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Toxicological and Cosmetic Functional Properties of Microwave-Assisted *Pereskia bleo* (Kunth) DC Leaves Extract

Intan Balqis-Wong[1], Siti Salwa Abd Gani[2,3,*], Uswatun Hasanah Zaidan[4], Mohd Izuan Effendi Halmi[5]

[1] *Halal Products Research Institute, Universiti Putra Malaysia, Putra Infoport, 43400 Serdang, Selangor, Malaysia*
[2]*Deparment of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Natural Medicine and Products Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[5]*Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
Corresponding Author: Siti Salwa Abd Gani

----------ABSTRACT----------

Cosmetic products are gaining attention due to the difficulty in identifying the toxicity status of animal-based ingredients commonly used in the market. Medicinal plants offer a natural and viable alternative for cosmetic formulations. Pereskia bleo (Kunt) DC, known locally in Malaysia as "Pokok Jarum Tujuh Bilah" is a medicinal plant from the Cactaceae family, traditionally used for treating various ailments because of its high antioxidant, anti-inflammatory, and antimicrobial properties. Despite its known medicinal uses, its potential as a cosmeceutical ingredient has not been widely studied. A non-conventional microwave-assisted extraction (MAE) method was used to prepare P. bleo leaf extract. The extract demonstrated significant cosmeceutical properties, showing 80.14% anti-collagenase, 74.04% anti-tyrosinase, and 48.03% anti-elastase inhibition activities. It also exhibited a sun protection factor (SPF) value of 23.74, indicating strong potential for use in functional cosmetics. The proximate composition of the leaves was analyzed, consisting of 20.26% crude protein, 3.55% crude fat, 11.13% crude fiber, 16.5% ash, 9.36% moisture, and 39.2% carbohydrates. Toxicity evaluations, including heavy metal analysis via ICP-MS and a brine shrimp lethality bioassay, showed that concentrations of arsenic, cadmium, chromium, lead, and mercury were all below the specific release limits set by FAO, WHO, and EFSA. No lethal concentration of minerals or heavy metals was detected, and the extract exhibited no cytotoxicity. These findings suggest that P. bleo leaves are safe and have excellent potential as a plant-based ingredient in cosmetic products due to their beneficial properties and safety profile.

KEYWORDS; *Microwave-assisted extraction, Pereskia bleo, Functional cosmetic, Toxicity*

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I. INTRODUCTION

Medicinal plants have long been used in traditional medicine due to their beneficial properties. However, verifying their safety and toxicity levels is crucial before recommending them for widespread medical use. *Pereskia bleo* Kunth DC (*P. bleo*), a member of the Cactaceae family, is widely used in traditional medicine for its purported anti-inflammatory, anticancer, and antioxidant properties [1]. *P. bleo* is known for its diverse medicinal and non-medicinal uses, particularly its leaves. The leaves, flowers, and fruits are often consumed raw to promote a healthy diet. The leaves are also dried, boiled, and consumed as a tea to detoxify the body, while fresh leaves are brewed into a refreshing drink believed to rejuvenate the body and relieve muscle pain.

In Panama, the plant is typically moulded and sun-dried, and the resulting dried form is traditionally mixed with water to treat gastrointestinal disorders [1,2]. In Colombia, crushed leaves have been used to purify drinking water. The plant has also been reported to treat a wide range of ailments, including headaches, stomachaches, inflammation, diabetes, cancer, hemorrhoids, hypertension, rheumatism, gastric pain, ulcers,

asthma, and wound infections [2]. Despite these traditional applications, recent cytotoxicity studies by Abd Malek et al. and Sim et al. reported no cytotoxic activity from the plant's extract against normal cell lines and non-cancer human fibroblast cell lines (MRC-5 cells), which are fibroblast cells isolated from human lungs [3,4]. However, the extract demonstrated strong cytotoxic effects against human nasopharyngeal epidermoid carcinoma cell lines (KB cells), a keratin-forming tumor cell line from epidermal carcinoma of the mouth.

Despite its popularity, the toxicological profile of *P. bleo* remains largely unexplored, raising concerns about potential cytotoxic effects and heavy metal contamination. With the growing global interest in herbal medicines, ensuring the safety of traditionally used plants like *P. bleo* is essential. There is a lack of scientific studies evaluating the plant's potential toxicity, particularly through *in vivo* bioassays and elemental composition analysis. Without this data, the safety and medicinal viability of *P. bleo* remains uncertain.

The brine shrimp lethality bioassay is a simple, cost-effective method widely used for preliminary toxicity screening of plant extracts. It provides a reliable indication of cytotoxicity by evaluating the lethal effects on *Artemia salina* nauplii, with results strongly correlating with cytotoxicity against cancer cell lines and other toxicological assays [5,6]. The assay determines the median lethal concentration (LC_{50}), which helps assess the toxic potential of plant extracts and predict their biological activity. In addition to *in vivo* bioassays, the presence of toxic elements like heavy metals in medicinal plants is a growing concern due to environmental contamination. Inductively coupled plasma mass spectrometry (ICP-MS) is a highly sensitive analytical technique used for the precise quantification of trace elements and heavy metals, such as arsenic, lead, cadmium, and mercury. These elements pose significant health risks if accumulated beyond permissible levels [7]. ICP-MS provides rapid, multi-elemental analysis with high precision and low detection limits, making it an ideal tool for evaluating the safety of herbal medicines.

Moreover, medicinal plants are increasingly recognized as valuable cosmeceutical sources. Cho et al. reported that these plants contain effective ingredients for skincare products, with therapeutic benefits that improve physical appearance [8]. According to Gupta et al., medicinal plants' high antioxidant bioactive compounds make them excellent candidates for cosmeceutical applications [9]. Despite the importance of *P. bleo* as a medicinal species, there has been no comprehensive report on its cosmeceutical properties. This study explores the plant's potential in cosmeceutical applications while addressing the existing knowledge gap regarding native Malaysian plants. The current study aims to evaluate the cytotoxicity and heavy metal content of *P. bleo* leaf extract through ICP-MS and brine shrimp lethality bioassay. Additionally, it seeks to determine the plant's functional cosmetic properties, including anti-collagenase, anti-tyrosinase, anti-elastase activities, and sun protective factor (SPF).

II. MATERIALS AND METHODS

Plant materials and extraction

Matured leaves of *P. bleo* (Kunth) DC were collected from a local village in Duyong, Melaka, Malaysia (Location: 2.2032° N, 102.3156° E). The plant species of the sample was identified by a botanist at the Institute of Bioscience, Universiti Putra Malaysia under voucher number KM 0102/24. Harvested leaves of *P. bleo* were brought to and processed in the laboratory. The leaves were manually cleaned, separated from their stems, and sun-dried for three days. Dried leaves were ground using a home-blender (Panasonic PSN-MXGM1011), and the ground leaf materials were then stored in a vacuum container and away from light until use. The sample extract was prepared by using the microwave-assisted extraction (MAE) method following Alara et al. procedures with minor modifications [10]. A domestic microwave oven (R662SLM, Sharp, Malaysia) with 20 L capacity and a digital control system for time and microwave power (10 to 110 watts) was used as the microwave. Extraction was done by measuring 1 g of ground leaf materials and placed in a 250 mL Erlenmeyer flask. The desired solvents in 10 mL volume and 60% concentration were added to the flask. The extraction process commenced at the 31 s extraction time and 50-watt microwave power. Following the extraction process, the extractant was filtered using Whatman No. 1 filter paper (90 mm, Sigma Aldrich, USA) and collected in a 100 mL Erlenmeyer flask. The extract was subsequently dried using a rotatory evaporator fitted with a controller and vacuum pump (Rotavapor® R-3000, Buchi, Switzerland). The dried extract was kept in a closed container and stored at 8 °C until use.

Proximate analysis

Proximate analysis of *P. bleo* was conducted to identify ash, crude fat, crude fiber, crude protein, and moisture content following procedures described by the Association of Official Analytical Chemists (AOAC, 2005) using the modified method from Niyi et al. [11]. Carbohydrates were also determined using a method introduced by AOAC, (1984). All proximate analyses were done in triplicate, and the results were calculated and expressed by means and standard deviations. In the present study, proximate analysis was determined under the ash content, crude fat content, crude fiber content, crude protein content, moisture content, and carbohydrate content.

Ash content

In determining ash content, a sample of the ground leaf (2.0 g) was placed in a pre-weighed crucible and heated in an oven for 12 h at 105 °C. The residue was slowly burned at 550 °C in a muffle furnace. Subsequently, the crucible was placed in a desiccator after an 8 h cremation process, and ash content was calculated using the following equation below:

$$\% \text{ Ash} = \frac{\text{Weight of Ash}}{\text{Weight of Sample}} \times 100$$

Crude fat content

For crude fat content determination of *P. bleo,* a 20 g sample was weighed and placed in a pre-weighed extraction thimble. The thimble was covered with cotton wool and placed in a Soxhlet extractor. A round bottom flask (RBF) filled with 160 mL petroleum ether was fixed into a Soxhlet apparatus. The reflux process was heated to 50 °C for 8 h, and the RBF was oven-dried at 100 °C for 24 h. The crude fat percentage was then determined by the following equation below:

$$\% \text{ Fat} = \frac{W_4 - W_3}{W_2 - W_1} \times 100$$

W_1 = Weight of empty thimble (g)
W_2 = Weight of empty thimble + sample (g)
W_3 = Weight of empty RBF (g)
W_4 = Weight of empty RBF + extracted lipid (after drying) (g)

Crude fiber content

The crude fiber content of *P. bleo* was determined by measuring 2.0 g of ground sample and added into a Berzelius beaker containing 150 mL sulphuric acid of 0.1274 M and was subsequently heated. After 30 min, the content was filtered using a Buchner funnel, and the Berzelius beaker was washed using distilled water. The sample was then transferred back into the Berzelius beaker, and the procedure was repeated by using 150 mL sodium hydroxide in 0.313 M concentration. At the end of the procedures, the content was filtered using a pre-weighed sintered glass, and the beaker was rinsed using distilled water and ethanol. The sintered glass with sample residue was dried in an oven set at 130 °C for 120 min. The crude fiber content was calculated using the following equation below:

$$\% \text{ Crude fiber} = \frac{W_a - W_b}{W_c} \times 100$$

W_a = Weight of sintered glass + filtered residue (after drying) (g)
W_b = Weight of sintered glass + ash (g)
W_c = Weight of sample (g)
W_a = Weight of sintered glass + filtered residue (after drying) (g)

Crude protein content

The crude protein content of *P. bleo* was determined by the Kjeldahl method. A Kjeltec 2300 Analyzer Unit (FOSS, Sweden) was used in the procedure. An amount of 2.0 g of ground sample was weighed and dispensed into the digestion tube of the analyzer. The tube was then heated up to 420 °C, and a Selenium tablet was added into the digestion tube as a catalyst to speed up the process. Subsequently, 12 mL of concentrated (98%) sulphuric acid was slowly added into the tube, and the sample was allowed to dissolve for 2 h. Next, 40% of sodium hydroxide was added to the alkali tank of the analyzer for a distillation process. A 250 mL Erlenmeyer flask filled with 4% boric acid, methyl red as an indicator, and bromocresol was placed at the collecting end of the distillation apparatus. At the end of the procedure, a digestion tube with diluted digest was connected to the distillation apparatus. The distillation process was run until 150 mL of distilled solution was collected. The collected solution was titrated with 0.1 N hydrochloric acid (HCl) until the solution turned violet as the endpoint. The total amount of HCl used was recorded. The following equation below was used to determine the percentage (%) of nitrogen:

$$\% \text{ Kjedahl Nitrogen} = \frac{V_s - V_b \times 14.01}{W \times 10}$$

V_s = the volume of standardized acid used in the titration of the sample
V_b = the volume of standardized acid used in the titration of blank
14.01 = the atomic weight of nitrogen
W = weight of the sample (g)
10 = factor to convert gram (g) to percent (%)

The percentage of crude protein in the sample was calculated using the equation below:
Percentage (%) of Crude Protein = % Kjedahl Nitrogen x F
F = Factor to convert nitrogen to protein
6.2 = Protein-Nitrogen conversion factor

Moisture content

A moisture analyzer (MX-50, A&D, Toshima-ku, Tokyo, Japan) was used to measure the moisture content of the ground leaf sample of *P. bleo*. In the procedure, 2.0 g of the ground sample was weighed and placed in the panhandle of the analyzer and heated to 105 °C using halogen lamps. Moisture content was calculated by using the following equation below:

$$\% \text{ Moisture} = \frac{W_b - W_a}{W_b} \times 100$$

W_a = The initial weight of the sample
W_b = The final weight of the sample

Carbohydrate content

The carbohydrate content of *P. bleo* was determined by using the equation below:

% Carbohydrate = 100 – (% Ash + % Crude Fat + % Crude fibre + % Moisture + % Crude Protein)

Toxicity evaluation

Mineral and heavy metal content

Determinations of minor (Al, Co, Cu, Fe, Mn, and Zn) and major (Ca, K, Mg, Na, and P) elements were carried out using Perkin-Elmer SCIEX Elan 9000 quadrupole-based inductively coupled plasma mass spectrometry (ICP-MS) (Perkin-Elmer SCIEX, Norwalk, CT, USA) apparatus. A microwave digestion system (ETHOS UP, Milestone Srl, Sorisole (BG), Italy) was used to digest 4 mL of ethanol extract of *P. bleo* with 5 mL of 65% v/v nitric acid (HNO3) and 30% v/v hydrogen peroxide (H2O2). The procedure was performed in triplicate. The microwave system took 10 min to achieve 180 °C and 15 min to maintain at that temperature. The sample was filtered using filter paper (0.45 m, Advantec M.F.S. Inc, Pleasanton (CA), USA), which produced a colorless solution. The samples were then diluted with up to 50 mL of distilled water.

Brine shrimp lethality bioassay

The brine shrimp lethality assay was performed to examine the cytotoxicity of *P. bleo* ethanol extracts. The assay was conducted using high-quality brine shrimps (*Artemia salina*), hatching eggs from sea salt premix (Ocean Nutrition Europe, Rijkmakerlaan, Essen, Belgium). The method was adapted from Krishnaraju et al. and Defoirdt et al. [12,13]. In the procedure, 200 mg of *A. salina* eggs were hydrated in 18 mL tap water in a 50 mL falcon tube. After an hour of hydration, the egg solution was mixed with 660 mL of 32% sodium hydroxide and 10 mL of 5% Clorox® (sodium hypochlorite). Subsequently (after 2 min), 14 mL of sodium thiosulphate (10 g/L) was added. The mixture was filtered through a sieve cloth and was rinsed using 20 mL of artificial seawater made by using sea salt 38 g/L. 1N sodium hydroxide (NaOH) was used to adjust the solution's acidity to pH 8.5. The shrimp eggs were then re-suspended and hatched in an 1 L conical-shaped vessel with sterile artificial seawater and were continuously aerated for 48 h. The shrimp eggs were allowed to hatch and develop into nauplii in an incubator shaker maintained at room temperature. Active nauplii free of eggshells were harvested from the hatching chamber's brighter area and used for the test. The nauplii were fed with 2 µL of autoclaved

yeast suspension. Ten nauplii were collected using a glass capillary and placed into 4.5 mL brine solution-filled vials. The experiments were performed with potassium dichromate as the positive control and autoclaved seawater as the negative control. The extract and brine solution concentrations were set from 1.95 to 1000 ppm. In each experiment, brine solution and plant extract were combined according to the concentration range and kept at room temperature for 24 h under the light, after which the surviving shrimp were counted. Percentage lethality was calculated by comparing the mean number of larvae that survived in the test and control tubes where the 50% lethal concentration (LC_{50}) was obtained. The mean survival rates of the larvae in control and extract-treated tubes were used to compute the percentage lethality. Values of LC_{50} were based on the best-fit technique.

Cosmeceutical properties of *P. bleo* extract

Anti-collagenase activity

Collagenase inhibition was determined by using a collagenase activity assay kit following the protocol of BioVission (K792-100). The kit and reagent, previously stored cold and away from light, were defrosted from -70 °C to room temperature. In the procedure, the test collagenase was first dissolved in Hank's balanced salt solution (HBSS). The sample was prepared by mixing 20 µL of optimized extract in DMSO and diluted in 50 µL of cold phosphate buffer saline as assay buffer. A 96-well microtiter microplate (Thermo Scientific, Massachusetts, USA) was used, and 10 µL of collagenase enzyme was added to each well. Incubation was allowed for 10 min at 37 °C. 100 µL of substrate mixture was prepared for each reaction. A master mix of 60 µL of collagenase assay buffer and 40 L of collagenase substrate (FALGPA) was prepared. 90 µL of collagenase assay buffer and 10 L of collagenase substrate mixture were ready as a blank for background control. Subsequently, 70 µL of collagenase assay buffer, 10 µL of collagenase substrate mixture, and 20 µL collagenase enzyme were mixed and set as a positive control. 2 µL inhibitor (1,10-phenanthroline, 1M), 10 µL of collagenase enzyme, and 88 µL of collagenase assay buffer were mixed and set as inhibitor control. 10 µL of collagenase enzyme, 2 µL of solvent used (dimethyl sulfoxide, DMSO), and 88 µL of collagenase assay buffer were mixed and set as solvent control. These mixtures were measured using a UV-VIS Microplate Reader (Spectra Max Plus 384, Molecular Devices Co. Ltd., USA). The absorbance was measured at wavelength 345 nm. The extracts' percentage inhibition of collagenase was determined by using the equations below:

$$\text{Collagenase Activity} = \frac{\frac{\Delta 0Dc}{\Delta T} \text{ x } 0.2 \text{ x } D}{0.53 \text{ x } V}$$

$\Delta 0Dc$ = Difference between $A_{345\ nm2}$ and $A_{345\ nm1}$
ΔT = Difference between T_2 and T_1
0.2 = Reaction volume (mL)
D = Sample dilution factor
0.53 = The millimolar extinction coefficient of FALGPA
V = Enzyme volume (mL)

$$\text{\% Collagenase inhibition} = \frac{A_0 - A_1}{A_0} \text{ x } 100$$

A_0 = The activity of positive control
A_1 = The activity of the extracted sample

Anti-tyrosinase activity

The determination of tyrosinase inhibition of *P. bleo* was performed by using a modified 96-well microplate method following the procedures of Haliloglu et al. [14] Tyrosinase enzyme is from a mushroom. A solution of sample extracts (10 mg/mL) was prepared using phosphate buffer at pH 6.8. A sample was diluted with DMSO at 0.1, 0.01, and 0.001 mg/mL concentrations. The experiment was assigned accordingly, where 14 wells were used: three wells of control (A), one well for blank (B), three wells for each sample extract (C), and one well for blank (D). In (A) 120 µL of 0.1 M phosphate buffer (pH 6.8) and 40 µL of tyrosinase (33.3 U/mL)

in phosphate buffer, (B) contained 160 µL of 0.1 M phosphate buffer (pH 6.8), (C) had 80 µL of 0.1 M phosphate buffer (pH 6.8), 40 µL of tyrosinase (33.3 U/mL) in phosphate buffer, 40 µL of sample diluted with a phosphate buffer solution that contains DMSO. (D) contained 120 µL of 0.1 M phosphate buffer (pH 6.8) and 40 µL of sample diluted with a phosphate buffer solution containing DMSO. A dilution of kojic acid with phosphate buffer (0.01 – 0.1 mg/ mL) was performed. Quercetin of 0.1 mg/mL in phosphate buffer was used as a reference. The contents were mixed accordingly and incubated at 23 °C for 10 min. Subsequently, 40 µL of 2.5 mM L-DOPA in phosphate buffer was added to each well. The mixtures were then incubated again at 23 °C for 15 min. The absorbance was measured at wavelength 475 nm using a UV-VIS Microplate Reader (Spectra Max Plus 384, Molecular Devices Co. Ltd., USA). The extracts' percentage inhibition of tyrosinase activity (Inh %) was determined by using the following equation below:

$$\text{Inh \%} = \left\{\frac{[(A-B)-(C-D)]}{(A-B)}\right\} \times 100$$

The experiment sets were run in triplicate. Tyrosinase inhibition activity of the extracts was expressed as mg of kojic acid equivalents (KAE) per g of *P. bleo* extract (mg of KAE/ g of extract). A calibration curve of kojic acid dilution in methanol was prepared. A standard curve was generated using the following equation below:

$Y = aX + b$, $R^2 = c$

Y = Percentage inhibition of tyrosinase
X = The concentration of the mixture (mg/ mL)

Anti-elastase activity

Elastase inhibition was determined using a neutrophil elastase colorimetric drug discovery kit with protocol guidance from Enzolifescience (BML-AK497). The kit and reagent, previously stored in cold storage and away from light, were defrosted from -70 °C to room temperature. In the procedure, 2 µL of inhibitor elastastinal was first diluted in 78 µL neutrophil elastase buffer solution (100 mM HEPES, pH 7.25, 500 mM NaCl, 0.05 % Tween 20 in DMSO) and yielded 100 µL of inhibitor solution. Then, 7.5 µL substrate (MeOSuc-AAPV-pNA) was diluted with 67.5 µL assay buffer to prepare a substrate solution. In a separate vial, 1 µL of neutrophil elastase enzyme was diluted with 89 µL of neutrophil elastase assay buffer. The elastase inhibition activity was measured. In the procedure, 20 L of the optimized extracts was diluted in 65 µL of neutrophil elastase assay buffer, in a 96-well microtiter microplate (Thermo Scientific, Massachusetts, USA). 10 µL of neutrophil elastase enzyme solution was added to the sample wells. The positive control was set by mixing 10 µL of neutrophil elastase enzyme with 85 µL of neutrophil elastase assay buffer. The inhibitor control was mixing 20 µL of inhibitor and 10 µL of elastase enzyme in 65 µL of neutrophil elastase assay buffer solution. The blank control was done by adding 95 µL of neutrophil elastase assay buffer into the appropriate well. All mixtures were then incubated for 30 min at 37 °C to let the elastatinal inhibitor interact with the neutrophil elastase enzyme. The assay started by adding 5 µL of the substrate (MeOSuc-AAPV-pNA) into all wells (sample extracts, blank control, inhibitor control, and positive control). Anti-elastase activities were measured using a UV-VIS Microplate Reader (Spectra Max Plus 384, Molecular Devices Co. Ltd., USA). Absorbance was measured at wavelength 405 nm. The plate was continuously read and monitored for 10 min, and the data were collected at 1 min intervals. A graph of absorbance of the tests against time (min) was plotted, and the slope reaction velocity was calculated. The extracts' percentage inhibition of elastase was determined by the equation below:

$$\text{Inhibition \%} = \frac{V_{Inhibition}}{V_{Control}} \times 100$$

$V_{Inhibition}$ = Velocity reaction of the sample extract or blank and inhibitor control
$V_{Control}$ = Velocity reaction of the positive control

Sun protective factor (SPF)

The photoprotective activity was conducted via *in-vitro* ultraviolet (UV) absorption analysis by determining the sun protective factor (SPF) for UVA, UVB, and UVC of the optimized extracts was done following the method reported by Dutra et al. [15]. In the procedure, 1 g of extract sample was measured and

transferred into a 100 mL volumetric flask. The sample extract was diluted with ethanol, sonicated using ultrasonication for 5 min, and filtered using cotton wool. The first 10 mL was removed, an aliquot of 5 mL was transferred to a 50 mL volumetric flask, and ethanol was used to dilute. Subsequently, a 5 mL aliquot was transferred to a 25 mL volumetric flask and diluted with ethanol to a volume of 25 mL. The absorbance was measured at a wavelength ranging from 200 to 450 nm. 1 mL aliquot of sample extract was added to a 1 cm quartz cell. 1 mL of ethanol was used as a blank control. The absorption data were analyzed within 290 to 320 nm at every 5 nm. The SPF was determined using the formula in the equation below:

$$SPF_{Spectrophotometric} = CF \times \sum_{290}^{320} EE\ (\lambda) \times I\ (\lambda) \times Abs\ (\lambda)$$

EE = Erythermal effect spectrum
I = Solar intensity spectrum
CF = Correction factor (= 10)
Abs = Absorption of sunscreen product

The values of (EE × I) are constants [16], as shown in Table 1 below.

WAVELENGTH, λ (nm)	EE × I (NORMALIZED)
290	0.015
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.018
Total	1
EE = Erythermal effect spectrum; I = Solar intensity spectrum	

Table 1. Normalized product functions used in the calculation of SPF

III. RESULTS

Proximate analysis

Proximate analysis is used to analyze the food substance in *P. bleo*, including moisture, ash, crude fat, crude fiber, crude protein, and carbohydrate content. Proximate compositions of *P. bleo* leaves are shown in Table 2. The main composition in the leaves is carbohydrate with 39.2 ± 0.3%. The second highest composition is protein with 20.26 ± 0.6% where it is the amount of nitrogen multiplied by the protein factor (14.01). The crude fat content is 3.55 ± 0.3%, while the crude fiber content is 11.13 ± 0.1%. The ash content value is 16.5 ± 0.7%. Lastly, the moisture content of *P. bleo* leaves is 9.36 ± 0.1%.

PROXIMATE COMPOSITION	AMOUNT (%)
Crude protein	20.26 ± 0.6
Crude fat	3.55 ± 0.3
Crude fibre	11.13 ± 0.1
Ash	16.5 ± 0.7
Moisture	9.36 ± 0.1
Carbohydrate	39.2 ± 0.3

Table 2. Proximate composition of *P. bleo* (Kunth) DC leaves

Toxicity evaluation

Mineral and heavy metal content

ICP-MS analysis was used to determine the mineral and heavy metal content in *P. bleo* leaves. Micromineral consists of copper (Cu), selenium (Se), zinc (Zn), Iron (Fe), chromium (Cr), manganese (Mn), nickel (Ni), and cobalt (Co) detected in *P. bleo,* and the tolerable upper intake level (UL) of each micromineral are listed in Table 3. The highest micronutrient in the leaves is iron (10265.42 ± 243.22 µg/L). Copper metal content is 46.61 µg/L, which is lower than the daily limit. Macronutrients consist of calcium (Ca), potassium (K), magnesium (Mg), and sodium (Na), and the result of *P. bleo* macrominerals and its tolerable upper intake

level (UL) are listed in Table 4. The macrominerals found include calcium (17.71 ± 0.01 mg/L), potassium (62.56 ± 1.93 mg/L), magnesium (12.95 ± 0.93 mg/L), and sodium (0.451 ± 0.04 mg/L).

ELEMENT	CONCENTRATION (µg/L)	UPPER LIMIT (µg/L)
Copper (Cu)	46.61 ± 0.17	5000
Selenium (Se)	0.53 ± 0.04	255
Zinc (Zn)	1184.40 ± 9.89	25000
Iron (Fe)	10265.42 ± 243.22	NM
Chromium (Cr)	31.72 ± 0.02	NM
Manganese (Mn)	3446.74 ± 28.78	8000
Nickel (Ni)	67.90 ± 5.37	NM
Cobalt (Co)	9.5 ± 0.34	NM
European Food Safety Authority (EFSA) panel on Dietetic Products, Nutrition, and Allergies (NDA), (2018). NM: Not mention		

Table 3. Microminerals in *P. bleo* leaves

ELEMENT	CONCENTRATION (mg/L)	UPPER LIMIT (mg/L)
Calcium (Ca)	17.71 ± 0.01	2500
Potassium (K),	62.56 ± 1.93	NM
Magnesium (Mg)	12.95 ± 0.93	250
Sodium (Na)	0.451 ± 0.04	NM
European Food Safety Authority (EFSA) panel on Dietetic Products, Nutrition, and Allergies (NDA), (2018). NM: Not mention		

Table 4. Macrominerals in *P. bleo* leaves

Heavy metals consist of copper (Cu), cobalt (Co), Iron (Fe), zinc (Zn), nickel (Ni), mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), beryllium (Be), bismuth (Bi), silver (Ag), aluminium (Al), lithium (Li), caesium (Cs), lead (Pb), barium (Ba), gallium (Ga), indium (In), manganese (Mn), rubidium (Rb), selenium (Se), strontium (Sr), vanadium (V), and uranium (U). The results of all the heavy metals in *P. bleo* and the specific release limit (SRL) of heavy metals in food referred from the Food and Agriculture Organisation (FAO), (1995), World Health Organisation (WHO), (2011), and European Food Safety Authority (EFSA), (2015) are listed in Table 5. All these values are below the specific release limit (SRL) and thus considered safe for consumption.

HEAVY METAL	CONCENTRATION (µg/L)	SRL (µg/L)
Aluminium (Al)	2270.33 ± 121.50	5000
Arsenic (As)	7.68 ± 0.36	10
Barium (Ba)	604.94 ± 6.59	1200
Beryllium (Be)	0.18 ± 0.01	10
Bismuth (Bi)	0.38 ± 0.02	NM
Cadmium (Cd)	0.6 ± 0.23	5
Cobalt (Co)	9.5 ± 0.34	20
Chromium (Cr)	31.72 ± 0.02	250
Caesium (Cs)	23.97 ± 0.35	NM
Copper (Cu)	46.61 ± 0.17	4000
Iron (Fe)	10265.42 ± 243.22	40000
Gallium (Ga)	6.65 ± 0.7	NM
Mercury (Hg)	0.063 ± 0.01	3
Indium (In)	< DL	NM
Lithium (Li)	5.32 ± 0.13	48
Manganese (Mn)	3446.74 ± 28.78	1800
Nickel (Ni)	67.90 ± 5.37	140
Lead (Pb)	15.01 ± 0.83	300
Rubidium (Rb)	2952.98 ± 71.34	NM
Selenium (Se)	0.532 ± 0.04	40
Silver (Ag)	0.21	80

Strontium (Sr)	1284.86 ± 0.51	NM
Thallium (Tl)	7.90 ± 0.29	NM
Vanadium (V)	7.57 ± 0.36	10
Uranium (U)	0.25 ± 0.01	30
Zinc (Zn)	1184.40 ± 9.89	5000

Table 5. Heavy metals in *P. bleo* leaves with a specific release limit (SRL) of heavy metal in food

Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was used to evaluate the toxicity of *P. bleo* leaves. The bioassay was done by using a method extracted from Krishnaraju et al. and the sample extracts were diluted according to the dilution series 1.95 to 1000 ppm [12]. Potassium dichromate was used as positive control and sea salt water was used as negative control. The brine shrimp mortality rate in dilution of *P. bleo* leaves extract, potassium dichromate, and sea salt water were presented in Table 6. The mortality rate of brine shrimp was found to be directly proportional to the concentration of potassium dichromate. *P. bleo* leaf extract did not show toxicity to the brine shrimp.

CONCENTRATION (ppm)	MORTALITY RATE (%)		
	POTASSIUM DICHROMATE	SEA SALTWATER	*P. bleo* LEAVES EXTRACT
1.95	3	0	0
3.91	10	0	0
7.81	17	0	0
15.63	20	0	0
31.25	40	0	0
62.5	80	0	0
125	100	0	0
250	100	0	0
500	100	0	0
1000	100	0	0

Table 6. The result of cytotoxicity activity of *P. bleo* leaves extract on brine shrimp nauplii

Cosmeceutical properties of *P. bleo* leaves extract

Anti-collagenase activity

The collagenase inhibition activity against the concentration of plant extract is presented in Table 7 and illustrated in Figure 1. The highest concentration (10 mg/mL) of *P. bleo* leaves extract exhibited 80.14 ± 0.01% inhibition of collagenase. The result for concentrations 5 mg/mL, 1 mg/mL, 0.5 mg/mL, and 0.1 mg/mL were 78.81 ± 0.01%, 74.18 ± 0.01%, 65.64 ± 0.01%, and 55.64 ± 0.01% respectively. Kojic acid as a reference with a concentration of 1 mg/mL was used as a reference and gave 77.19 ± 0.01% inhibition activity.

SAMPLE	R^2	REMAINING COLLAGENASE ACTIVITY (%)	INHIBITION COLLAGENASE ACTIVITY (%)
P. bleo leaves extract	0.9302	19.86	80.14
Inhibitor (1,10-Phenanthroline)	0.961	17.21	82.79
Negative (control)	0.9284	100	0

Table 7. Inhibition of collagenase activity in *P. bleo* leaves extract

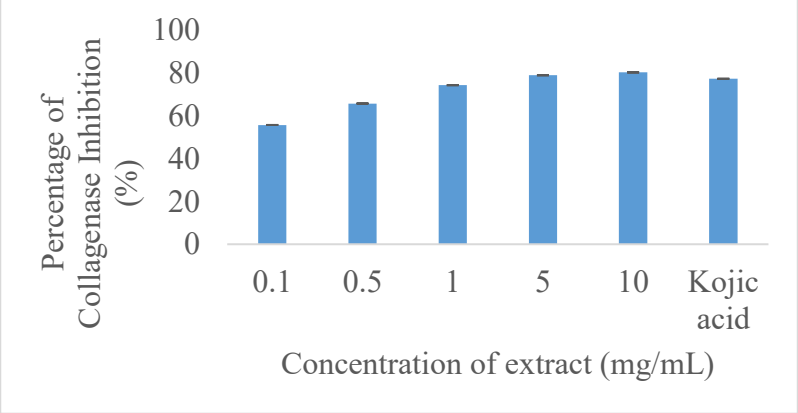

Figure 1. Percentage of collagenase activity in *P. bleo* leaves extract

Anti-tyrosinase activity

Results showed that *P. bleo* leaf extract with a concentration of 1 mg/mL gives 74.04 ± 0.03% of tyrosinase inhibition. Kojic acid is used as a reference and gives 76.4 ± 0.01% of tyrosinase inhibition. The tyrosinase inhibition percentage for kojic acid and *P. bleo* is shown in Figure 2.

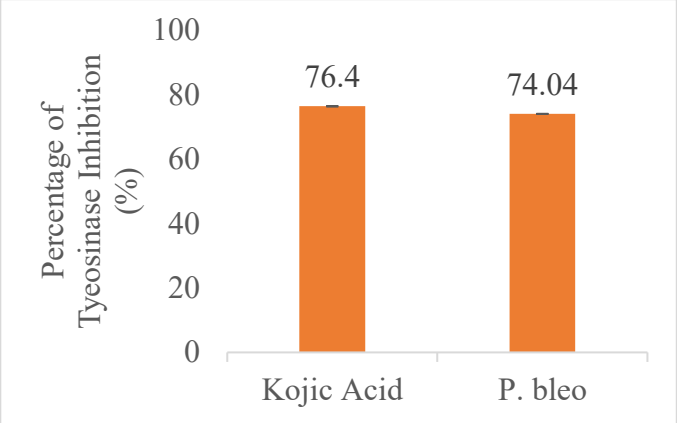

Figure 2. Percentage of tyrosinase activity in *P. bleo* leaves extract

Anti-elastase activity

Results showed that the concentration of 100 ppm gives the highest inhibition activity with 48.03 ± 0.01%, followed by 10 ppm with 34.41 ± 0.01% and 1 ppm with 22.22 ± 0.02%. The inhibition activity is shown in Table 8 and illustrated in Figure 3. 100 ppm ascorbic acid was used as a positive control, and the elastase inhibition activity was 40.66 ± 0.01%.

SAMPLE	R^2	REMAINING ELASTASE ACTIVITY (%)	INHIBITION ELASTASE ACTIVITY (%)
P. bleo extract	0.9939	51.97	48.03
Inhibitor (Elastatinal)	0.9913	38.71	61.29
Negative (control)	0.9962	100	0

Table 8. Inhibition elastase activity in *P. bleo* leaves extract

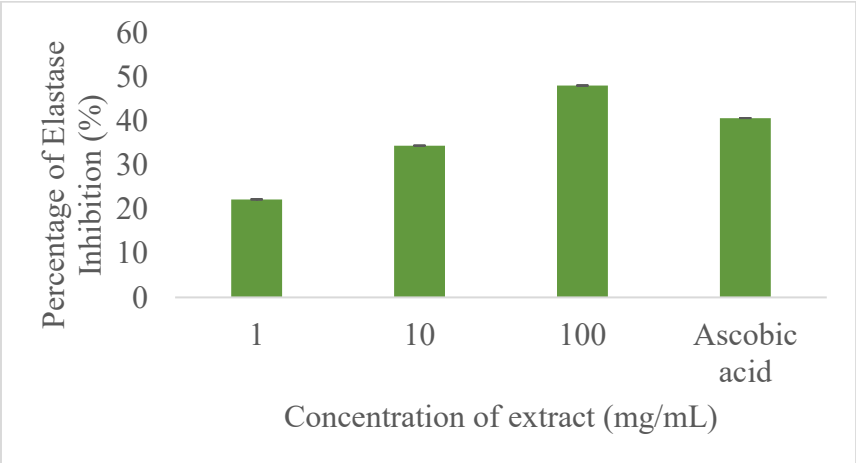

Figure 3. Percentage of elastase activity in *P. bleo* leaves extract

Sun protective inhibitor (SPF)

In this experiment, the transmission spectrum of the extract and two other commercial UV sunscreen active ingredients as reference gave great photo-protective activity where strong spectrophotometric absorption was observed in the UVC and UVB area in Figure 4. The results for the calculated SPF value of *P. bleo* leaves extract and avobenzone and benzophenone as two commercial UV sunscreen active ingredients are tabulated in Table 9. All the samples were diluted to 1 mg/mL for the SPF analysis. The SPF value for *P. bleo* leaves extract is 23.74 while the SPF value for avobenzone and benzophenone are 36.90 and 34.60 respectively. The SPF value of *P. bleo* leaves extract is higher than the minimum recommendation of SPF-15 set by the FDA, indicating its potential as a natural sunscreen.

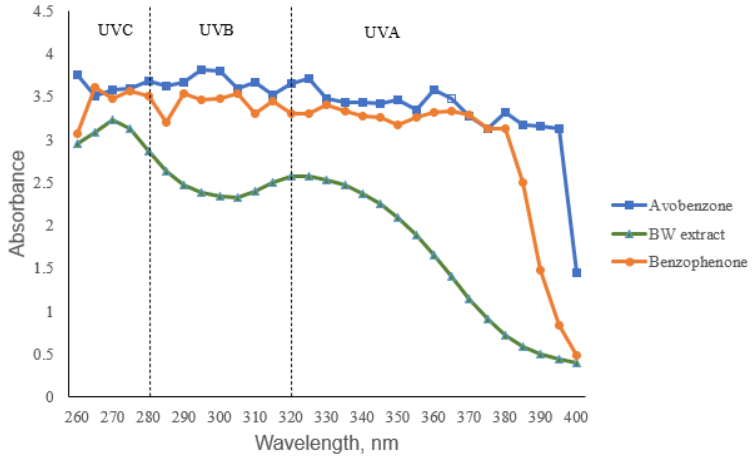

Figure 4. Spectrometric absorption profile of *P. bleo* leaves extract

NO.	WAVELENGTH (nm)	EE VALUE	SAMPLE EXTRACT	AVOBENZONE	BENZOPHENONE
1.	290	0.015	2.4786	3.6638	3.5411
2.	295	0.0817	2.3835	3.8153	3.4654
3.	300	0.2874	2.3404	3.8076	3.4809
4.	305	0.3278	2.3326	3.6041	3.534
5.	310	0.1864	2.4068	3.6774	3.3087
6.	315	0.0839	2.5051	3.5277	3.4444
7.	320	0.018	2.5695	3.6629	3.304
SUN PROTECTIVE FACTOR (SPF)		23.7422	36.8977		34.6029

Table 9. SPF value of *P. bleo* leaves extract

IV. DISCUSSION

Proximate analysis

The carbohydrate content of *P. bleo* leaves is the highest among all compositions, which aligns with same findings by López-Barraza et al. [17]. The protein content (20.26 ± 0.6%) is notably high, and studies showed that the *Pereskia* family contains a high level of protein compared to other vegetables like kale (1.6%) and beans (18 – 20%) [2]. However, most previous reports claim to obtain a low amount of protein in the plant [18,19].

Crude fat (3.55 ± 0.3%) shows in the number of steroids found in the leaf extract. Crude fiber content is the dietary fiber present in the leaves. The crude fat and crude fiber (11.13 ± 0.1%) values are consistent with previous reports on plant species in the *Pereskia* family. Ash content represents the amount of minerals or inorganic components present in the plant and acts as a cofactor in the metabolic process [11]. These minerals are also useful in improving health. The ash content (16.5 ± 0.7%) has similar value with *Pereskia aculeata* by de Castro Campos Pinto and Scio [2] which is 16.1 ± 0.1. However, rose cactus (*P. bleo*) mucilage that was reported by Farhanah et al. and Hong and Ibrahim, (2012) to give 28.1 ± 0.16% and 28.67 ± 0.05% respectively [18,19]. Meanwhile, the ash content result from another *Acanthopanax trifoliatus* was reported by Ganogpichayagrai and Suksaard to be 2.95 ± 0.04 [20]. This shows that the ash content for *P. bleo* leaves is at the average level and is still considered a decent amount.

Moisture content is sometimes taken as an estimation of the quality of the food. It also helps in the solubility of hydrophilic carbohydrates [19,20]. Moisture content is occasionally used to estimate the quality of food. High moisture content promotes the growth of microorganisms such as mould and fungi. The moisture content (9.36 ± 0.1%) is quite low compared to the *Acanthopanax trifoliatus* leaves sample which is 74.62 ± 0.38% by Ganogpichayagrai and Suksaard [20], and rose cactus (*P. bleo*) mucilage with 13.69 ± 0.45% by Hong and Ibrahim [19].

Toxicity evaluation

Mineral and heavy metal content

ICP-MS (inductively coupled plasma mass spectrometry) is commonly used to analyze minor or trace components in food. ICP-MS can determine multiple elements in a short time, low limit of detection and high precision. The analysis is used to assess the nutritional value of food and to assess the health hazards associated with a shortage or excess of vital nutrients in food [21]. The leaves of *P. bleo* are usually consumed as salad and concoction by locals, because of this it is wise to evaluate the minor elements and heavy metals that are present in the leaves of the plant [1].

Minerals are essential in human daily nutrition and help in metabolic functions, growth and formation of bones, the electrolytic balance of the body fluid, and the nervous system's functions. Mineral deficiency and aberrant mineral levels can cause body functional disturbance [22]. Minerals are divided into two: micronutrients and macronutrients. The essential elements known as micronutrients are essential for a variety of metabolic processes and are strongly related to human growth and general health. The lack of these metals will influence the human body system and may create physiological problems [23]. A tolerable upper intake level (UL) is the greatest level of chronic daily consumption of nutrients (from all sources) that is not expected to cause a risk of deleterious health effects to humans [24]. The UL for each mineral was extracted from the European Food Safety Authority (EFSA) panel on Dietetic Products, Nutrition, and Allergies (NDA).

The highest microelement in the leaves is iron metal (10.27 ± 0.24 mg/L) and it is an essential metal for metabolic function in the human body. The deficiency of this metal that acts as an enzymatic catalyst in most of the biochemical reactions will cause a decrease in the rate of metabolic function reaction. Iron is also required to produce haemoglobin. The iron obtained is lower than the recommended safety limits for consumption which is 80 to 110 mg/L and required to take at least 19.3 – 20.5 m for men and 17.0 – 18.9 daily for women. Copper metal acts as an antioxidant and is important in the production of haemoglobin, metabolism activity in connective tissue, growth of bone, and transportation of iron. It is advised to take 19.3 – 20.5 mg of for women and 17.0 – 18.9 mg of copper for men daily [25]. The current study showed that *P. bleo* leaves extract contains 46.61 µg/L, which is lower than the daily limit. Therefore, the leaf of *P. bleo* that contains iron is safe to consume by the locals.

Major minerals or known as macrominerals required by the body more than 100 mg in daily intake and represent 1% of the human body weight, unlike microminerals that require less than 100 mg daily. Macrominerals are essential in the production of cell tissues, enzymes in cellular and basal metabolism, and the electrolytic balance of the body fluid [26,27]. Potassium metal is the highest macromineral (62.56 ± 1.93 mg/L) in the leaves and it has been reported before that *P. bleo* produces two times more potassium compared to tomato which is known to be rich in potassium. A rich potassium diet was reported to be good in the reduction of blood pressure, and this may be the reason for consuming the leaves of *P. bleo* to treat hypertension traditionally [1]. Adult males need 3400 mg of potassium daily, while adult females require 2600 mg daily [25]. The result of potassium content is lower than the suggested daily intake for both male and female adults, and therefore it is within the safe limit. Calcium mineral helps in maintaining bone health and blood pressure. An adult requires about 2500 mg of calcium every day [25]. In the current study, calcium is the second highest micromineral (17.71 mg/L) detected in the leaf extract of *P. bleo*. The amount of the metal is below the limit of daily intake and therefore is not dangerous. All the minerals are below the tolerable upper intake level (UL) and therefore, *P. bleo* leaves are safe to consume.

Heavy metal poisoning in medicinal plants has been a concern for researchers. Contamination of the plant by heavy metals can happen from polluted irrigation and contaminated soil [28]. Metal poisoning is induced by a disruption in the cellular metabolic pathway. The heavy metal from contaminated food will be ionized in the acidic condition within the stomach and react with protein and enzymes that will lead to more health problems [29]. The heavy metals are not biodegradable and able to stay in the body for a long period due to long biological half-lives [28]. Heavy metal accumulation in the kidneys and livers will affect a range of biochemical processes, potentially leading to cardiovascular, neurological, renal, and bone disease [29]. The level of all heavy metals detected in the extract are low concentration and below the SRL. Therefore, *P. bleo* leaves are safe to consume daily.

Brine shrimp lethality bioassay

Traditionally *P. bleo* leaves are used to maintain a healthy diet and as a tonic to re-energise the body. Therefore, primary toxicity screening on *P. bleo* leaves extract was conducted through brine shrimp lethality bioassay. This experiment will determine the safety of the extract for internal or external usage. *In vivo* brine

shrimp lethality bioassay is well known to be the simplest test to evaluate the toxicity of plant extracts and requires a short time and low cost. The bioassay will also assist in detecting fungal toxins and, the presence of heavy metals and pesticides [12]. From the results, it showed that *P. bleo* leaf extract did not cause any toxicity on the brine shrimp. Sim et al. reported that *P. bleo* leaves methanolic extract did not show any cytotoxicity against MRC-5 cells [30]. However, few studies reported that methanolic extract showed significant cytotoxic activity against breast carcinoma cells (T47-D cell line) and KB cells but no cytotoxic activity on normal cells [2].

Cosmeceutical properties of *P. bleo* leaves extract

Anti-collagenase activity

Anti-collagenase kit is an analysis that helps to investigate the ability of *P. bleo* leaf extract to inhibit collagenase activity. BioVission's collagenase activity assay kit measures the collagenase activity using synthetic peptides (FALGPA) that resemble the structure of collagen. The *in vitro* anti-collagenase activity assay was done by using 1,10-Phenanthroline as an inhibitor and negative control. From the result of 1 mg/mL of *P. bleo* leaves extract is capable of inhibiting the collagenase just as much as kojic acid (77.19 ± 0.01%). This showed that *P. bleo* leaves extract has the potential as a good source of anti-aging agent. This is due to polyphenol and flavonoid compounds present in the extract.

Plant extracts have been proven multiple times to be a good source of collagenase inhibitors. The presence of flavonoid compounds and phenolic compounds is reported to be excellent anti-collagenase inhibitors [31]. Azahar et al. reported that an optimized mixture of Melastomataceae leaf extract gave good collagenase inhibition activity against premature skin aging due to the presence of bioactive compounds that decrease the activity of collagenase enzyme and act as natural collagenase inhibitor agents [32]. Shukri et al. reported that leaves extract of *Persicaria odorata* contains flavonoid compounds like rutin and (-)-epigallocatechin 3-gallate that are excellent antioxidant compounds and act as a good anti-collagenase inhibitor [33]. This is because of the presence of metal chelators from the leaf extract that bonded to the active site of the enzyme and inhibit the substrate (FALGPA) from breaking down by the enzyme collagenase.

The anti-collagenase activity of *P. bleo* leaves extract is notable, with a high inhibition rate (80.14 ± 0.01%) at 10 mg/mL, indicating its potential as an anti-aging agent. The flavonoid and polyphenol compounds present in the extract are likely responsible for this activity, as they are known to inhibit collagenase, a key enzyme involved in skin aging.

Anti-tyrosinase activity

P. bleo leaves extract demonstrated significant tyrosinase inhibition (74.04 ± 0.03%) at a concentration of 1 mg/mL comparing to tyrosinase inhibition of kojic acid as reference (76.4 ± 0.01%), suggesting its potential as a skin-lightening agent. Kojic acid is well known to be a strong tyrosinase inhibitor and is always used as an ingredient in skin-lightening products to reduce hyperpigmentation [34]. From the result, it showed that *P. bleo* leaf extract has the potential to be a tyrosinase inhibitor.

Plant extracts have been proven multiple times to be a good source of tyrosinase inhibitors. It is reported by Azmi et al. that peptides compound inhibits tyrosinase activity and decompose pigmentation resulting in lighter skin [35]. Younis et al. reported that *Stenocarpus sinuatus* leaf extract contains a high amount of vitamin E. Vitamin E is known to be a good antioxidant compound and is reported to be able to reduce pigment appearance [36]. *P. bleo* leaves extract was reported by Abd Malek et al. to contain vitamin E which may be the reason for good tyrosinase inhibition results. Rutin and (-)- epigallocatechin 3-gallate flavonoid compounds may also be responsible for the inhibition activity [37].

Anti-elastase activity

Anti-elastase inhibition activity was conducted by using a neutrophil elastase colorimetry drug discovery kit where elastatinal is used as an inhibitor and negative control. Elastatinal will break down neutrophil elastase enzyme and *P. bleo* leaves extract was used as an inhibitor to prevent the decomposition of enzyme from happening. The moderate elastase inhibition (48.03 ± 0.01%) observed in *P. bleo* leaves extract and compared to inhibition activity in positive control ascorbic acid (40.66 ± 0.01%) that indicates its potential as a skin care ingredient. Azahar et al. reported that the inhibition activity of *Melastoma* sp. extract is 70.78% and Vijayakumar et al. stated that red pitaya peel extract gave 87.62% of elastase inhibition activity with the same kit [32,38].ability to protect skin from elastase, an enzyme that contributes to skin aging.

Sun protective inhibitor (SPF)

The *in vitro* SPF analysis was done on *P. bleo* leaf extract by using a method from Dutra et al. [15]. The sunscreen activity was observed by recording the transmission spectrum of the ethanol solution *P. bleo* leaves extract in the range of 260 to 400 nm and SPF calculations are based on the absorbance derived from the spectrum. Figure 4 shows that *P. bleo* leaves extract has good potential as a photoprotector, and it can be observed that the maximum absorption wavelength for the extract in UVC and UVB are 270 nm and 280 nm respectively. This demonstrates that the leaf extract has a high ability to prevent sunburn caused by UVB radiation [15]. UVB is more energetic compared to UVA and the UV light at wavelength 280 nm caused more damage compared to the light at 340 nm so the capacity of sunscreen to block UVB rays is more essential in preventing the harmful consequences of sun exposure [39,40].

The SPF value of *P. bleo* leaves extract (23.74) exceeds the minimum recommendation of SPF-15 set by the FDA, indicating the risk of harmful effects on the skin can be reduced. It was reported that plants that are rich in flavonoids will be a good sunscreen. This is because flavonoid compounds are good at absorbing ultraviolet radiation [41]. The cyclic and aromatic ring structure of the flavonoid compounds will absorb the radiation specifically in the wavelength range between 240 nm – 285 nm and 300 nm – 550 nm [42]. It has been reported that *P. bleo* leaves extract contains a high number of flavonoid compounds so these compounds may be the reason for the high SPF value of the extracts [43]. The result from the chemical compound profile proved that *P. bleo* leaves extract contains a high number of flavonoid compounds like rutin and (-)- epigallocatechin 3-gallate which may be the reason for good cosmeceutical analysis results.

V. CONCLUSION

P. bleo leaves have been consumed raw to maintain a healthy diet or drink as a concoction to detox the body. The toxicity of the leaf extract was analyzed using ICP-MS and *in vivo* brine shrimp lethality bioassay. The result from ICP-MS showed that the level of minerals present in the leaves are all safe level and the heavy metals present are below the specific release limits adopted by the Food and Agriculture Organisation (FAO), (1995), World Health Organisation (WHO), (2011) and European Food Safety Authority (EFSA), (2015). Therefore, the leaves are safe to consume as salad and tonic by locals. The ability of *P. bleo* leaf extract in cosmeceutical properties was investigated by using optimized leaf extract to inhibit the collagenase, tyrosinase, and elastase activities. From the analyses, it was observed that the leaf extract is a good collagenase and tyrosinase inhibitor with 80.14% and 74.04% inhibition activity but only showed 48.03% activity for elastase inhibition activity. The photoprotective analysis was also conducted on the *P. bleo* leaves extract. The SPF value of the extract is 23.74 which is higher than the suggested SPF value recommended by FDA. Therefore, *P. bleo* leaf extract has a high potential as a valuable cosmetic ingredient.

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