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Effects of Thanaka (*Hesperethusa crenulata*) Stem Bark Extract on Collagen Activation and Anti-Melanogenesis for Cosmetic Applications

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ABSTRACT

Thanaka (*Hesperethusa crenulata*) stem bark has been utilized as natural cosmetics, particularly in Myanmar since a thousand years ago. However, information on bioactive compounds is still limited. This research aimed to quantify the marmesin levels in the extracts of Thanaka stem bark using High-Performance Liquid Chromatography (HPLC). Thanaka stem bark samples were obtained from diverse markets in Myaing Township, Magway Region (S1), Monywa Township (S2), and Shwebo Township (S3) from Sagaing Region. Moreover, antioxidant activity, cytotoxicity, collagen production, and anti-melanogenic effects were investigated. The marmesin content in Thanaka stem bark extracts derived from S1, S2, and S3 were 12.86 ± 0.48, 1.38 ± 0.09, and 7.61 ± 0.68 mg/g dry extract, respectively. The results of Pearson's correlation coefficient revealed a significant relationship between antioxidant activity and total phenolic content. S1 exhibited the most favorable IC_{50} values for DPPH and ABTS which were 56.43 ± 1.49 µg/mL, and 14.04 ± 0.05 µg/mL, respectively. Additionally, it demonstrated a corresponding FRAP value of 337.62 ± 11.00 mM in terms of Ferrous sulfate equivalent per gram dry extract. Cell assays revealed no toxicity in immortalized human epidermal keratinocytes (HaCaT cells) treated with extracts at concentrations below 1000 µg/mL. Moreover, human foreskin fibroblasts (HFF-1 cells) exhibited enhancement in collagen production without any observed toxicity. Furthermore, Thanaka stem bark extracts could reduce melanin content and provide moderate tyrosinase inhibition in murine melanoma (B16F10) cells without toxicity. In conclusion, Thanaka stem bark extract demonstrates promising biological activities for development as a multifunctional skin care product.

***Keywords*:** Thanaka stem bark, marmesin, keratinocytes, human fibroblasts, melanocytes, collagen production

Introduction

Thanaka (*Hesperethusa crenulata* (Roxb.) M. Roem or *Naringi crenulata* (Roxb.) or *Limonia crenulata* Roxb) belongs to the family Rutaceae, which is widely grown in Southeast Asia[1] and India.[2] It is a spinous, small tree, about 8-12 m tall, with imparipinnate winged-shaped, glabrous leaves, axillary racemose white flowers, berry-globose fruit, and dull brownish-yellow bark.[3] In folk medicines, all parts of the plant are used: leaves for treatment of epilepsy; root for body pain, vomiting, and dysentery; stem powder as anti-acne and anti-aging; bark for puerperal fever; and fruit decoction as an antidote to insect poison and anthelmintics.[1,2]

Traditionally, Thanaka stem bark has been ground into powder on a round flat stone by mixing with water and applied directly to the face by Myanmar women as a natural facial cosmetic for over a thousand years.[4,5] Its application is also found in some parts of northern Thailand. It is commonly called Krajae in Thailand, Thanaka in Myanmar, [6] and Mahavilvam in Tamil, India. [2]

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It is easily available in the market and is utilized for a variety of cosmetic and dermatological purposes, such as sun protection, in cases of acne and scar concerns, skin lightening, anti-pruritus, skin coolness, and mosquito repellent.[4]

The application of Thanaka in the form of a moist, powdery paste on the cheeks may result in the presence of yellow patches, which could be considered undesirable by some individuals. Hence, nowadays, skincare products containing Thanaka extracts, such as moisturizing creams, face serum, and sunscreen products, are formulated to fulfill customer demand. These formulations incorporate additional ingredients like collagen, hyaluronic acid, vitamins, herbs, and flowers, accompanied by pleasant fragrances.[7]

Preliminary phytochemical characterization of the ethanol extract of Thanaka (stem, bark, and leaf) revealed the involvement of carbohydrates, lipids, protein, reducing sugar, phenol, flavonoid, tannin, saponin, triterpenoid, alkaloid, and quinone. [2] Moreover, plenty of coumarins were found in Thanaka.[8–10] Coumarins possess several biological properties such as antimicrobial, anti-inflammatory, anti-cancer, [11] antioxidant, anticoagulant, and antiallergic.[12] Marmesin, a natural coumarin compound, is abundant in the families of Umbelliferae, Rutaceae, Moraceae, Apiaceae, and Leguminoseae. Especially, marmesin is well known for its antifungal activity, radical scavenging activity,[13] anti-inflammatory, anti-hepatotoxic, and reducing cancer cell proliferation.[14] Additionally, marmesin has been reported to have similar tyrosinase (a key enzyme in the process of melanin synthesis) inhibitory power to arbutin (a skin whitening agent).[15] Moreover, the active compound marmesin presented in Thanaka has a wide range of UVA radiation absorption properties. [16]

Radical oxygen species (ROS) are known to trigger collagen degradation, leading to the development of wrinkled and saggy skin.

However, the detrimental effects of ROS can be countered by antioxidants, which effectively reduce oxidative stress and restore the depleted collagen levels caused by these free radicals.[17] Moreover, UV-induced oxidative stress accelerates melanin biosynthesis.[18] UV radiation promotes the secretion of α melanocyte-stimulating hormone (α-MSH) in keratinocytes and triggers melanogenesis.[19] Skin boosts melanin pigment production to protect against UV rays, but excessive melanin synthesis could cause some dermatological problems such as blemishes, solar lentigo, freckles, and melasma, as well as melanoma. [20] However, melanogenesis in melanocytes can be suppressed by antioxidants and tyrosinase inhibitors.[18]

Despite current understanding regarding the bioactivities of Thanaka extract, certain aspects remain underexplored. Therefore, this study aimed to determine the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities associated with Thanaka extract, including the marmesin content in the ethanol extract of Thanaka stem bark using high-performance liquid chromatography (HPLC).

Furthermore, the cytotoxic effects of Thanaka extracts were evaluated on various types of human skin cells, with a particular focus on immortalized human keratinocytes (HaCaT), human foreskin fibroblasts (HFF-1) involved in collagen synthesis including murine melanoma (B16F10) cells known for anti-melanogenic activity. This investigation could provide an inclusive understanding of the safety and biological abilities of Thanaka extract for its potential application in the cosmetic industry.

Materials and Methods

Materials

Materials used in our studies, including Folin-Ciocalteu reagent, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Quercetin, Trolox, Dimethyl sulfoxide (DMSO), Picric acid, Direct Red 80, Human collagen type I, α-MSH, L-dopa, Kojic acid, and bovine serum albumin, were ordered from Sigma-Aldrich, USA. Other materials included gallic acid (Merck, Germany), ascorbic acid (Fisher Scientific), marmesin (MedChemExpress, Suite Q, Monmouth Junction, USA), acetonitrile (HPLC grade), Bradford reagent, and Triton X-100 (PanReac AppliChem, Germany). And Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), 1% antibiotic-antimycotic, and trypsin were purchased from Gibco, while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Life Technologies, USA. Cell lines such as HaCaT, HFF-1, and B16F10 were purchased from ATCC, USA.

Plant collection, identification, and extraction

The Thanaka stem bark specimens (S1-S3) were purchased from local markets in three different areas of Myanmar in August 2021, specifically from Myaing Township, Magway Region (S1), Monywa Township, Sagaing Region (S2), and Shwebo Township, Sagaing Region (S3). The stem bark samples (S1-S3) were identified as *Hesperethusa crenulata* (Thanaka or Krajae), Herbarium no. 3687-3689 by Assoc. Prof. Dr. Suppachai Tiyaworanant, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. The stem bark samples were cleaned with tap water and then dried. Subsequently, the barks were cut into small pieces and further pulverized into powder for plant extraction. The powder (10 g) was individually macerated in 95% ethanol (100 mL) and then shaken in an incubator shaker (Kuhner shaker, Switzerland) at 150 rpm at room temperature for two cycles of 12 h each. The solutions were filtered through the Whatman No.1 filter paper using the previously established method.[21] All the filtered solutions for each respective sample were evaporated using a rotary evaporator (Buchi, Thailand) and freeze-dried (Labconco, USA). The yield of all extracts was recorded. The extracts were put into glass bottles and kept at -4°C for further experimental analysis.

Determination of UV absorption spectrum

The UV spectra of all extracted samples were examined in this study. Thanaka extract concentrations of 10 µg/mL were prepared using 95% ethanol, and their UV absorptions were measured at 200-400 nm range by a UV spectrophotometer (Thermo Fisher Scientific, USA).

TLC fingerprint determination of Thanaka stem bark extracts

The TLC fingerprint analysis was conducted on all extracted samples and a standard marmesin. The TLC Silica gel 60 F_{254} aluminium plate (10 cm × 10 cm) was used as the stationary phase, while a mobile phase composed of chloroform-methanol (9.5:0.5) was employed.[13] The resulting chromatographic spots were visualized under both 254 nm and 365 nm wavelengths using a spectroline fluorescence analysis cabinet, USA.

Determination of total phenolic content and total flavonoid content

The modified Folin-Ciocalteu method was utilized to quantify the total phenolic content.[22] The standard gallic acid solutions (two-fold serial dilution) were prepared in distilled water from a 1 mg/mL stock solution to establish a calibration curve. The 20 µL of each extract (1 mg/mL) or standard solution was mixed with 1:10 diluted Folin-Ciocalteu reagent (100 µL) in a 96-well microplate and followed by a 6-min incubation. Subsequently, an 80 µL solution of 7% sodium carbonate was added to the mixture and kept for 30 min at room temperature under dark conditions. The mixture was measured at 765 nm.

For total flavonoid content determination, a two-fold serial dilution of standard quercetin in ethanol was used to obtain the standard calibration curve. 20 µL of each extract (1 mg/mL) or standard solution and 20 µL of 10% aluminium chloride solution were added into 96-well plates. Then, distilled water (180 µL) and 1 M sodium acetate (20 µL) were added to the mixture. After 30 min incubation at room temperature, the absorbance was observed at 415 nm. The total flavonoid content was determined from the standard calibration curve. [23]

Method validation for HPLC analysis

The HPLC analytical method for marmesin quantification in Thanaka (*Hesperethusa crenulata*) extracts was developed based on the previous method with some modifications.[24] The HPLC (Agilent Technologies 1260 Infinity, USA) connected with a quaternary pump, an auto-injector, and a UV-VIS detector was used. A C18 column (250 x 4.6 mm, 5 µm) (Phenomenex) was employed and maintained at 35°C to separate the analytes. Solvents A (water) and B (acetonitrile) were utilized as mobile phases, filtered through a 0.2 µm nylon membrane, and sonicated for 30 min to remove the gas before analysis. The gradient used was solvent B: 0.00−3.00 min, 20.0% B; 3.00–8.00 min, 20.0–25.0% B; 8.00–18.00 min, 25.0% B; 18.00–19.00 min, 25.0–45.0% B; 19.00–35.00 min, 45.0% B; 35.00–36.00 min, 45.0–70.0% B; 36.00–45.00 min, 70.0% B; 45.00–50.00 min, 70.0–20.0% B. The flow rate was set at 1.0 mL/min while the volume injected was 10 µL and samples were detected at a wavelength of 338 nm that exhibited the maximum absorbance of the external standard by UV spectrophotometry.

The validation of the HPLC method used to determine marmesin from Thanaka stem bark extract was conducted following the guidelines outlined in the ICH guideline, 2005.[25] The key validation parameters included linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision. Linearity was evaluated by constructing a linear standard calibration curve, plotting various concentrations of the standard against peak area. The LOD and LOQ were evaluated from the calibration curve using the equation 3.3 σ/S for LOD and 10 σ/S for LOQ, where σ represents the standard deviation according to regression statistics and S represents the slope. The accuracy and precision were evaluated across three distinct concentration levels: low (15.0 µg/mL), middle (35.0 µg/mL), and high (55.0 µg/mL). The experiments were conducted to evaluate both intra-day and inter-day variability with the %RSD of less than 2%.

Determination of marmesin by HPLC

The quantification of marmesin content in Thanaka stem bark extracts was performed using a validated HPLC method. The standard stock solution (1 mg/mL) was serially diluted to the varied concentrations in methanol to construct a calibration curve in the concentration range of 10 – 70 µg/mL. All the prepared sample solutions and standard solutions were filtered through 0.22 µm syringe filters. The quantification of marmesin content was then determined using a standard calibration

curve, and the results were expressed as milligrams per gram of dry extract (mg/g DE).

Antioxidant activity assay

The assessment of DPPH radical scavenging activity was conducted as follows: A 100 µL volume of 0.20 mM DPPH methanolic solution was combined with an equal volume of each extract or standard ascorbic acid at different concentrations. The mixture was then incubated for 30 min in a dark place at room temperature. The distilled water (DI) was used as a blank, while the control was DI with a DPPH solution. [26] The absorbance of the samples was measured at 517 nm using a microplate reader from Perkin Elmer, USA. The inhibition percentage of DPPH was calculated using the following equation (1):

$$\%\text{Inhibition} = A_{(C)} - [\, A_{(+S)} - A_{(blank)}] \,/\, A_{(C)} \times 100 \quad (1)$$

Where $A_{(C)}$ was the control absorbance (DPPH solution with DI), $A_{(+S)}$ was the sample absorbance (sample with DPPH solution) after the reaction, and $A_{(blank)}$ was the absorbance of DI alone. Consequently, the IC_{50} value was obtained by plotting %inhibition against the corresponding sample concentrations.

The ABTS assay was carried out with slight adjustments. The $ABTS^+$ solution was prepared by combining 7 mM ABTS and 2.45 mM potassium persulfate in a 2:1 v/v ratio. This combination was allowed to sit at room temperature for about 16 h without being exposed to light. Then it was diluted with distilled water to achieve the absorbance value of 0.70 ± 0.02 at 734 nm. A 150 µL of the diluted $ABTS^+$ solution was mixed with 50 µL of each extract or standard (Trolox). The mixture was then incubated for 10 min at room temperature away from light. Subsequently, the absorbance was detected at 734 nm. The IC_{50} value was derived by plotting %inhibition against the respective sample concentration.[27]

The FRAP assay was performed based on the method described by Chaves et.al. (2020) [28] with slight modifications. The FRAP reagent was prepared by a combination of 300 mM sodium acetate buffer solution (pH 3.6), 10 mM TPTZ, and 20 mM ferric chloride ($FeCl_3$) hexahydrate in a proportion of 10:1:1 (v/v/v). Subsequently, 50 µL of each extract (1 mg/mL) and FRAP reagent (150 µL) were mixed up in 96-well plates. The mixture was incubated for 40 min in the dark, following the absorbance measurement at 593 nm. Ferrous sulfate ($FeSO_4$) was used to construct the calibration curve and the values were expressed as millimolar of $FeSO_4$ equivalent per gram of dry extract (mM $FeSO_4$ equivalent/g DE).

Cytotoxicity assay

Immortalized human keratinocytes (HaCaT) cell lines, human foreskin fibroblasts (HFF-1) cell lines, and murine melanoma cell lines (B16F10) were cultured separately in Dulbecco's Modified Eagle Media (DMEM) with supplementary materials (10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution). The cells were maintained in an incubator at 37°C with 5% CO_2. The cytotoxic effect was studied using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described by Cha et.al. [29] with some modifications. The cell density 1.5×10^5 cells/mL was seeded in 96-well plates and incubated for 24 h at 37°C with 5% CO_2. The stock extract solution was prepared in DMSO. The final concentration of DMSO was maintained at 0.5% for HaCaT and HFF-1 cells at 10 µg/mL-1000 µg/mL concentrations of each sample in DMEM. For B16F10 cells, the final concentration of DMSO was 0.25% when using concentrations ranging from 10 µg/mL to 500 µg/mL of each sample.

After an initial 24-hour incubation period, the cells were treated with different concentrations of extracts. The treatment was continued for 24 h for HaCaT and HFF-1 while 48 h for B16F10 cells. The medium was subsequently aspirated, and 100 µL of MTT solution was substituted into each well, resulting in a final concentration of 0.5 mg/mL. After 3 h incubation at 37°C, formazan crystals were dissolved by the addition of 100 µL dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm. The percentage of survival cells was calculated by the following equation (2) and compared to the negative control (unexposed cells).

$$\text{Cell viability (\%)} = (A_{(+Sample)} \,/\, A_{(-Sample)}) \times 100 \quad (2)$$

Collagen content determination of HFF-1

HFF-1 cells were exposed to different concentrations of extracts in serum-free DMEM containing 0.5% DMSO at 37°C with 5% CO_2 for 24 h. The serum-free DMEM with 0.5% DMSO without extract was a control. After 24 h incubation, 100 µL of supernatant medium from each sample was then transferred to new 96-well plates. A 100 µL aliquot of medium and 100 µL of 0.5 M acetic acid were mixed with 1 mL of Sirius red solution (saturated with picric acid). The mixture was then sonicated for 30 min, subsequently centrifuged at 12000 rpm, 25°C for 5 min. Then, the supernatant was removed, and the pellets were dissolved in 0.1 M hydrochloric acid (1 mL). Next, the mixture was centrifuged again at 12000 rpm, 25°C for 5 min. After centrifugation, 1 mL of 0.5 N sodium hydroxide was introduced to the collected pellets and mixed thoroughly using a vortex mixer. The absorbance was measured at 540 nm and the collagen content was determined using the standard calibration curve for collagen type I.[30,31]

Melanin content determination on B16F10

The cellular melanin content was examined following a previously reported method with some modifications. [32] In brief, B16F10 cells were cultivated in 24-well plates at a density of 5×10^4 cells/well. 24 h later, the cells were treated with different concentrations of the extract in DMEM with 0.5% DMSO and 500 nM α-melanocyte stimulating hormone (α-MSH) for an additional 48 h. Subsequently, the cells were collected and centrifuged at 12,000 rpm for 5 min. The resulting pellets were reconstituted in 200 µL of 1 N NaOH containing 10% DMSO, followed by cell lysis at 80°C for 1 h. Melanin content in supernatant was determined at 405 nm using a microplate reader (Perkin Elmer, USA).

Cellular tyrosinase inhibition on B16F10

The intracellular tyrosinase activity was analyzed as previously described by Kanpipit et al.[32] with some modifications. The cell density 5×10^4 cells/mL was seeded in 24-well plates and incubated for 24 h at 37°C with 5% CO_2. Cells were exposed to extract concentrations of 25, 100, and 250 µg/mL in DMEM with 0.5% DMSO and α-MSH (500 nM). After another 48 h, cells were collected by centrifugation. Then, cells were rinsed with ice-cold PBS (pH 7.4) and lysed using 200 µL of 0.1% Triton X-100 in PBS (pH 6.8). The protein content was assessed by the Bradford method. 100 µL of lysed cells containing 200 µg/mL protein were mixed with 100 µL of 0.1% L-DOPA, and incubated at 37°C for 20 min. The absorbance was detected using a microplate reader at 475 nm, and the cellular tyrosinase percentage was calculated.

Statistical analysis

Data were mentioned as an average of three times performance with ± standard deviation and analyzed by one-way ANOVA by the IBM SPSS Statistics version 28.0.1.0 software. Pearson's correlation coefficient (R) was used for the assessment of correlation. A significance level of $p < 0.05$ was used to denote statistical differences.

Results and discussion

Sample collection and preparation of extracts

Figure 1 illustrates the Thanaka stem bark extracts (S1, S2, and S3). The percentage yields of extraction from S1, S2, and S3 were determined to be 10.00%, 5.20%, and 6.10%, respectively (Table 1).

Determination of UV absorption spectrum

The UV spectra characteristics of each extract in the UV region (200 nm - 400 nm) are presented in Figure 2. Although all Thanaka stem bark extracts displayed similar patterns, there were differences in the intensities of absorption within the UV region (200 nm - 400 nm).

TLC fingerprint determination of Thanaka stem bark extracts

The chromatographic spots were visualized under 254 nm and 365 nm wavelengths (Figure 3). However, the marmesin band was not observed under 254 nm (Figure 3A). The marnesin's bands were observed under 365 nm as the blue bands. The R_f-value of marmesin was observed at 0.55 (Figure 3B).

Phytochemical contents and antioxidant activity of Thanaka (Hesperethusa crenulata)

For phytochemical contents, total phenolic content (TPC) and total flavonoid content (TFC) of Thanaka extracts were determined. The TPC of extracts from S1, S2, and S3 were found to be 102.61 ± 5.61, 97.37±1.27, and 92.95 ± 5.36 mg GAE/g dry extract, respectively. S1 displayed the highest total phenolic content (TPC) with no significant difference compared to S2. However, no significant differences were observed in total flavonoid content (TFC) among the samples S1, S2, and S3 (Table 1).

The antioxidant capacities of the bioactive chemicals found in plants can be studied using a variety of analytical techniques. In this research, we employed commonly used methods such as DPPH, ABTS, and FRAP assays to evaluate the capability of all samples in scavenging free radicals. The DPPH and ABTS assays involve a reaction mechanism that can be attributed to a combination of single electron transfer and hydrogen atom transfer. On the other hand, the FRAP assay only depends on the single electron transfer mechanism. [33]

Table 1 displays the antioxidant activities of S1, S2, and S3, as assessed by the DPPH and FRAP assays. The results indicate no significant differences among the three samples. However, when evaluated using the ABTS assay, S1 demonstrated significantly higher free radical scavenging activity with an IC_{50} value of 14.04 ± 0.05 µg/mL whereas S2 and S3 exhibited IC_{50} values of 15.55 ± 1.06 µg/mL and 15.89 ± 0.75 µg/mL, respectively.

Compared to the positive control, S1, S2, and S3 exhibited relatively weaker antioxidant activities. The IC_{50} value for the positive control vitamin C in the DPPH assay was 4.06 ± 0.43 µg/mL, whereas the IC_{50} value for the positive control Trolox in the ABTS assay was 6.50 ± 0.27 µg/mL.

In a previous study, the biological activities of Thanaka stem bark extract were examined using six different solvents for extraction: hexane, dichloromethane, ethyl acetate, methanol, 85% ethanol, and water. Among these solvents, 85% ethanol displayed the most potent DPPH radical scavenging capability. Additionally, the extracts obtained from Thanaka bark displayed notable anti-inflammatory properties, significant antioxidative effects, moderate inhibition of tyrosinase activity, and slight antibacterial activities. However, the extracts varied in their potencies, indicating different levels of effectiveness for each biological activity. [5] Previous research has demonstrated that the biological effects of plant extracts are predominantly associated with their antioxidant characteristics and chemical composition, especially total phenolic components. [34]

The antioxidant activity of Thanaka stem bark can be different depending on factors such as the specific origin or variety of the plant material, as well as the type of extraction solvents used.

HPLC method validation

Table 2 shows the validated parameter for marmesin, the linear range, LOD, and LOQ of the established method with a correlation coefficient (R^2) value of 0.9998. In terms of accuracy, the recovery value was determined, while the %RSD of the intra-day (repeatability) and inter-day (immediate precision) for the precision analysis were evaluated. The mean recovery ranged from 95.35% to 108.01%, while the %RSD for precision was 0.57% – 1.25% (Table 2). Thus, based on these results, it could be concluded that the implemented method was accurate and precise.

Quantification of marmesin by HPLC

The modified HPLC method was used to determine the marmesin content in Thanaka extracts. All samples exhibited a peak with a similar retention time compared to the standard, with no interruption in eluting the analytes, and efficient separation was observed in all chromatograms (Figure 4). Marmesin had a retention time of 16.666 – 16.679 min in samples (S1-S3), while the standard marmesin was 16.658 min. S1 had the highest marmesin concentration (12.86 ± 0.48 mg/g dry extract), followed by S3 and S2 at 7.61 ± 0.68 and 1.38 ± 0.09 mg/g dry extract, respectively ($p < 0.05$) (Table 1). The phytochemical contents of plants could vary depending on several factors, such as soil condition, season, climate, use of pesticides and fertilizers, age of the plant, harvesting time, and post-harvest processing (storage).[35,36] Although S2 resulted in non-significantly different total phenolic and flavonoid content and free radical scavenging capacity compared to S3, a significantly low marmesin content was observed. It might be assumed that the antioxidant activity may be due to the chemical constituents in Thanaka extract rather than marmesin. To our knowledge, this is the first report on the quantitative determination of marmesin in the 95% ethanolic extract of Thanaka stem bark. Furthermore, there is limited research on bioactive compounds present in Thanaka. Therefore, future research should be encouraged to ensure the quality control of the commercial powder of Thanaka available on the cosmetic market.

(A) Sample 1 (B) Sample 2 (C) Sample 3

Figure 1: Thanaka stem bark (*Hesperethusa crenulata*): (A) Sample 1 (S1), (B) Sample 2 (S2) and (C) Sample 3 (S3).

Table 1: Total phenolic content, total flavonoid content, and antioxidant activity of Thanaka (*Hesperethusa crenulata*) extracts from sample S1-S3

Sample	%Yield	TPC (mg GAE/g DE)	TFC (mg QE/g DE)	DPPH(IC_{50}) (µg/mL)	ABTS(IC_{50}) (µg/mL)	FRAP (mM $FeSO_4$)/g DE)	Marmesin (mg/g DE)
S1	10.00	102.61 ± 5.61^{b}	16.14 ± 0.83^{a}	56.43 ± 1.49^{b}	14.04 ± 0.05^{b}	337.62 ± 11.00^{a}	12.86 ± 0.48^{c}
S2	5.20	97.37 ± 1.27^{a,b}	15.10 ± 0.78^{a}	56.30 ± 5.89^{b}	15.55 ± 1.06^{c}	330.83 ± 5.70^{a}	1.38 ± 0.09^{a}
S3	6.10	92.95 ± 5.36^{a}	15.90 ± 0.37^{a}	57.21 ± 2.85^{b}	15.89 ± 0.75^{c}	316.90 ± 26.30^{a}	7.61 ± 0.68^{b}
Vitamin C	-			4.06 ± 0.43^{a}			
Trolox	-				6.50 ± 0.27^{a}		

The values are expressed as mean ± standard deviation (Mean ± SD) of three measurements.

$^{a, b, c}$ means the significant difference in the same column (Duncan's test, $P < 0.05$). TPC, total phenolic content; GAE, gallic acid equivalent; DE, dried extract; TFC, total flavonoid content; QE, quercetin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP, ferric reducing antioxidant power; IC_{50}, 50% of the maximal inhibitory concentration.

Table 2: Validation parameters for calibration curve, limit of detection (LOD), and quantification (LOQ)

Parameters	Results

Linearity)n=5(	
-linear equation	y = 26.66x – 8.69
Coefficient of determination)R^2(	0.9998
Range)µg/mL(	10 – 70
LOD)µg/mL( )n=9(	1.11
LOQ)µg/mL( )n=9(	3.37
Precision)%RSD(	
Intra-day)n=5(	0.79 – 1.25
Inter-day)n=15(	0.57 – 0.91
Accuracy)%Recovery((n=5(	95.35 – 108.01

*The correlation coefficient was calculated using the average of three measurements

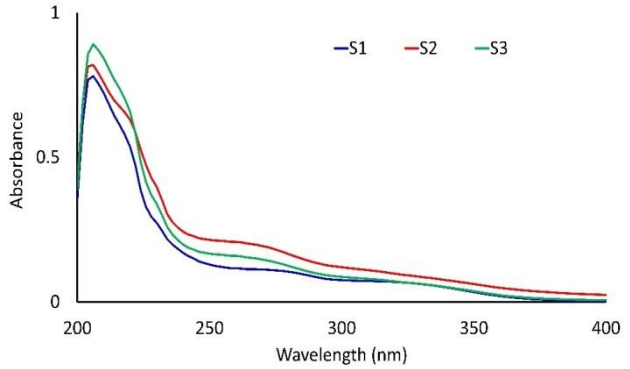

Figure 2: UV spectra of Thanaka stem bark extracts: S1, S2, and S3 (10 µg/mL in 95% ethanol).

Correlation between total phenolic content, total flavonoid content, marmesin content, and antioxidant activity

Previous studies have reported a correlation between antioxidant activity and total phenolic compounds.[37,38] This study aligns with the previously published papers due to a correlation observed between the results of the phenolic concentration and antioxidant activity assay tested. According to Pearson's correlation coefficient (R-value), an intense negative correlation was found between the high phenolic compounds of the samples and the low IC_{50} values of DPPH and ABTS, R = -0.972 and R = -0.906, respectively (Table 3). In addition, phenolic content showed a strong positive correlation (R = 0.970) with ferric reducing power (FRAP assay). There was no correlation among flavonoid content, DPPH, and FRAP assays, but a moderate relation between flavonoid compounds and ABTS was indicated (R = -0.650). A high correlation was observed between DPPH and ABTS (R = 0.780). The FRAP assay exhibited strong correlation with the DPPH IC_{50} value (R = -1.000) than with the ABTS IC_{50} value (R = -0.776). Therefore, the antioxidant capacity of Thanaka is influenced by its total phenolic content. The hydroxyl groups constituted in phenolic compounds contribute radical scavenging activity by transferring a hydrogen atom to free radicals, which thereby become stable form.[39] Besides, antioxidant strength differs based on the number and position of hydroxyl groups linked to the aromatic ring of phenols.[40] Moreover, marmesin content exhibited a strong correlation with TFC (R = 0.968) as marmesin belongs to the flavonoid group. Furthermore, marmesin content showed strong negative correlation with the IC_{50} value of the ABTS assay (R = -0.819), but a very low correlation was detected for the DPPH and FRAP analyses.

Cytotoxicity assay

The cytotoxic effects of all samples on human epidermal keratinocytes (HaCaT) cell lines, human foreskin fibroblasts (HFF-1) cell lines, and murine melanoma (B16F10) cell lines were investigated using the MTT assay. Cytotoxicity of S1-S3 was observed in HaCaT cells when the concentration reached 1000 µg/mL, resulting in a 60% cell viability (Figure 5). Interestingly, all samples showed no toxic effect (cell viability >80%) at all concentrations tested on HFF-1 cells (Figure 6A). After 24 and 48 h of exposure, all samples showed increased cytotoxicity on B16F10 cells at 500 µg/mL, resulting in a cell viability of approximately 65%, (Figure 7A and 7B). One previous experiment demonstrated the very low cytotoxic effect (IC_{50} values >12 mg/mL) of 85% ethanol, methanol, and water extracts of Thanaka bark in human skin melanoma A-375 cell lines. [5] Thus, it may be assumed that murine melanoma cell lines (B16F10) seem to have less tolerance to Thanaka extract compared to human skin melanoma A-375 cell lines. This research revealed that Thanaka extract is not toxic to skin cells including fibroblasts (HFF-1) and keratinocytes (HaCaT) at the concentration below 1000 µg/mL which is considered a significantly high concentration with negligible toxicity.

Collagen synthesis on fibroblasts (HFF-1)

The dermis layer of the skin is mainly composed of type I collagen, which functions for skin firmness, elasticity, and flexibility. The maintenance of the collagen level could lead to healthy skin.[41] In this study, a slight collagen-inducing effect was found in some samples at the studied doses (10 – 1000 µg/mL) higher than control, especially S1 at 500 µg/mL showed the highest collagen content of 44.16 µg/mL (Figure 6B). Nevertheless, the collagen-producing amounts of all samples were lower than the positive control (vitamin C) ($p < 0.05$).

Melanin content and tyrosinase inhibitory activity

Melanin biosynthesis occurs inside membrane-bound organelles called melanosomes in melanocytes situated in the basal layer of the epidermis and is subsequently carried to the epidermal keratinocytes.[42] In the melanogenic pathway, enzymes such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2) regulate melanin biosynthesis, [43] whereas tyrosinase plays a pivotal role and catalyzes the hydroxylation of L-tyrosine (monophenolase activity) to 3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA (diphenolase activity) to dopaquinone. [44,45]

Table 3: Correlation analysis (R) between total phenolic content, total flavonoid content, marmesin, and antioxidant activity

	TPC	**TFC**	**Marmesin**	**DPPH (IC_{50})**	**ABTS (IC_{50})**	**FRAP**
TPC	1.000					
TFC	0.268	1.000				
Marmesin	0.500	0.968	1.000			
DPPH (IC_{50})	-0.972	-0.032	-0.280	1.000		
ABTS (IC_{50})	-0.906	-0.650	-0.819	0.780	1.000	
FRAP	0.970	0.026	0.274	-1.000*	-0.776	1.000

* is expressed significant correlation coefficient at the 0.01 level

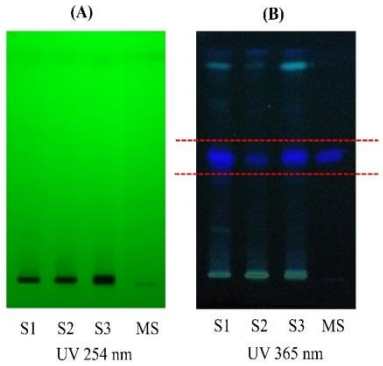

Figure 3: TLC fingerprint of Thanaka stem bark extracts (S1, S2, and S3) compared to standard marmesin (MS) using chloroform-methanol (9.5:0.5) as a mobile phase: (A) observation under UV 254 nm and (B) observation under UV 365 nm.

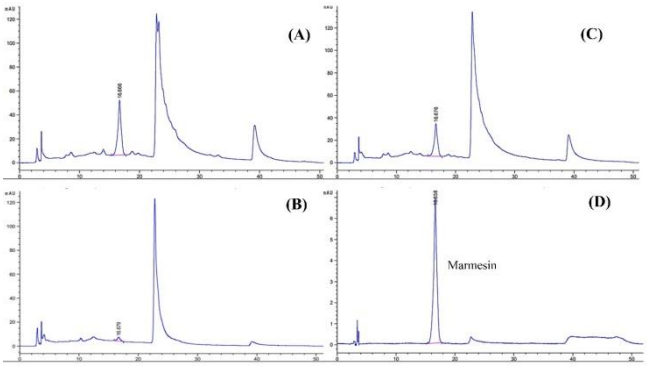

Figure 4: HPLC chromatograms detected by UV-VIS detector at 338 nm of 95% ethanol extract of Thanaka (*Hesperethusa crenulata)* stem bark: (A) S1, (B) S2, (C) S3, and (D) Standard marmesin. (Extracts concentration 5 mg/mL and standard concentration 10 µg/mL). The retention time of standard marmesin is 16.658 min).

Arbutin is a skin-lightening agent with tyrosinase inhibition. [46] One study reported that arbutin content ranged from 0.268% ± 0.015 to 1.711% ± 0.120 of the crude extract weight in twelve Thanaka stem wood samples from different sources [21] Another finding revealed that the amount of arbutin in the aerial part of Thanaka was 1.06 ± 0.06 mg/g of sample.[47] Chang *et al*.[15] illustrated the similar mushroom tyrosinase inhibitory activities (IC_{50}) of marmesin and arbutin (168.0 µM and 140.0 µM respectively), but their inhibitory activity is 10 times lower than kojic acid. Similarly, Shu et.al reported that marmesin provides a weaker tyrosinase inhibition compared to kojic acid. [48]

This research was the pioneer to assess cellular melanin synthesis and tyrosinase inhibition. The extract concentrations of 25, 100, and 250 µg/mL were selected based on the cytotoxicity results. As shown in Figure 8A, the cellular melanin content of all samples exhibited a dose-dependent decline after 48 h, indicating an obvious reduction compared to the control (medium with 500 nM α-MSH). The maximum melanin reduction was observed at the highest tested concentration. 250 µg/mL of S1 and S3 exhibited higher inhibition compared to positive control (100 µg/mL kojic acid). The remaining melanin content of cells exposed to S1, S2, and S3 at 250 µg/mL was 83.07 ± 2.26%, 128.65 ± 6.27%, and 77.87 ± 5.08% respectively, while kojic acid (a well-known tyrosinase inhibitor with depigmented action) showed 114.58 ± 12.65%. Regarding the tyrosinase inhibitory effect, the inhibitory capacity at the concentrations of 25, 100, and 250 µg/mL ranged from 26.56 ± 0.26% to 51.44 ± 1.39% for S1, 19.88 ± 1.98% to 33.08 ± 3.73% for S2, 19.88 ± 2.84% to 45.52 ± 1.15% for S3, while kojic acid (100 µg/mL) showed 70.89 ± 1.84% inhibition (Figure 8B). Despite the lowest marmesin content, S2 resulted in no significant differences at concentrations (25 µg/mL and 100 µg/mL), but lower inhibition at 250 µg/mL compared to S3. All samples demonstrated different inhibitory capacities (melanin synthesis and tyrosinase activity), which may be due to the combined effect of bioactive compounds present in Thanaka. However, all samples exhibited lower inhibitory strength on tyrosinase, but higher suppression of melanin production compared to the positive control (kojic acid). One previous study showed a similar finding, where tyrosinase inhibitory activity of lecithin was slightly higher than α-Tocopheryl ferulate (α-TF). However α-TF indicated significantly greater melanin reduction compare to lecithin on normal human melanocytes.[49] Therefore, the anti-melanogenic effect of Thanaka extract might be not only through inhibition of tyrosinase but also through other enzymatic pathways. Further anti-melanogenic mechanism studies will be needed in the future.

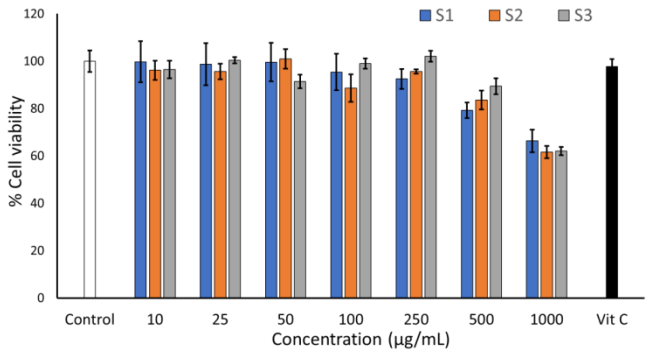

Figure 5: Cell viability of the ethanol extracts from Thanaka (*Hesperethusa crenulata*) stem bark (S1 – S3) 10 – 1000 µg/mL and positive control (25 µg/mL of vitamin C) on HaCaT cells. Control is medium with 0.5% DMSO from untreated cells. Data represent mean ± SD of three repetitions.

Conclusion

This research demonstrated the varying marmesin content in Thanaka (*Hesperethusa crenulata*) stem bark obtained from different sources. Additionally, the validated HPLC method proved suitable for identifying and quantifying marmesin compounds in the Thanaka stem bark. Our findings ensure that the phenolic compounds involved in Thanaka are the key contributors to antioxidant activity due to their strong correlation with antioxidant properties. In murine melanoma (B16F10) cells, a reduction in melanin content was observed at the non-toxic doses, indicating moderate tyrosinase inhibitory activity. Furthermore, this study confirmed that Thanaka stem bark extract is considered safe for skin cells, with a mild collagen-inducing effect and anti-hyperpigmentation action, which could potentially make it a valuable ingredient for natural skincare products.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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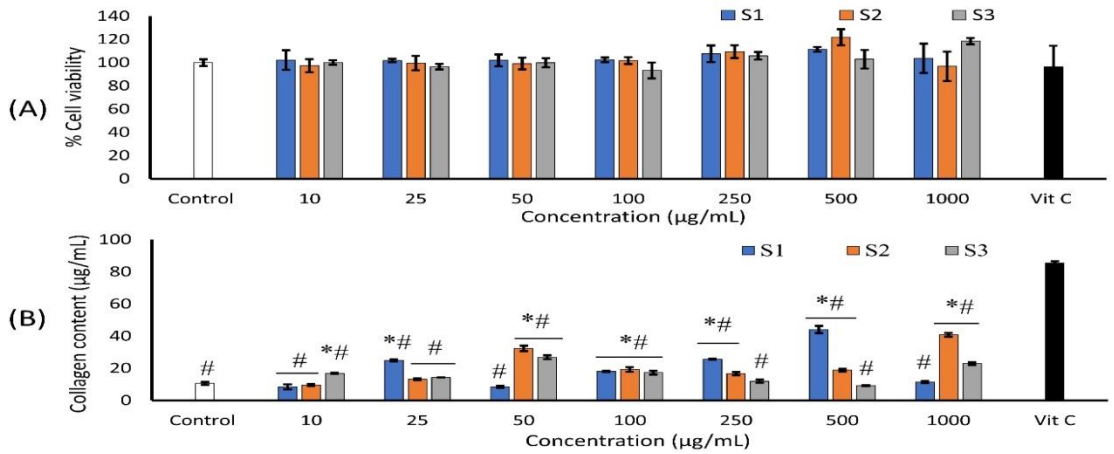

Figure 6: The effects of the ethanol extracts from Thanaka (*Hesperethusa crenulata*) stem bark (S1 – S3) 10 – 1000 µg/mL and the positive control (50 µg/mL vitamin C) on HFF-1 cells. (A) %cell viability (B) collagen content production. Control is medium with 0.5% DMSO from untreated cells. Data represent mean ± SD of three repetitions. *, # indicate significant differences compared to control and positive control (vitamin C), respectively, as determined by Tukey's post-host test ($p < 0.05$).

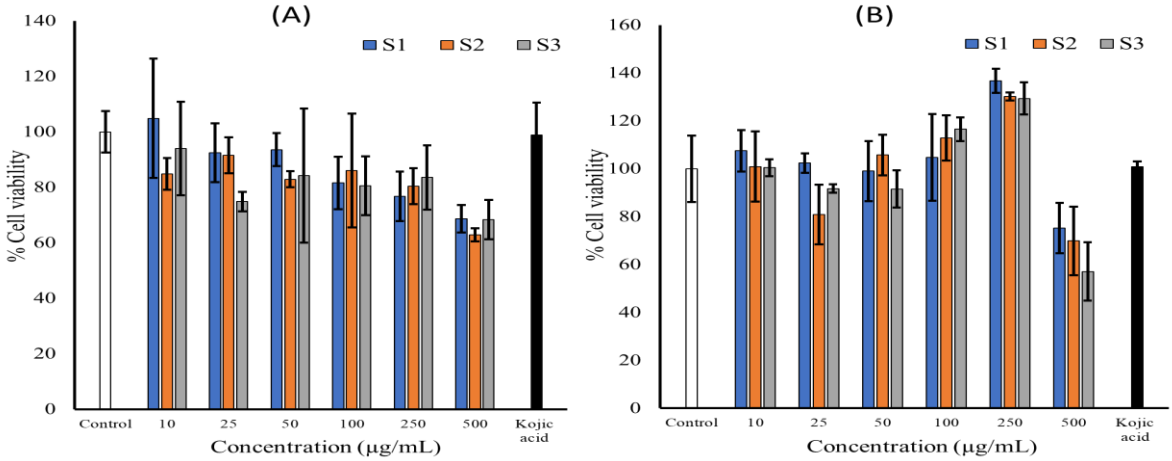

Figure 7: Cell viability of the ethanol extracts from Thanaka (*Hesperethusa crenulata*) stem bark (S1 – S3) on B16F10 cells. (A) 24 h incubation and (B) 48 h incubation using 10 – 500 µg/mL of each extract and the positive control (100 µg/mL kojic acid). Control is medium with 0.25% DMSO from untreated cells. Data represent means ± SD of three repetitions.

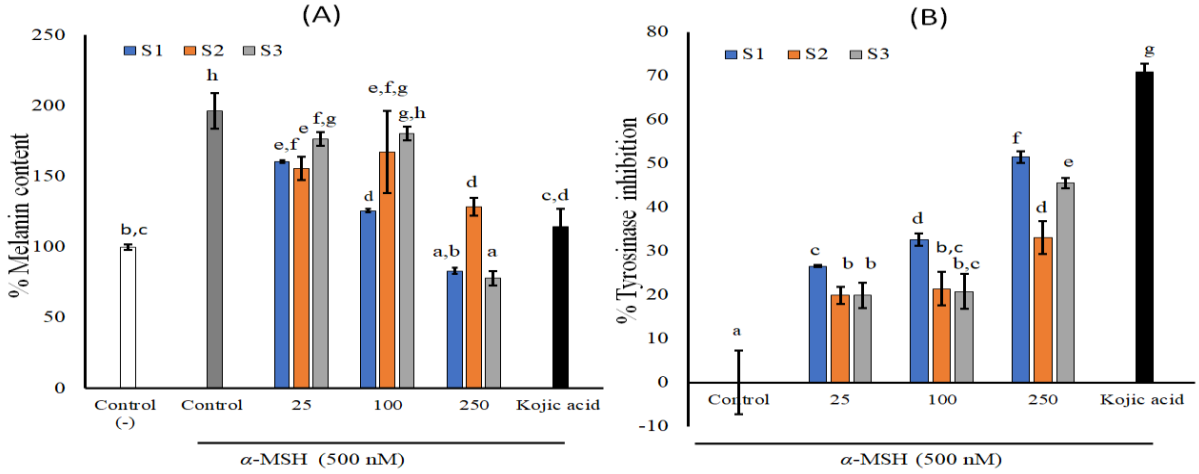

Figure 8: Effect of the ethanol extracts from Thanaka (*Hesperethusa crenulata*) stem bark (S1-S3) on B16F10 cells. (A) melanin content and (B) cellular tyrosinase activity. Extracts (25–250 µg/mL) and the positive control (100 µg/mL kojic acid) were exposed to 500 nM *α*-MSH-stimulated cells for 48 h. Control (-) is medium with 0.5% DMSO from non-stimulated cells and control is medium with 0.5% DMSO from stimulated cells. Data represent mean ± SD of three repetitions. Different letters depict significant differences (Duncan's test, $p < 0.05$).

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