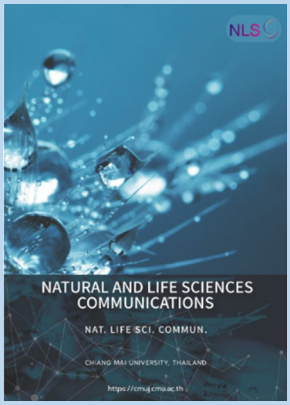
Research article



Multifunctional Biological Activities and Cytotoxic Evaluation of *Bouea macrophylla* for Cosmetic Applications

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ABSTRACT

This study aimed to investigate the biological activities, cytotoxicity, phytochemical constituents and stability profile of *Bouea macrophylla* Griff. peel extracts for cosmeceutical applications. Extraction using maceration or fractionation was optimized using various solvents; ethyl acetate, ethanol, 50%v/v ethanol. The antioxidant activities were determined using DPPH and ABTS radical scavenging, lipid peroxidation inhibition and FRAP assays. Anti-tyrosinase activity was also performed for implying skin depigmentation effects. Total phenolics content, total flavonoids content and total anthocyanin content were also investigated. In addition, *ex vivo* cytotoxicity and anti-inflammatory effects of the selected extract were studied. Moreover, high performance liquid chromatography (HPLC) technique was used to analyze and quantify phytochemical components of the extract to study the stability profile. The results revealed that the highest percentage yield was shown in hydroethanolic extract (BPHE). Regarding phytochemical contents, BPHE contained 83.91 ± 0.00 mg gallic acid equivalent (GAE)/g extract, 12.98 ± 0.01 mg quercetin equivalent (QE)/g extract. Additionally, BPHE exhibited the strongest antioxidant and anti-tyrosinase properties along with good anti-inflammatory effects. Furthermore, BPHE had no cytotoxicity to human fibroblast cells. The HPLC results showed two major peaks in BPHE, i.e., gallic and ellagic acids. Accordingly, *B. macrophylla* peel extract could be a promising bioactive ingredient to develop further as anti-aging cosmetic and cosmeceutical products.

Keywords: *Bouea macrophylla* Griff. peel extract, Antioxidant, Anti-tyrosinase, Anti-inflammatory, Cytotoxicity

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INTRODUCTION

It has been proven that oxidative stress contributes to increased production of reactive oxygen species (ROS) which is chiefly associated to skin aging and elevated tyrosinase enzyme, melanogenesis-related proteins and mRNA levels in melanocytes leading to increased melanogenesis (Chen, Liu, Zhao, and Qiu, 2021). Likewise, skin inflammation triggered by pro-inflammatory mediators which is also induced by ROS during stressful situations plays a crucial role in skin aging and a number of skin problems (Checa and Aran, 2020). As a result, current cosmetic and cosmeceutical products are composed of active agents which potentially ameliorate oxidative stress, inflammation as well as skin hyperpigmentation (Salvioni et al., 2021). It has been ubiquitously reported that several natural products, herbs, or even waste materials from agricultural, food and beverage industries have been extensively applied in cosmetics owing to their great potentials of antioxidation, anti-inflammation and depigmentation. In general, most natural ingredients containing secondary metabolites such as phenolics, flavonoids, tannins, amino acids and vitamins which have shown a multiple of beneficial effects for treating several skin problems (Santos, Corrêa, and Chorilli, 2015). Consequently, a number of scientific research have attempted to explore promising cosmetic ingredients from natural resources.

Marian plum (*Bouae macrophylla* Griff.) has been considered one of the economic crops of Thailand widely grown in several areas especially in Nakhon Nayok, Phitsanulok and Ang Thong Provinces. Three different cultivars in Thailand are categorized by fruit characteristics and tastes including sour *B. macrophylla*, sweet *B. macrophylla* and mayong chid (*B. oppositifolia*). Among all cultivars of Thai *B. macrophylla*, the sweet *B. macrophylla* is regarded as an important local economic fruit that Thai farmers grow for commercial purposes from February to March due to its unique sweet taste. Also, the sweet *B. macrophylla* fruit is widely consumed owing to its lower selling price compared with B. oppositifolia. Related research has revealed that the ethanolic extracts from leaves of *B. macrophylla* in different types including sour *B. macrophylla*, sweet *B. macrophylla* and *B. oppositifolia* cultivated in Nakhon Nayok province contain high total phenolic contents (Thummajitsakul & Silprasit, 2017). Furthermore, methanolic extracts of ripe and unripe fruits of *B. macrophylla* were reported for their high concentrations of alkaloids, flavonoids, saponins, sterols, triterpenes, phenolics, tannins and vitamin C. Additionally, the ethanolic extract of sweet *B. macrophylla* seed from ripe fruit presents a strong antioxidant property, which was equivalent to Trolox, a water-soluble form of tocopherol (Dechsupa, Kantapan, Tungjai, & Intorasoot) [0]. From these reports, the extracts of the *B. macrophylla* fruit, leaves and seeds were important sources of natural antioxidants (Sukalingam, 2018). However, no evidence regarding chemical compositions, biological properties, or safety profiles of Thai sweet *B. macrophylla* peels, considered as a waste product from fruit processing industries, has been revealed until now. Therefore, the peel of sweet *B. macrophylla* is interesting to investigate regarding as bioactive substances and biological potential for cosmeceutical applications.

As a consequence, the aims of this study were to investigate the biological activities of *B. macrophylla* peel extract for the first time in terms of antioxidant, anti-tyrosinase and anti-inflammatory properties along with cytotoxicity concerning human skin fibroblast cells implying a safety profile. Moreover, pre-formulation studies, in terms of phytochemical contents and stability of the extract, were evaluated. These findings possibly add economic value to agricultural waste and provide a promising active ingredient for cosmeceutical applications.

MATERIALS AND METHODS

Chemical materials

Solvents used for extraction including ethyl acetate and ethanol were analytical grade. Solvents used for HPLC analysis as acetonitrile, methanol and phosphoric acid (H_3PO_4) were HPLC grade. The 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), linoleic acid, 2,2'-azobis (2-amidonopropane) dihydrochloride (AAPH), ammonium thiocyanate (NH_4SCN), tyrosinase from mushroom, 4-amino-3-hydroxyphenylalanine (L-tyrosine) and 3,4-dihydroxy-L-phenylalanine (L-dopa) were purchased from Fluka (Buchs, Switzerland). Gallic acid (GA), ellagic acid (EA), L-ascorbic acid, kojic acid and Trolox were purchased from Sigma-Aldridge Inc. (Schnelldorf, Germany). Beta-arbutin was purchased from Namsiang Co., Ltd. (Thailand).

Plant materials

The sweet *B. macrophylla* fruits were harvested from the *B. macrophylla* plantation in Phitsanulok Province, Thailand. The peels of sweet *B. macrophylla* were then collected. The plant materials were identified and authenticated the Medicinal plant innovative center, Faculty of Pharmacy, Chiang Mai University with herbarium code of 0023323. The peels were cleaned to remove the any dirt on the surface and cut into small pieces. They were incubated at 50°C for 24 hours in a hot-air oven. The dried plant materials were ground into fine powder using an electric blender before extraction and stored in an airtight container until use.

Plant extraction

The dried sweet *B. macrophylla* peels were extracted using solvents presenting different polarities by conventional maceration and solvent fractionation techniques relying on extracting potential and polarities for polyphenol extraction. Firstly, the dried plant was macerated in three cycles of 95% v/v ethanol and 50% v/v ethanol for 48 hours. Before use, the sample was mixed with solvents at 1:2 (w/v) ratio, and the mixture was then filtered through Whatman No.1 filter paper. The 95% v/v ethanolic solution was evaporated using a rotary evaporator (Buchi® Rotavapor R-300, Thailand) at 45°C to obtain crude ethanolic extract (BPE), whereas the 50% v/v ethanolic extract solution was evaporated to remove ethanol before being spray-dried with 2% w/v maltodextrin as a carrier (Buchi® Mini Spray dryer B-290, Thailand) at 140°C inlet and 100°C outlet temperatures to produce hydroethanolic extract (BPHE). Secondly, the dried plants were fractionated firstly with ethyl acetate, followed by fractionation with 95% v/v ethanol, respectively. The concentrated ethyl acetate fraction (BPEA) and the ethanolic fraction (BPEE) were then obtained using evaporation. The crude extracts were stored in airtight containers and kept in a refrigerator (SF-C697, Sanyo, Japan) at 4°C for further use.

The percentage yield was expressed as the mass of crude extract obtained per dried weight of *B. macrophylla* peels and was calculated by Truong et al. (2019) [0] according to the equation below.

$$\text{Percent yield (\%)} = \frac{crude\ extract}{dried\ weight\ of\ plants} \times 100 \quad (1)$$

Phytochemical screening testing

Obtained extracts of *B. macrophylla* peels were evaluated for the presences of alkaloids, flavonoids, phytosterol, saponins, tannins and phenolics. The color changes indicated positive results following standard procedures.

Testing for alkaloids

Each extract was dissolved in 3 ml of ethanol, then 5 ml of 2 N of hydrochloric acid (HCl) was added and the mixture was heated in water bath. The mixture was then filtered and 3 ml of mixture was treated with a few drops of Dragendorff's reagent. A black precipitate indicated the presence of alkaloids. Absolute ethanol served as a negative control while Ergotamine tartrate served as a positive control (Parbuntari et al., 2018).

Testing for flavonoids

Each extract was dissolved in 5 ml of ethanol. Then, a few drops of conc. HCl and magnesium ribbon were added and a pink color was observed within 3 minutes, indicating the presence of flavonoids. Quercetin served as a positive control while absolute ethanol was a negative control (Singh and Saxena, 2017).

Testing for phytosterols

Each extract was dissolved in chloroform and filtered. Then 1 ml of acetic acid anhydride was added and mixed thoroughly. After that, the reaction mixture was acidified by 2 ml of concentrated sulfuric acid (H_2SO_4). The presence of purple-red ring at the interface of two layers or blue-green color in the acetic acid anhydride layer indicated the presence of phytosterols (Pradnya and Alka, 2014).

Testing for saponins

Each extract was dissolved with 20 ml of deionized water and boiled in a water bath. After that, the mixture was cooled. The solution was then shaken vigorously in a graduated cylinder for 15 minutes. The presence of stable foam indicated the presence of saponins (Weli et al., 2018).

Testing for tannins and phenolics

The extract was dissolved in 10 ml of 0.9% w/v NaCl solution and heated by sonicator for 15 minutes, followed by filtration. Then, 1% w/v of gelatin salt reagent was added. The white precipitation indicated the presences of tannins. Positive test was also confirmed by addition of a few drops of 1% w/v ferric chloride ($FeCl_3$) solution to the mixture. The presence of phenolics demonstrated a bluish-black color. A positive control was GA, while a negative control was deionized water

Determination of total phenolic content

Quantification of total phenolic content (TPC) was carried out using the Folin-Ciocalteu colorimetric method with modifications (Poomanee et al., 2018). The extract was dissolved in 50% v/v ethanol (0.5 ml). Then, the reagent mixture, consisting of 2 ml Folin-Ciocalteu reagent (Merck®, Germany), 7.5 ml sodium carbonate solution (Na_2CO_3), and 4 ml distilled water was mixed with the extract solution and left at room temperature. After 30 minutes, the absorbance was measured using a UV-VIS spectrophotometer (Shimadzu, UV-2600i, Japan) at a wavelength of 765 nm. The GA calibration curve, which is a regression of GA concentration (X) and absorbance (Y) was produced. The TPC values of the extracts was calculated based on the regression equation of Y = 0.0057X + 0.0177; R^2 =0.9920) and expressed in terms of mg gallic acid equivalent per gram of extract (mg GAE/g extract).

Determination of total flavonoid content

The total flavonoid content (TFC) of the extracts was determined using the method of Poomanee et al. (2015) with modifications, and the quercetin calibration curve was constructed. Each extract was dissolved in absolute ethanol, then, the extract solution (1 ml) was mixed with 5% w/v of sodium nitrite ($NaNO_2$)

(0.3 ml) and DI water (4 ml) in a test tube and left for 5 minutes. Then, 10% w/v of aluminum chloride ($AlCl_3$) (300 µl) was added and left at room temperature for 5 minutes, followed by 1 M sodium hydroxide (NaOH) (2 ml). After mixing, the solution was incubated for 15 minutes. The absorbance was measured at 510 nm using a UV-visible spectrophotometer. The quercetin calibration curve, a regression of quercetin concentration (X) and absorbance (Y) was produced. The concentration of TFC in the test samples was calculated from the calibration plot (Y = 0.0011X + 0.0067, R^2 = 0.9929). The TFC values of the extracts were expressed as mg quercetin equivalent per gram of extract (mg QE/g extract).

Determination of total anthocyanin content

Total anthocyanin content (TAC) was determined by the pH-differential method following the procedure of Lee et al. (2005). The presence of anthocyanin was characterized by a shift in color from pink-red to blue-violet. Briefly, 1 ml of extract solution was transferred to a 10 ml volumetric flask with potassium chloride buffer (pH 1.0) and sodium acetate (pH 4.5), then incubated for 15 minutes. The absorbance of the mixture was separately measured at 520 and 700 nm using a UV-Vis spectrophotometer. Anthocyanin pigment expressed as milligrams per liter (mg/L), was converted to mg of TAC per 100 g sample. The TAC was calculated based on cyanidin-3-glucoside equivalents using the equation below.

$$\text{Anthocyanin pigment (mg/L)} = \frac{\text{A} \times \text{MW} \times \text{DF} \times 10^3}{\varepsilon \times 1} \quad (2)$$

where A is the absorbance, MW is the molecular weight of cyanidin-3-glucosode (449.2 g/mol), DF is the dilution factor, ε is the molar absorptivity (26,900 $L.mol^{-1}.cm^{-1}$), and 1 is the cell path length (1 cm).

Determination of antioxidant activities

DPPH radical scavenging assay

The DPPH assay was performed according to the modified method of Poomanee et al. (2018). Each extract was dissolved in absolute ethanol to create different concentrations ranging from 0.3125 to 10 mg/ml. The reaction mixture consisting of 20 µl of the extract solution and 180 µl of 120 mM DPPH solution was kept in the dark for 30 minutes and the absorbance was then measured using a microplate reader (Spectra-Max® M3, USA) at a wavelength of 520 nm. Percentage inhibition on DPPH radicals was calculated using the equation below.

$$\text{Percentage inhibition (\%)} = \left[\frac{\text{Ac}-\text{As}}{\text{Ac}}\right] \text{x } 100 \quad (3)$$

where Ac is an absorbance of the control and As is the absorbance of the sample. In addition, the concentration of the extract that scavenge 50% of DPPH radicals (IC_{50}) was reported based on the regression of the extract concentrations concerning the % inhibition. The effects of the extracts were compared with those of reference standards including Trolox, GA, EA and L-ascorbic acid.

ABTS radical scavenging assay

The ABTS radical scavenging activities of the extracts were determined according to the method of Poomanee et al. (2018). Each extract was dissolved in 50%v/v ethanol in DI water to provide a concentration ranging from 0.15625 to 5 mg/ml. The ABTS reagent was prepared by mixing 7 mM ABTS reacting with 140 mM potassium persulfate ($K_2S_2O_8$) and incubating in the dark at room temperature for about 16 hours. Then, the obtained ABTS radical solution was diluted with DI water at a ratio of 1:100. To determine the scavenging activity, 1000 µl ABTS

reagent was mixed with 10 µl of the sample in a 96-well flat micro-plate and then incubated at room temperature for 6 minutes. After incubation, the absorbance was measured at 734 nm using a microplate reader. The result was expressed as Trolox equivalent antioxidant capacity (TEAC) value of each extract (mg TEAC/mg extract) and IC_{50} value and compared with those of GA, EA and L-ascorbic acid.

Lipid peroxidation inhibition assay

The inhibitory effects of the extracts on lipid peroxidation were studied. The amount of peroxides generated from linoleic acid oxidation were evaluated using the thiocyanate method according to the modified method of Poomanee et al. (2018). The reaction mixture was prepared by thoroughly mixing 0.3 ml of the extract solution at different concentrations (0.3125-5.0 mg/ml), 1.3% w/v linoleic acid, phosphate buffer (PBS) pH 7.0, DI water and 46.35 mM AAPH solution and incubating at 45°C for 4 hours in the dark. Using the ferric-thiocyanate technique, the mixture consisted of 20 mM of ferrous chloride ($FeCl_2$) acidic solution, 10% w/v ammonium thiocyanate (NH_4SCN) solution and 75% v/v methanol was combined with the incubated reaction mixture for 3 minutes. The absorbance was then measured at 500 nm using a microplate reader. Percent inhibition on linoleic acid peroxidation was calculated as previously stated. In addition, IC_{50} value of each extract was reported. Trolox, GA, EA and L-ascorbic acid were used as reference standards.

Determination of ferric reducing antioxidant power (FRAP)

The FRAP assay was used to evaluate the reducing potentials of the extracts by decreasing the Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ, which was performed according to methods of Poomanee et al. (2018) described below. Briefly, freshly prepared FRAP reagent was comprising 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$ and DI water. The reaction was started by adding an extract solution (100 µl), DI water (900 µl) and FRAP reagent (2,000 µl) and incubated at room temperature in the dark for 30 minutes. The absorbance was then measured at 593 nm using a UV-visible spectrophotometer. The calibration curve was plotted between different concentrations of $FeSO_4$ and the absorbance. Trolox, GA, EA, and L-ascorbic were used as positive controls. The results were expressed as equivalent concentration 1 (EC_1) as the concentration of an antioxidant with a reducing power of 1 mM of $FeSO_4$. Lower values of EC_1 indicate greater reducing power activity. Ferrous sulfate ($FeSO_4 \bullet 7H_2O$) was used as a standard antioxidant agent.

Determination of tyrosinase inhibition activity

Anti-tyrosinase activity implying the skin whitening effect of the extract was performed according to the modification method of Poomanee et al. (2015). L-DOPA and L-tyrosine served as substrates in the reaction. Briefly, 70 µl of 1.66 mM of mushroom tyrosinase enzyme solution (Sigma-Aldrich, Singapore), 70 µl extract solution (0.3125 to 10 mg/ml) and PBS (pH 6.8) were mixed in a 96-well plate and incubated at room temperature for 10 minutes. Then, 70 µl of the substrates in a concentration of 0.85 mM were added. The enzyme activity at 20 minutes was measured using a microplate reader at 492 nm. L-ascorbic acid, beta-arbutin and kojic acid were used as positive controls. Percent inhibition on tyrosinase was calculated using the equation below.

$$\text{Tyrosinase inhibition (\%)} = \left[\frac{Ac - As}{Ac}\right] \times 100 \quad (4)$$

where Ac is an absorbance of control and As is an absorbance of sample. The IC_{50} value was also determined.

Determination of anti-inflammatory activity using the nitric oxide (NO) inhibition assay

The NO release of the RAW 264.7 cell line was measured as nitrite production using the Griess reaction following the method of Torres-Rodríguez et al. (2016). Briefly, the RAW 264.7 cell line, which was formerly cultured in DMEM containing 10% v/v FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml), was seeded at a density of 2 x 10^5 cells on a six-well plate and incubated for 48 h at 37°C in 5% CO2 atmosphere. After incubating, the cells were activated with lipopolysaccharide (LPS), and treated with different extract concentrations (0.00005, 0.0005, 0.005, 0.05, and 0.5 mg/ml) or triamcinolone acetonide, serving as the standard and incubated for 24 h. Then 100 µl of cell culture medium was transferred to a 96-well plate and mixed with an equal volume of Griess reagent (1% w/w sulfanilamide and 0.1% w/w N-1-(napthyl) ethylenediamine dihydrochloride in 2.5% w/w H_3PO_4). The plate was then incubated for 5 minutes at room temperature and the absorbance was measured at 550 nm using an Ultra Microplate Reader (Biotek, Winooski, VT, USA). The presence of nitrite was assessed and the results were expressed as Percent NO reduction calculated using the equation below.

$$\text{NO reduction (\%)} = \left[\frac{\text{Abs of control} - (\text{Abs of sample} - \text{Abs of blank})}{\text{Abs of control}}\right] \text{x } 100 \quad (5)$$

where Abs of the control is absorbance of cells treated with medium containing LPS; Abs of the sample and Abs of the blank are absorbances of cells treated with the extract mixed medium with LPS or without LPS, respectively.

Determination of cytotoxicity in human fibroblast cells

The sulforhodamine B (SRB) assay was used to assess cell viability as in the related studies of Vichai & Kritara (2006). The cytotoxicity of the selected extract was tested in human fibroblast cell lines. Before treatment, the cells were seeded in a sterile 96-well plate at a cell density of 1.9 × 10^4 cells/well and incubated at 37°C in 5% CO_2 saturated humidity condition for 24 h. After that, the cells were stimulated by different concentrations of extract or GA at 0.0001, 0.001, 0.01, 0.1 and 1 mg/mL and incubated overnight. The cells were then rinsed and stained with SRB solution and left at room temperature for 30 minutes before washing with 1% v/v acetic acid. The medium was then withdrawn and 200 ul of 10 Mm Tris base solution (pH 10.5) was added to each well, followed by 5 minutes of shaking on a vortex shaker to solubilize the protein-bound dye. In this study, sodium lauryl sulfate (SLS) was prepared in the same way as the extract. Eventually, the optical density (OD) was measured at 540 nm. The results were expressed as the ratio by standardizing the desired OD level to the control OD level. The absorbance values were calculated percent of cell viability using the equation below.

$$\text{\% Cell viability} = \left[\frac{\text{OD of sample}}{\text{OD of control}}\right] \text{ x } 100 \quad (6)$$

where OD of the sample is the absorbance of cells treated with sample; OD of the control is absorbance of DMSO-treated cells.

High performance liquid chromatography (HPLC) fingerprint analysis

Gradient HPLC elution was performed to elucidate fingerprint analysis of the selected extract as described in the related study of Dechsupa et al. (2018). A methanolic solution of the extract was prepared at 1 mg/ml and then filtrated through a 0.22 µm syringe filter. The UV-wavelength of 270 nm was operated for detection. The chromatographic column was Inertsil ODS-3 reverse phase C-18 column (5 µm, 4.6 × 250 mm, GL Science Inc. USA). Phytochemical constituents of the extract were identified using the following mobile phases; mobile A:

acetonitrile and mobile B: 0.01% v/v phosphoric acid (pH 2.84). The time program for gradient elution was conducted as follows: 0–10 minutes, 90% of B; 11 to 30 minutes, 10% of B; 31 to 50 minutes, 10% of B.

Determination of extract stability

The extract solution was dissolved in DI water at a concentration of 1 mg/ml and stored in a transparent glass vial. Stability testing was conducted at different storage conditions: 4°C, room temperature with light and without light as well as 45°C for 30 days and an accelerated condition as heating-cooling for 6 cycles (4°C 24 hr. and 45°C 24 hr.). After storing, physical appearances such as color and precipitation were observed. In addition, antioxidant properties in terms of DPPH scavenging activity and tyrosinase inhibition of the extract were investigated and compared between after storing and at the initial phase. The stability of phytochemicals of the extract was also determined using HPLC.

Statistical analysis

All experiments were performed in triplicates and the results were expressed in terms of mean ± standard deviation (SD). Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) with multiple comparisons by Tukey in SPSS statistics Software Version 17.0 (IBM Co. Ltd., NY, USA). To compare stability between after storing and the initial phase, the independent t-test was used. *P*-value less than 0.05 implied a statistically significant difference.

RESULTS

Bouea macrophylla Griff. peel extracts

Percentage yields and characteristics of the obtained extracts are shown in Table 1. The maceration of dried *B. macrophylla* peels using various solvents exhibited differences in their characteristics of the *B. macrophylla* crude extracts. The maximum yield was obtained from BPHE, followed by BPEE, BPE, and BPEA, respectively.

Table 1. The percentage yields and characteristics of *B. macrophylla* peel extracts.

Samples	Types of solvents	Extraction yield (%w/w)	Characteristics
BPEA	Ethyl acetate fraction	2.81 ± 1.70^{d}	Dark brown, viscous liquid
BPHE	Hydroethanolic extract	45.46 ± 4.20^{a}	Brown-yellow, dry power
BPEE	Ethanolic fraction	28.06 ± 3.50^{b}	Light brown, viscous liquid
BPE	Crude ethanolic extract	20.18 ± 3.09^{c}	Brown, viscous liquid

Note: Each value was showed as mean ± standard deviation (SD) of triplicate (n = 3), Superscript letters (a,b,c,d) indicate significant differences ($P<0.05$) between sample analyzed by One-Way ANOVA with multiple comparison using Tukey test.

Phytochemical screening of the extracts

This study performed the preliminary phytochemical screening of the different extracts from *B. macrophylla* peels showing the presences of secondary metabolites including phytosterol, saponin, tannin, and phenolic extracts as shown in Table 2. Most chemical components of the *B. macrophylla* peels were tannins and phenolics owing to high yields of BPE, BPEE, and BPHE, whereas phytosterols were found in only BPEA and BPE. These phytochemical compounds could offer biological activities of the plants.

Table 2. The phytochemical components of *B. macrophylla* peel extracts.

Samples	Phytochemical compounds					
	Alkaloids	Flavonoids	Phytosterols	Saponins	Tannins	Phenolics
BPEA[1]	-	-	+	-	-	-
BPHE[2]	-	-	-	+	+	+
BPEE[3]	-	-	-	-	+	+
BPE[4]	-	-	+	-	+	+

Note: [1]BPEA: Ethyl acetate fraction, [2]BPHE: Hydroethanolic extract, [3]BPEE: Ethanolic fraction, [4]BPE: Crude ethanolic extract; Positive (+) and negative (-) signs indicate the presence and absence of phytochemical compounds respectively.

Total phenolic, flavonoid, and anthocyanin contents of the extracts

The result demonstrated the extracts were composed of phenolic and flavonoid constituents in significantly different amounts as shown in Table 3. The highest TPC was found in BPE followed by BPHE, BPEE, and BPEA. In the case of flavonoids, BPEA presented the highest TFC among all extracts. Moreover, the anthocyanin was not detected in the extracts.

Table 3. Total phenolic and total flavonoid contents of *B. macrophylla* peel extracts.

Samples	Total phenolic content (mg GAE/g extract)*	Total flavonoid content (mg QE/g extract)**
BPEA	76.75 ±0.09^{d}	31.95 ±0.10^{a}
BPHE	80.25 ±0.01^{b}	6.04 ±0.01^{d}
BPEE	78.50 ±0.09^{c}	6.63 ± 0.01^{b}
BPE	87.35 ±0.09^{a}	6.33 ±0.01^{c}

Note: * mg GAE/ g extract is mg gallic acid equivalent per gram of extract.
** mg QE/ g extract is mg quercetin equivalent per gram of extract.
Superscript letters (a,b,c,d) indicate significant differences (P<0.05) between sample analyzed by One-Way ANOVA with multiple comparison using Tukey test

Antioxidant activities of *B. macrophylla* peel extracts

Regarding antioxidant assays in terms of DPPH and ABTS scavenging, linoleic acid peroxidation along with FRAP assays revealed the potential of the *B. macrophylla* peel extracts to attenuate oxidative stress through comprehensive pathways. Concerning the various extracts, BPE, BPEE and BPHE exerted a notable DPPH scavenging properties, whereas BPEA was not as strong as the other extracts as shown in Table 4. However, these are less potent than Trolox, GA, EA, and L-ascorbic acid (P < 0.05). Each extract also presented antioxidation through the ABTS scavenging assay. Considering the two assays, among all extracts, BPHE revealed the highest free radical scavenging activity because it showed the lowest IC_{50} on ABTS radicals and a low IC_{50} on DPPH radicals with no significant difference from BPEE and BPE. Furthermore, according to the linoleic acid peroxidation assay, BPHE extract exhibited the best inhibitory effect on lipid peroxidation indicating a similar manner to the results of ABTS and DPPH assays. In the FRAP assay, the abilities to reduce TPTZ-Fe3+ to TPTZ-Fe2+ of all extracts were shown in Table 4 for which the reducing effect of BPHE was the greatest.

Table 4. Effects of *B. macrophylla* peel extracts in various solvents on different antioxidant models and anti-tyrosinase activity.

Samples	Free radical scavenging			Linoleic acid peroxidation IC_{50} (mg/mL)	FRAP assay EC_1 (mg/mL)	Anti-tyrosinase activity IC_{50} (mg/mL)
	DPPH IC_{50} (µg/mL)	ABTS TEAC (mg Trolox/mg extract)	ABTS IC_{50} (µg/mL)			
BPEA	477.48 ±59.46 ^{c}	0.05 ±0.01 ^{d}	18.09 ±1.86^{c}	0.31 ±0.10 ^{c}	0.125 ±0.000 ^{g}	ND
BPHE	56.55±3.63 ^{b}	0.19 ±0.01 ^{d}	2.05 ±0.20^{b}	0.12 ±0.05 ^{b}	0.019 ±0.001 ^{a}	1.66 ±0.84 ^{b}
BPEE	54.54 ±8.52 ^{b}	0.09 ±0.04 ^{d}	4.13 ±1.55^{b}	0.19 ±0.05 ^{bc}	0.066 ±0.001 ^{e}	ND
BPE	64.69 ±1.49 ^{b}	0.11 ±0.01 ^{d}	4.18 ±0.04^{b}	0.22 ±0.04 ^{bc}	0.035 ±0.001 ^{c}	ND
Trolox	8.32 ±0.10 ^{a}	0.94 ±0.03 ^{c}	0.43 ±0.02^{a}	0.013 ±0.01 ^{a}	0.071 ±0.000 ^{f}	-
Gallic acid	0.95 ±0.03 ^{a}	4.41 ±0.15 ^{a}	0.08 ±0.01^{a}	0.025 ±0.00 ^{a}	0.039 ±0.000 ^{d}	-
Ellagic acid	2.48 ±0.28 ^{a}	0.86 ±0.10 ^{c}	0.57 ±0.07^{a}	ND	0.070 ±0.000 ^{f}	-
L-ascorbic acid	5.83 ±2.31 ^{a}	3.46 ±0.20 ^{b}	0.12 ±0.01^{a}	0.34 ±0.09 ^{c}	0.024 ±0.000 ^{b}	0.10 ±0.00 ^{a}
Beta-arbutin	-	-	-	-	-	0.09 ±0.00 ^{a}
Kojic acid	-	-	-	-	-	0.02 ±0.00 ^{a}

Note:Superscript letters (a,b,c,d,e,g) indicate significant differences (P<0.05) between sample analyzed by One-Way ANOVA with multiple comparison using Tukey test; ND = not detected, EC_1 is equivalent concentration, which represented ferric reducing ability equivalent to 1 mM $FeSO_4$.

Tyrosinase inhibition activity of *B. macrophylla* peel extracts

The *B. macrophylla* peel extracts were studied for inhibiting tyrosinase enzyme through the substrate of L-tyrosine. Table 4 indicates that only BPHE was capable of inhibiting tyrosinase through the L-tyrosine pathway which exhibited an IC_{50} value of 1.66 ± 0.84 mg/ml, whereas other extracts revealed no activity. In compared with the reference standard compounds that are commonly used as active components in skin lightening cosmetic products, such as beta-arbutin, kojic acid and L-ascorbic acid, BPHE was regarded to display low tyrosinase inhibitory activity.

Anti-inflammatory activity of the selected extracts

Before assessing anti-inflammatory efficacy, the cytotoxicity of the chosen extracts on the RAW 264.7 cell line was firstly assessed. The result of BPHE showed no toxicity to the cells. After that, the anti-inflammatory effect of BPHE on LPS-stimulated RAW 264.7 macrophages was evaluated in a term of inhibitory effect on NO production. Triamcinolone acetonide is a commonly used as an anti-inflammatory drug. As shown in Figure 1. BPHE could inhibit NO production from LPS-induced macrophages up to 24.76 ± 3.73% at concentrations of 0.5 mg/ml, while triamcinolone acetone can inhibit NO production was 28.56 ± 3.96% at a concentration of 0.5 mg/ml. Furthermore, the extract presented no significant difference from triamcinolone acetonide at all doses (P > 0.05).

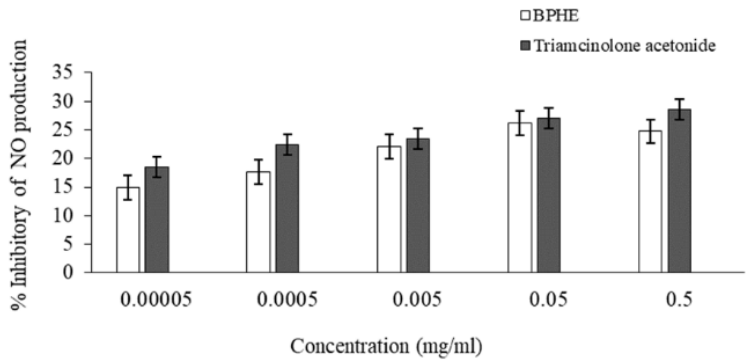

Figure 1. Percent inhibition on nitric oxide (NO) of the hydroethanolic extracts (BPHE) and ellagic acid on LPS-induced RAW 264.7 cell line; (Mean ± SD; n = 4)**.**

In vitro cytotoxicity in human fibroblast cells of the selected extract

Owing to the greatest biological properties of BPHE, this extract was chosen for investigation of cytotoxicity. The results shown in Figure 2, revealed percent cell viability following the treatment with BPHE extract and ellagic acid at different concentrations ranging from 0.0001 to 1 mg/ml. It was shown that the BPHE and ellagic acid were not hazardous to human fibroblast cells at all doses. The percent cell viability of the BPHE-treated cells and ellagic acid-treated cells varied 95.95 - 110.85% and 90.85 - 111.90%, respectively. The positive control of a cytotoxic agent was sodium lauryl sulfate (SLS), which shown toxicity against human fibroblast cells at concentrations of 0.1 and 1 mg/ml with percent cell viability of 46.32 ± 3.44% and 24.06 ± 1.61%, respectively.

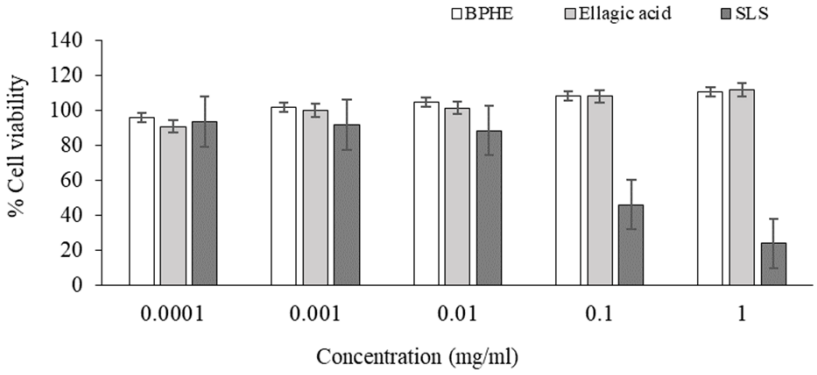

Figure 2. Cytotoxicity of the hydroethanolic extract (BPHE) and ellagic acid on human fibroblast cells comparing with sodium lauryl sulfate (SLS); (Mean ± SD; n = 4).

HPLC fingerprint of the selected extracts

The chemical compositions of the BPHE were determined using the HPLC. The chromatograms as shown in Figure 3, revealed that gallic acid and ellagic acid were identified in BPHE at retention times of 7.07 ± 0.00 minutes and 23.12 ± 0.01 minutes, respectively. It is worth noting that gallic acid and ellagic acid could be used as marker of the extract which might determine the biological property of the extract. The gallic acid and ellagic acid content of BPHE extract contained 5.90 ± 4.24 mg and 2.40 ± 1.41 mg per gram of extract. In addition, the amounts of gallic acid and ellagic acid were used to be indicators for stability testing.

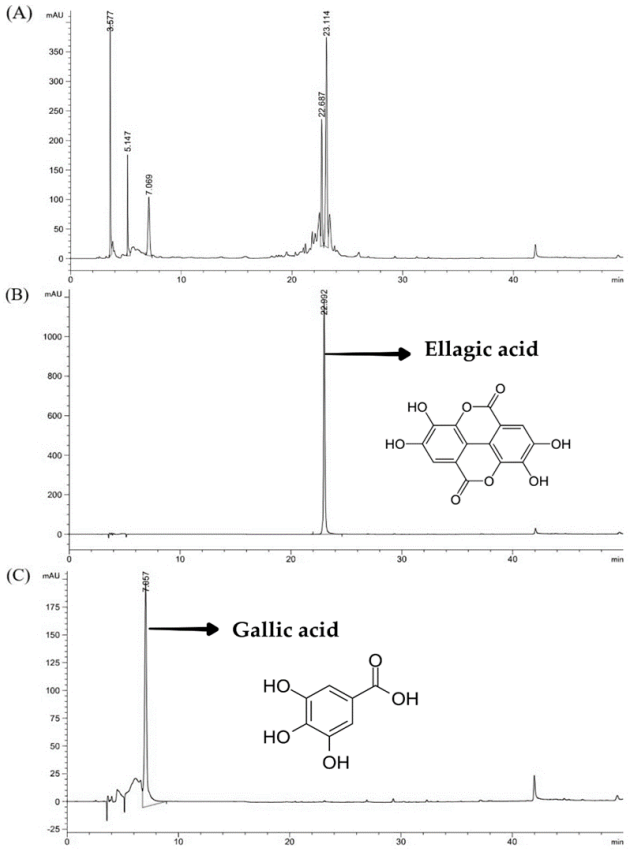

Figure 3. HPLC chromatograms of (A) BPHE, (B) Ellagic acid (40 ppm), and (C) Gallic acid (80 ppm).

Stability test of the selected extracts

The stability profile of BPHE in terms of DPPH scavenging and anti-tyrosinase properties at various conditions was explored. After storage under heating-cooling (HC) for 6 cycles as an accelerated condition and 45°C conditions for 30 days, the physical appearances of the samples changed slightly in which darker yellow in color and no precipitation were presented compared to the initial.

Figure 4 illustrated DPPH scavenging property of BPHE compared to the initial after storage in various conditions. Antioxidant effects of all samples after storage under all including 4°C, high temperature (45°C), HC, room temperature under light (RTL) and without light (RTD) were significant decreased from the initial ($P < 0.05$). Likewise, the tyrosinase inhibitory activity of the extract was significantly decreased in all conditions. In particular, under 45°C and RTL, the highest reductions of the effect from the initial were observed. Additionally, chemical stability of BPHE containing gallic acid and ellagic acid were shown in terms of percent reduction from the initial as shown in Figure 5. The highest reduction of ellagic acid were found after storage under HC and RTL while the highest reduction of gallic acid were found after storage under 45°C and RTL which were in correspondence with the attenuation of its biological effect.

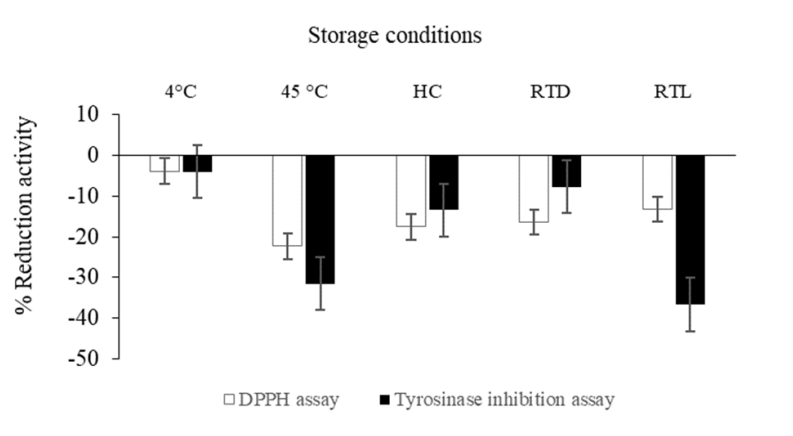

Figure 4. The percentage reduction DPPH antioxidant activity and tyrosinase inhibitory activity of BPHE after stability testing compared to the initial testing.

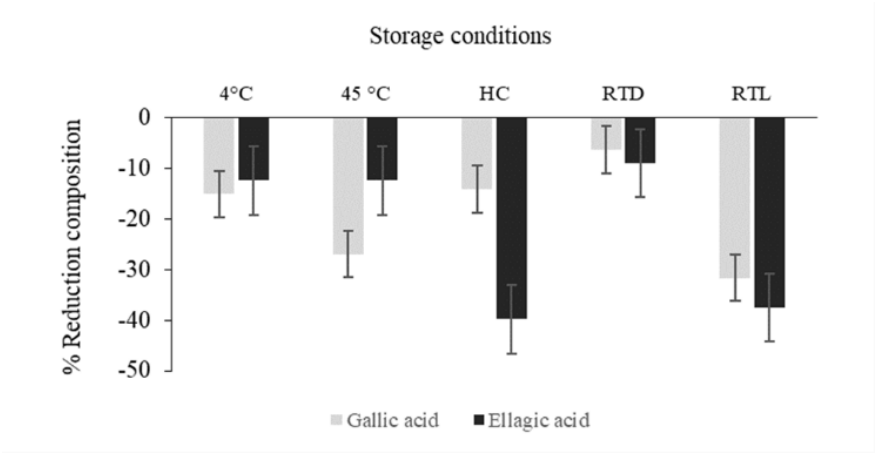

Figure 5. The percentage reduction composition of chemical stability of BPHE from day 0 at various conditions.

DISCUSSION

In the present study, *B. macrophylla* peel which was a waste product from fruit consumption and processing, was firstly investigated for its biological potentiality in terms of antioxidant, anti-tyrosinase and anti-inflammatory activities in order to be applied as a cosmeceutical active ingredient. Initially, the extracting solvents were optimized. Maceration is one of the conventional extraction methods that is easy and inexpensive because it only requires a basic instrument (Tambun, Alexander, and Ginting, 2012). In addition, this technique was widely utilized to extract thermally unstable compounds (Zhang, Lin, and Ye, 2018). Importantly, solvents used for extraction have a crucial influence on the types and quantities of secondary metabolites from the medicinal plants (Rebey et al., 2012). It was reported that ethyl acetate, water and aqueous combinations of ethanol, methanol and acetone are the most commonly used solvents for polyphenols extraction from plant materials (Ajila et al., 2010). Three extracting solvents presenting different polarities as ethyl acetate, ethanol and 50% v/v ethanol were included in our experiment. Our findings revealed that *B. macrophylla* peel, which were extracted by 50% v/v ethanol using maceration method gave the maximum percent yield implying that hydroethanolic solvent is the most efficient solvent for extracting *B. macrophylla* peel rather than the other solvents. This indicates that a great quantity of polar polyphenols resided in the

peel. In addition, it was reported that the mixture of water and ethanol potentially augmented the extracting capacity since plant tissue is easily swelled by protic solvent. Subsequently, the structures of plant protein such as cell wall were denatured and loosen by ethanol that enhance the penetration of solvent molecules (Zuorro, Iannone, and Lavecchia, 2019). A previous study also reported that *B. macrophylla* seeds extracted from 50% v/v ethanol showed the highest yield compared to others from different concentrations of ethanol in water (25%, 75% and 95% v/v), which was in correspondence with our results (Dechsupa et al., 2018). The most found polyphenol compounds in plant extracts are phenolics, flavonoids and tannins which were highly capable of neutralizing free radicals and showed strong antioxidant capacity (Prabh and Vasantha, 2011; Arina and Harisun, 2019; Adam, Razali, Arapoc, Aziz, and Marsiddi, 2021). In previous study, Sukalingam et al. (2018) reported that methanolic extracts of ripe and unripe fruits of *B. macrophylla* contained the highest concentrations of alkaloids, flavonoids, saponins, sterols, triterpenes, phenolics, tannins and vitamin C, followed by ethanolic, aqueous and hexane extract, respectively. Likewise, our phytochemical screening results demonstrated that phytochemical compositions of *B. macrophylla* peel extracts were phytosterols, saponins, tannins and phenolics depending on polarity of the solvents and contributing to their promising biological activities. It has been widely proven that most of the tannins and phenolics are extracted effectively by ethanol or polar solvents than by non-polar solvents which was correlated to our results (Nawaz et al., 2020). Meanwhile, phytosterols and flavonoids present in a lipophilic manner can be easily extracted by nonpolar solvents such as ethyl acetate, petroleum ether and hexane (Uddin et al., 2018). Moreover, the presence of phytosterols in crude BPE but not in BPEE indicated that fractionation using ethyl acetate following by ethanol could partially purified the extract. In addition, it is worth noting that our results of total phenolic and flavonoid contents of BPEA were not in correspondence with those of phytochemical screening test which might provide false negative results of flavonoids and phenolics in the BPEA.

Our findings firstly demonstrated that extracts of *B. macrophylla* peel have strong antioxidant activities owing to the presences of phenolics and flavonoids. Polyphenol compounds are well-known for their antioxidant activity preventing human, animal and plant cells against the harmful effects of free radicals (Ghasemzadeh and Ghasemzadeh, 2011). The antioxidant capacity of polyphenols is due to the hydroxyl (-OH) moiety existing in the chemical structure that have the capacity to inhibit or prevent a target molecule from ROS peroxide, hyperoxide and lipid peroxyl (Pourmorad, Hosseinimehr, and Shahabimajd, 2006; Yamagishi and Matsui, 2011; Wu et al., 2011). In addition, the high amount of polyphenolic compounds was in correspondence with higher antioxidant activity since there were more active compounds to scavenge the available free radicals (Shahidi, Janitha, and Wanasundara, 1992). The extracts of *B. macrophylla* peel have highest ability to scavenge ABTS free radicals followed by DPPH radicals due to IC_{50} values reported from ABTS assay was apparently lower than DPPH assay. This finding could be explained that ABTS radical is more sensitive to antioxidant compounds due to faster kinetics reacting with the hydroxyl contained compound (Ilyasov et al., 2020). Furthermore, the experimental systems of ABTS and DPPH assays were different in a term of physical property of the radicals. The ABTS-radical scavenging system was high polarity since aqueous solution serves as a dissolving solvent of the free radicals, whereas DPPH-radical scavenging system was low polarity owing to its lipophilicity in nature (Lee et al., 2015). This finding also implied that the active substances in the extracts might be probable to be hydrophilic substances which was corresponding to the characteristic of BPHE. Furthermore, the oxidation of cellular lipids is known as lipid peroxidation that is the chain reactions of oxidative degradation of lipids. It plays an important role in the degradation of cellular functions leading to various degenerative disease and even skin aging problems (Ayala, Muñoz, and Argüelles, 2014). Our results demonstrated that BPHE had the highest inhibitory capacity on lipid peroxidation.

Moreover, BPHE has the greatest ability of ferric reducing antioxidant power. Therefore, BPHE has outstanding antioxidant activities compared to other extracts.

Melanin is the key characteristic that causes dark spots, freckles, melisma and melanomas. Due to melanin production consists of complex oxidation and enzymatic processes, with tyrosinase being one of the most specific enzymes (Ayala et al., 2014; Sun, Guo, Zhang, and Zhuang, 2017). Besides, ROS are also important in melanogenesis and the catalyzed reactions of tyrosinase that produce dopaquinone lead to an increase in melanin synthesis (Zuo et al., 2018). Various skin whitening agents possess anti-melanogenic properties *via* antioxidant properties and directly inhibitory effects or regulatory action on tyrosinase activity. Hence, tyrosinase inhibitors are promising targets in cosmetics and skin pigmentation treatments. From our results, the tyrosinase inhibitory activity of *B. macrophylla* extract was also revealed of which only BPHE exerted this effect. Therefore, BPHE had a potential skin-whitening agent through both antioxidant and anti-tyrosinase effects.

Inflammation reaction which leading to extensive skin deterioration is also attributed to oxidative stress. In addition, the cell responding to cytotoxic compounds is usually followed by the production of inflammatory cytokines (Cui et al., 2018). Nitric oxide (NO) is regarded as one of the major inflammatory mediators contributing the prolong inflammatory and immune responses (Sharma et al., 2007). The bioactive compounds can inhibit the activity of inducible nitric oxide synthase (iNOS) and scavenge free radicals (Wiegand and Hipler, 2009). This research also investigated the potential of *B. macrophylla* peel extract to regulate NO production and secretion from lipopolysaccharide (LPS)-activated RAW 264.7 cells, a mouse macrophage cell line. RAW 264.7 cells are frequently used to investigate the anti-inflammatory effects of pharmaceutical agents (Adebayo, Ondua, Shai, and Lebelo, 2019). The ability of the BPHE to inhibit NO production from LPS-induced RAW 264.7 cells was shown at a concentration of 0.05 mg/ml. These results strongly supported an anti-inflammatory activity of *B. macrophylla* peel extract. Taken together, BPHE could be a promising active ingredient for cosmeceutical applications.

Cosmetics and personal care products should be concerned about safety and devoid of dangerous compounds that harm to humans and other living things (Hwang et al., 2018). Although natural substance has beneficial effects and many comprehensive mechanisms related to free radicals and oxidative stress, yet it is important to consider the safety assessment. Our study revealed that the cytotoxicity of the extract on human fibroblasts through sulforhodamine B (SRB) assay. It was reported that BPHE and ellagic acid which was a one of the major compounds in BPHE at concentrations of 0.0001^{-1} mg/ml had no toxicity to human fibroblasts. Therefore, the *B. macrophylla* peel extracts were safe for human use.

Aside from safety assessment, standardized profiles are the most important step for quality assurance in the natural cosmetics which is simplified by chemical and chromatographic procedures (Seo et al., 2016; Barthe et al., 2021). Phytochemical compositions in the study of Dechsupa et al. (2018) revealed that *B. macrophylla* seed kernel extracts demonstrated eighteen compounds by using HPLC at a wavelength of 270 nm. Furthermore, gallic acid and ellagic acid were successfully identified. In present study, BPHE was chosen for HPLC fingerprint analysis due to its significant antioxidant, anti-tyrosinase as well as its anti-inflammatory properties. The HPLC chromatogram of BPHE exhibited two identified peaks including gallic acid and ellagic acid. These compounds could be used as a BPHE biomarker for further product development and quality control. However, other phytochemical compounds found in the extract might play a role in its biological potential which were interesting for further investigation.

According to the stability testing, the results suggested that both decline in gallic acid and ellagic acid might have an effect on the decrease in antioxidant property and tyrosinase inhibition of the extract. These results possibly provided the information for further developing of an innovative cosmetic formulation in order to overcome these stability limitations of the extract. Moreover, the storage

condition of the extract should be controlled in terms of low temperature and light-protection system.

CONCLUSIONS

This study firstly reported the multifunctional biological activities of B. macrophylla peel extracts in terms of antioxidation, anti-tyrosinase and anti-inflammatory effects along with the phytochemical constituents, safety and stability profiles for cosmetic applications. Our study revealed that hydroethanolic extract of *B. macrophylla* peel exerted the greatest biological properties with a good safety profile in human fibroblasts. Gallic acid and ellagic acid were identified in this extract. These findings implied that *B. macrophylla* peel extract has high potential to be a bioactive ingredient for cosmeceutical applications. However, the results of stability test suggested that cosmeceutical applications of *B. macrophylla* peel extract might require the development of optimized delivery systems to enhance its stability especially under light and high temperature conditions.

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AUTHOR CONTRIBUTIONS

Pawarisa Maneechai chiefly conducted the experiments, performed the statistical analysis and wrote the manuscript with data visualization by Pimporn Leelapornpisid and Worrapan Poomanee who designed all of the experiments and revised the manuscript. All authors have read and approved of the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in this work.

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