition of *A.indica* and α-MSH extracts to test cellular tyrosinase activity can inhibit the process of pigment formation.

DISCUSSION

The purpose of this study was to investigate the effect of *A. indica* leaf extract on melanogenesis in B16F10 melanoma cell inhibition. In this experiment when studying the toxicity of *A. indica* water extract on cells we found no significant toxicity. As shown in determination of total phenolic compounds content analysis result, *A. indica* water extract contains a high level of phenolic and flavonoid compounds which have been reported to have multiple biological effects, including antioxidant activity[21]. Flavonoids have potential sources of antioxidant compounds, which are important in the plant for normal growth development and defense against injury[22]. Therefore, it is possible that the properties of the above two components result in protection against cell damage. This results correlate with previous study that reported some compound from the leaves of *A.indica* were isolated with an EtOH extract were evaluated for their inhibitory activities against melanogenesis in B16 melanoma cells, induced with α-melanocyte-stimulating hormone (α-MSH)[23] exhibited inhibitory activities in reduction of melanin content.

One method to determine the possible mechanism by which *A. indica* leaf extract is to observe its effect on tyrosinase activity which is the enzyme responsible for the synthesis of melanin[24]. The inhibition of tyrosinase activity is the most common target for melanogenesis

inhibitors. In this study, direct inhibitory effect of *A. indica* leaf extract on tyrosinase activity was observed in mushroom tyrosinase activity assays. The results indicated that *A. indica* water extract has no direct effect on inhibiting mushroom tyrosinase activity. The results indicated that *A. indica* water leaf extract had no direct effect on inhibiting mushroom tyrosinase activity. Nevertheless, this study shows that A. *indica* water leaf extract could inhibit cellular tyrosinase activity. This would concur with a former investigation revealing the mechanism involved in the melanogenesis inhibitory by some constituent in A. *indica* hexane leaf extract showed a reduction of protein level expressions which was followed by a decrease in the expression of tyrosinase[25]. Presently, the important compound in *A. indica* water leaf extract to control the cellular function through relevant process such as regulations of the mechanism remains unknown. It is still possible that the *A. indica* water leaf extract could inhibit cellular tyrosinase activity resulting in further inhibition of melanogenesis leading to skin depigmentation. However, further studies on pharmacology is necessary to confirm the results. The results from these experiments indicate that the *A.indica* leaf extract can inhibit melanin production by reducing the activity of the tyrosinase enzyme, which is the target of the inhibition of melanin production. It was found that *A.indica* leaf extract is not toxic to cells. Therefore, it is possible to develop *A.indica* leaf extracts to be used to treat disorders caused by excessive melanin production, such as melasma or other hyperpigmentation symptoms, which may be used as a component in skin whitening cosmetics. This work has thus provided a further evidence of the importance of *A. indica* leaf extract and its constituents as potential melanogenesis inhibitory agents.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support provided by Thammasat University Research Fund under the TU Research Scholar, Contract No.056/2561. We would like to thank Miss Debbie Vik for helping us in correcting our English.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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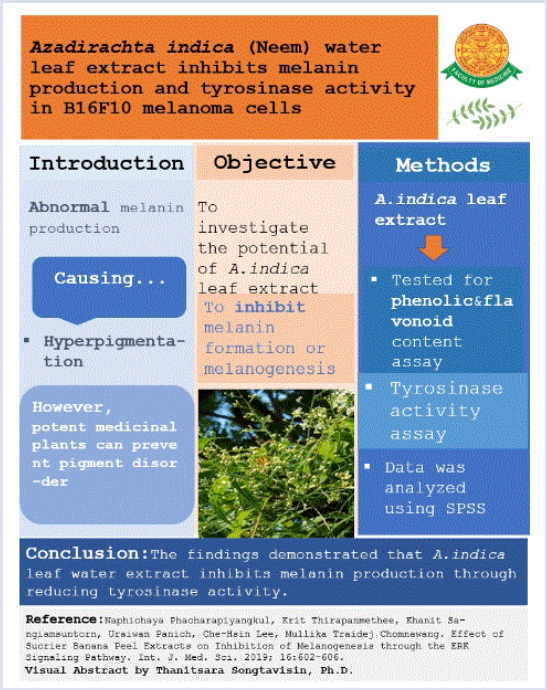
GRAPHICAL ABSTRACT



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Cite this article: Songtavisin T, Pratoomthai B, Gangnonngiw W, Naowaboot J. *Azadirachta indica* (Neem) Water Leaf Extract Inhibits Melanin Production and Tyrosinase Activity in B16F10 Melanoma Cells. Pharmacogn J. 2021;13(4): 1030-1035.