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EVALUATION OF THE FREE RADICAL SCAVENGING ACTIVITIES OF ETHANOL EXTRACTS OF *ANNONA MURICATA* (SOURSOP) LEAVES AND BARK FOR COSMETIC EMULSIONS

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Plant extracts offering skin health benefits such as antioxidant and photoprotective properties are increasingly used in cosmetic formulations. The antioxidant properties of ethanol extracts of *Annona muricata* (soursop) leaves and bark were assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. The leaf extract yielded 448 µg GAE/mL (micrograms of gallic acid equivalent per milliliter) total phenolic content, 119 µg QE/mL (micrograms of quercetin equivalent per milliliter) total flavonoid content and 101 µg TAE/mL (micrograms of tannic acid equivalent per milliliter) total tannin content, while the bark extract yielded 575 µg GAE/mL total phenolic content, 24.2 µg QE/mL total flavonoid content and 126 µg TAE/mL total tannin content. The 50% inhibitory concentration (IC_{50}) values reveal the free radical scavenging properties of the samples as follows: ascorbic acid (104 µg/mL) > bark extract (128 µg/mL) > leaf extract (198 µg/mL), showing that ascorbic acid, a commercial antioxidant, exhibited the most effective free radical scavenging property. The free radical scavenging abilities of these extracts may be attributed to their rich phytochemical contents. Cosmetic emulsions formulated with the plant extracts were oil-in-water emulsions stable for 60 days at room temperature (30 °C), in an incubator (40 °C) and in a refrigerator (7 °C). The results showed that *A. muricata* leaf and bark extracts could potentially be applied as natural free radical scavenging ingredients in antioxidant and anti-aging cosmetic formulations for photoprotective skincare through ultraviolet (UV) radiation shielding.

Keywords: plant extracts, antioxidant, free radical scavenging, cosmetic emulsions, photoprotective skincare

1. Introduction

The high market value for cosmeceuticals based on natural or organic products has necessitated intensive research to support the production of green cosmetic products in the industry. Natural products provide an enduring, fascinating and untapped source for the development of new skincare ingredients [1]. Plant extracts have been optimized as active ingredients for many decades in the pharmaceutical, nutraceutical and cosmeceutical industries. Plant extracts are one of the main sources of active ingredients in these industries. Several review and research articles have addressed the growing interest in cosmeceuticals formulated from plant extracts with bioactive properties [1]-[7]. These extracts are obtained from whole plants or their vegetative parts such as fruit, leaves, roots, bark, stems, branches, seeds or flowers and may contain plant chemical compounds such as phenols, flavonoids, tannins, saponins, terpenoids, alkaloids and vitamins that promote biological effects [5],[8]. These plant extracts have become functional ingredients in cosmeceuticals that facilitate moisturization, skin repair as well as antioxidant, antimicrobial, anti-inflammatory, photoprotective, anti-aging, wound-healing and hyperpigmentation inhibition properties of cosmetic formulations. They are also active free radical scavengers and can improve skin tone, texture and appearance [8].

Sources of reasonable aetiology of imbalance in the prooxidant/antioxidant system – which ultimately leads to skin coloration and aging through the buildup of oxidative stress in the skin - are ultraviolet (UV) radiation, visible light as well as the degradation of natural resources, including ozone and particles [9]. These compounds trigger aging by facilitating the synthesis of reactive oxygen/nitrogen species (ROS/RNS). Practices and often the inevitable exposure of skin to UV radiation have both useful and harmful effects on human health. The skin absorbs vitamin D when it is irradiated with ultraviolet B (UVB) for a short period of time, which plays an important role in maintaining the calcium balance in the body [10]. Excessive exposure of the skin to sunlight and UV

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radiation is harmful due to the production of free radicals or direct photochemical changes of various skin biomolecules [11]. However, human cells contain innate protective agents which fight against the harmful effects of these reactive species produced by sunlight, but the protective effect of these internal antioxidants is limited when very high amounts of the reactive oxygen species are present [11].

The failure of such protective agents in the human body to effectively protect against high levels of ROS/RNS necessitates cosmeceuticals that contain plant-based chemicals capable of enhancing the protective activities of antioxidants in the body. The efficacy of potent constituents in cosmeceuticals that can elicit the important role of enhancing antioxidants in the body is due to the bioactivities of the chemical entities in these substances. This has led to an upsurge in research activities to support the discovery of natural products with antioxidant properties [8],[12]. The most prominent non-enzymatic antioxidant in human skin is L-ascorbic acid, also commonly known as vitamin C. Findings have shown that many plants such as *Annona muricata,* also known as soursop, are organic sources of this antioxidant [2],[6],[13].

Annona muricata is a member of the family *Annonaceae* and is the tropical deciduous tree that produces the biggest fruit of the Annona genus. It is widespread in North America, Europe, Asia and Africa [14]. The leaves, bark, roots, fruit and seeds of soursop are used by natives to treat several conditions, including hypertension, inflammation, hyperglycemia, gastrointestinal disorders, respiratory diseases and carcinoma [15]. They contain plant chemicals such as tannins, saponins, terpenoids, alkaloids, glycosides and phenols, which are particularly effective against microorganisms and exhibit excellent antioxidant activities [14],[15]. Soursop is also rich in phenolic and flavonoid moieties exhibiting great antioxidant activity [16]-[18]. Although *Annona muricata* leaves and bark contain a variety of bioactive substances, they have not yet been fully optimized in the field of cosmetics [19]. This research, therefore, assesses the usefulness of *Annona muricata* leaf and bark extracts as potent constituents in cosmeceutical formulations, impacting the skincare industry in the following ways: i) providing a deeper insight into the potential of *Annona muricata* leaf and bark extracts as active ingredients in skincare formulations, ii) demonstrating the free radical scavenging properties of *Annona muricata* leaf and bark extracts, and iii) developing organic cosmeceuticals to help retard aging related to oxidative stress in society.

2. Experimental

2.1. Sample collection and identification

Annona muricata leaves and bark were obtained from Onna, a Local Government Area in Akwa Ibom State in Nigeria, preserved in polyethylene bags and labelled before being transported to the laboratory for identification and preparation. The samples were identified and a voucher specimen deposited in the herbarium catalogued with the voucher number AKSUH/A0038.

2.2. Extraction of Annona muricata (soursop) leaf and bark samples

The *Annona muricata* leaves and bark were washed with water, sliced into tiny pieces and air dried for 14 days at room temperature (30 °C). The air-dried samples were ground using a fabricated manual blender to obtain coarsely powdered samples which were weighed using a top loading balance, placed in big glass jars and macerated with absolute ethanol (99.9 %) for 72 hours at 30 °C while being stirred every 4 hours using a glass rod. The extracts were filtered using cotton wool stuffed in a plastic funnel and the filtrate concentrated in a rotary evaporator at 35 °C under a pressure of 10 kPa before completely removing the solvent via evaporation in a water bath at 40 °C. The percentage yield (%) of crude ethanol extract was calculated based on the mass of the dry samples using the following equation:

$$Yield\ (\%) = \frac{A1}{A2} \times 100 \qquad (1),$$

where *A1* denotes the mass of ethanol extract (g) and *A2* stands for the mass of the dry coarse powdered sample (g).

2.3. Quantitative determination of the phytochemicals

2.3.1. Determination of the total phenolic content

The total phenolic content (TPC) of the extracts was calculated using Folin-Ciocalteu's phenol reagent (Sigma-Aldrich) [20]. 10 mg of each of the plant extracts was dissolved in 10 mL of methanol (AnalaR, 99.9 %) to produce a 1 mg/mL solution. 0.1 mL of the 1mg/mL extract was added to 0.5 mL (1/10 dilution with water) of Folin-Ciocalteu's phenol reagent and 1 mL of distilled water in a vial. The solution was homogenized and incubated at 25 °C for 1 minute before adding 1.5 mL of 20 % sodium carbonate (Na_2CO_3). The mixture obtained was shaken vigorously and incubated in darkness for 120 minutes at room temperature (30 °C). The absorbance of the reaction mixture was determined at 760 nm by a UV-2500 Spectro UV-Vis Spectrophotometer (EMCLAB Instruments, Duisburg, Germany) using distilled water as the blank. A set of reference standard solutions of gallic acid (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 µg/mL) was prepared and a standard curve plotted. The absorbance of each sample was assessed relative to the standard curve plotted from gallic acid solutions. The total phenolic content was determined from the linear regression equation of the gallic acid calibration curve in micrograms of gallic acid equivalent per milliliter (µg of GAE/mL) of extract.

2.3.2. Determination of the total flavonoid content

The total flavonoid content (TFC) of the extracts was calculated based on a colorimetric approach [21]. 0.5 mL of the 1 mg/mL extract and 2 mL of distilled water were added to a vial and mixed briskly to achieve homogeneity. 0.15 mL of 5% (w/v) sodium nitrite solution was added to the bottle and the reaction mixture incubated at ambient temperature for 6 minutes. Then 0.15 mL of 10% (w/v) $AlCl_3$ was added to the resulting mixture and the mixture incubated for another 6 minutes before adding 2 mL of 1M NaOH. The volume of the reaction mixture was topped up to 5 mL by adding 0.2 mL of distilled water before homogenizing and incubating the reaction medium for 15 minutes. The absorbance of the reaction mixture was determined at 510 nm by a UV-2500 Spectro UV-Vis Spectrophotometer (EMCLAB Instruments, Duisburg, Germany) using distilled water as the control. A set of reference standard solutions of quercetin (10, 100, 200, 300, 400 and 500 µg/mL) was made and a standard curve generated. The absorbance of each sample was assessed relative to the standard curve generated from quercetin. The total flavonoid content of the samples was determined from the linear regression equation of the quercetin calibration curve in micrograms of quercetin equivalent per millimeter (µg of QE/mL) of extract.

2.3.3. Determination of the total tannin content

The total tannin content (TTC) of the extracts was calculated using Folin-Ciocalteu's phenol reagent [22]. 0.1 mL of 1 mg/mL extract was added to a 10 mL volumetric flask containing 7.5 mL of distilled water and 0.5 mL of Folin-Ciocalteu's phenol reagent. 1 mL of 35% Na_2CO_3 solution was added to the flask and the volume of the mixture topped up to 10 mL with distilled water. The mixture was mixed forcefully and incubated at 25 °C for 30 mins. A set of analytical standard solutions of tannic acid (50, 100, 150, 200 and 250 µg/mL) accordingly was made following the aforementioned procedure. The absorbance of the examined and standard solutions were determined against the control at 725 nm by a UV-2500 Spectro UV-Vis Spectrophotometer (EMCLAB Instruments, Duisburg, Germany) with distilled water employed as the control. The tannin content was obtained from the linear regression equation of the tannic acid calibration curve presented in microgram of tannic acid equivalent per milliliter (µg of TAE/mL) of extract.

2.4. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The antioxidant activity of the extracts was determined by carrying out a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay [23]. A fresh solution of DPPH (0.004 % w/v) was prepared in methanol (purple color). In the same way, a 1 mg/mL stock solution of the raw extract was dissolved in methanol. 1.0, 0.8, 0.6, 0.4 and 0.2 mL of the stock solution was diluted with methanol up to a final volume of 1 mL to obtain concentrations of 1000, 800, 600, 400 and 200 µg/mL, respectively. 1 mL of the freshly primed 0.004 % (w/v) DPPH solution was added to each vial containing different concentrations of the extract, mixed thoroughly and incubated for 30 mins at ambient temperature in the absence of light. The transformation of the purple color of DPPH into yellow was measured by determining the absorbance at 517 nm using a UV-2500 Spectro UV-Vis Spectrophotometer (EMCLAB Instruments, Duisburg, Germany). The free radical scavenging activity of the extract was determined by the following equation:

$$DPPH\ scavenging\ effect\ (\%) = \\ = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad (2),$$

where A denotes the absorbance at 517 nm.

The free radical scavenger known commercially as ascorbic acid of the same amount as well as serial dilutions were made and applied for comparative purposes or as a positive reference. The DPPH solution without the extracts was employed as the control and methanol utilized as the blank. An inhibitory concentration of 50 % (IC_{50}) was obtained from the graph of the DPPH scavenging activity (%) of ascorbic acid and the extracts against the amount of the samples (µg/mL). The IC_{50} reading refers to the concentration (µg/mL) of the extract/standard that causes the initial concentration of DPPH radicals to halve. Lower IC_{50} values are indicative of a greater antioxidant effect.

2.5. Formulation of cream with Annona muricata (soursop) leaf and bark extracts

The creams were primed based on a technique in the literature with slight modifications [24]. The compositions of the cream vehicle and the improved creams (cream containing the plants extract) are presented in *Table 1* while a schematic diagram detailing the preparation of the emulsions displayed in *Figure 1*. Since Formulation 1 (FL1) exhibited the characteristics of a cream while Formulation 2 (FL2) those of a lotion, FL1 was selected as the most suitable base for the improved creams.

The oil phase was made by placing all its constituents (*Table 1*) into a clean beaker and heating it to a temperature of 75 °C. In the same way, the constituents of the aqueous phase (*Table 1*), except the extract and fragrance, were mixed in a different beaker and heated to a temperature of 75 °C. The oil phase was gradually added to the water phase while being thoroughly mixed by a hand mixer until a temperature of 35°C was attained. Now the extract was added while constantly being stirring by an electric blender to ensure homogeneity. Eventually, two drops of fragrance oil (0.1 mL) were added and the preparation mixed briskly to obtain the final product which was cooled to room temperature while constantly stirred. The prepared cream formulations were stored at room temperature in air-sealed plastic containers in the absence of light.

Table 1: Ingredients for the formulation of the cream base and modified creams

	Formulation of cream (% weight)				
	Cream bases		Modified creams		
	FL1	FL2	BC	LC	BLC
Oil-phase (ingredients)					
Paraffin oil	4.0	2.0	4.0	4.0	4.0
Petroleum jelly	2.0	4.0	2.0	2.0	2.0
Emulsifying wax	7.0	6.0	7.0	7.0	7.0
Stearic acid	2.0	2.5	2.0	2.0	2.0
Cetyl alcohol	2.0	2.5	2.0	2.0	2.0
Aqueous-phase (ingredients)					
Glycerin	2.0	2.5	2.0	2.0	2.0
Perfume	0.1	0.1	0.1	0.1	0.1
Preservative (Sodium Benzoate)	0.5	0.5	0.5	0.5	0.5
Soursop extract	-	-	1.0	1.0	0.5/0.5
Deionized water	80.5	80.0	79.5	79.5	79.5

BC = Bark extract cream, LC = Leaf extract cream and BLC = Bark and leaf extract cream

Table 2: Percentage yield and color of the extracts

Extract	Yield (%)	Physical appearance
Leaf	12.5	Green
Bark	5.31	Brown

2.6. Characterization of the formulated cream bases and modified creams

2.6.1. Physical characteristics

Physical features (color and phase separation) were observed and visually assessed after 60 days.

2.6.2. Determination of pH

The pH of the prepared creams was measured soon after their formulation and at ambient temperature. 2 g of the cream was weighed into a beaker before being diluted with 18 mL of distilled water, mixed thoroughly and a pH electrode directly placed into the solution to measure the pH at 30 °C. The pH measurements of each cream were repeated three times.

2.6.3. Types of emulsions

A drop dilution method was employed to evaluate the emulsion type [25]. 1 g of emulsion was placed in a test tube and diluted with 10 mL of distilled water. An emulsion that cannot mix with water verifies it is of type W/O, and vice versa.

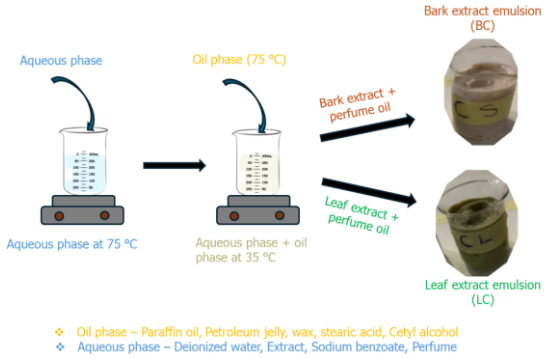

Figure 1: Schematic diagram of the preparation of the emulsions

2.6.4. Stability tests

Stability tests on the cream formulations were carried out under various conditions to examine their impact on the consistency of the emulsions [26]. These examinations were carried out on the creams stored at 7 °C in a refrigerator, 30 °C in the laboratory and 40 °C in an incubator. The physicochemical properties (color, creaming, pH and emulsion type) of the creams kept at 7, 30 and 40 °C over 60 days were evaluated at different intervals.

3. Results and discussion

3.1. Yield and physical appearance of ethanol extracts of A. muricata

The yield and physical appearance of ethanol extracts of *A. muricata* leaves and bark are shown in *Table 2*. The ethanol extracts of *A. muricata* leaves and bark produced yields of 12.48 and 5.31 %, respectively. The leaf extract was green while the color of the bark extract was in accordance with the color of the samples and the major phytochemicals found in them.

3.2. Phytochemical composition of the extracts

Total phenolic, flavonoid and tannin contents of the extracts were determined using the regression equation of the calibration lines of gallic acid, quercetin and tannic acid (*Figures 2a-2c*) in that order. Their values are presented in *Table* 3.

Although the results in *Table 3* indicate that the concentration of phenolics and tannins in the bark extract is greater than in the leaf extract, the flavonoid content of the leaf extract was greater. These outcomes are in line with those reported in another study in which the phenolic content of leaf samples was found to be greater than the flavonoid content [27]. The elevated phenolic,

Table 3: Total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC) of *Annona muricata* leaf and bark extracts

Extract	TPC (µg GAE/mL) ± S.D.	TFC (µg QE/mL) ± S.D.	TTC (µg TAE/mL) ± S.D.
Leaf	448 ± 3	119 ± 3	101 ± 1
Bark	575 ± 4	24.2 ± 2.5	126 ± 1

S.D. stands for the standard deviation

Table 4: IC_{50} values of ascorbic acid as well as ethanol extracts of *A. muricata* leaves and bark

Samples	IC_{50} value (µg/mL)
Ascorbic acid	104
A. muricata leaf extract	198
A. muricata bark extract	128

flavonoid and tannin contents of the leaf and bark extracts of *Annona muricata* show that the leaf and bark extracts are comprised of bioactive plant compounds, which are promising reservoirs of various kinds of medicinal bioactive substances [28]. When integrated into cosmeceuticals, these bioactive plant compounds may exhibit antioxidative, antimicrobial and anti-inflammatory effects, rendering them suitable substitutes for synthetic compounds found in cosmetics. Phenolic compounds are valuable components in the cosmetics industry due to their antioxidant, antimicrobial and anti-aging properties as well as soothing agents [29]. Since flavonoids are notable for antioxidant and soothing properties, they can be applied in topical formulations to protect the skin [30]. Given that free radicals facilitate premature aging and skin trauma, plant extracts with antioxidant properties may be able to shield the skin from the effects of these free radicals [29]. The topical application of antioxidative bioactive preparations can protect the innate antioxidant framework of the skin protecting it against oxidative stress and sun-induced aging in the long term.

3.3. DPPH free radical scavenging assay of A. muricata leaves and bark

The DPPH free radical scavenging activity and IC_{50} values of ethanol extracts of *A. muricata* leaves and bark in comparison with ascorbic acid are presented in *Figure 3* and *Table 4*, respectively, suggesting a notable antioxidative effect.

Ascorbic acid produced the most effective antioxidant activity with an IC_{50} value of 104 µg/mL, followed by the *A. muricata* bark extract with 128 µg/mL and the leaf extract with 198 µg/mL. A lower IC_{50} value suggests a greater ability to scavenge free radicals. The

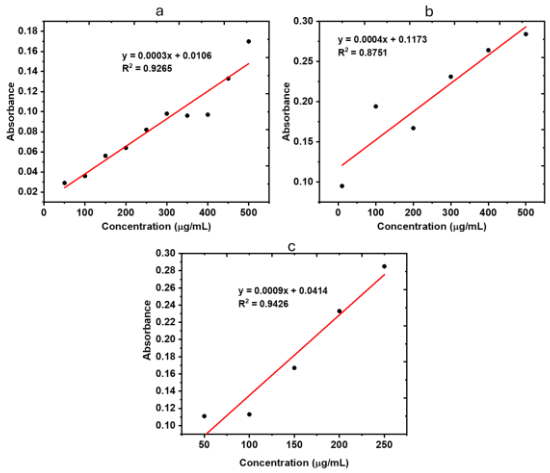

Figure 2: Calibration lines of a) Gallic acid, b) Quercetin and c) Tannic acid

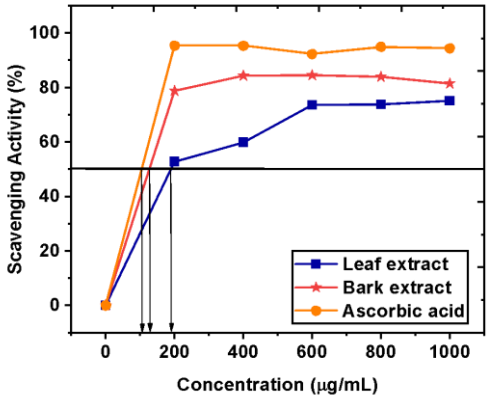

Figure 3: DPPH free radical scavenging activities of ascorbic acid as well as ethanol extracts of *A. muricata* leaves and bark

antioxidant potencies of these extracts could be credited to the availability of bioactive plant compounds (phenolics and flavonoids) in the extracts that are capable of suppressing free radicals that can damage the skin. Tannin, one of the bioactive plant compounds found in the extract, is said to tighten skin as well as prevent infections, resulting in healthy skin [31]. The phytochemical composition of these extracts shows that the free radicals in these extracts are associated with their phenolic and tannin content, where their incorporation into cosmeceutical preparations may facilitate the scavenging of free radicals on the skin, thereby mitigating the impact of sun-induced aging and the consequent emergence of wrinkles.

3.4. Physicochemical properties of the creams

All the prepared emulsions were identified to be of the oil-in-water type following a dilution test, where they were dispersed in water, and were consistent for a period

Table 5: Physicochemical properties of the freshly produced creams and after 60 days

Samples	pH		Color		Type of emulsion	Stability
	Initial	Day 60	Initial	Day 60		
F1 Cream base	5.20	5.20	White	White	o/w emulsion	Stable
F2 Cream base	5.30	5.30	White	White	o/w emulsion	Stable
CL Cream (Cream with Leaf extract)	5.40	5.30	Dark green	Dark green	o/w emulsion	Stable
CS Cream (Cream with Bark extract)	6.00	5.90	Light brown	Light brown	o/w emulsion	Stable
CLS Cream (Cream with both extracts)	5.30	5.30	Dark brown	Dark brown	o/w emulsion	Stable

o/w = oil-in-water

of 60 days as shown by the physicochemical features of the emulsions in *Table 5*. This suggests that water is the continuous phase while oil is the dispersed phase, in accordance with other findings [32]. The pH of the emulsions ranged from 5.20 to 6.00, the highest pH was recorded for the emulsion prepared with the leaf extract. The physical appearance, e.g. color, odor and texture, of the emulsions did not change over 60 days at the modulating temperatures (30 °C room; 40 °C incubator and 7 °C refrigerator - only the results at room temperature are presented in *Table 5*), confirming that the emulsions were consistent. Oil-in-water (o/w) emulsions are frequently used in cosmetic carrier systems because they moisturize and enhance the skin condition by forming a protective layer on it. The pH of 5.02 - 6.00 shows that both the creams with and without the extracts are dermatologically safe since this is within allowed limits for use on skin [2], [6].

4. Conclusions

Ethanol extracts of the leaves as well as bark of *Annona muricata* were prepared and used as ingredients for the preparation of oil-in-water emulsions to assess their potential as active ingredients in cosmeceutical formulations. The results obtained revealed that the extracts contain variable concentrations of phytochemicals such as phenolics, flavonoids and tannins, that is, the bark extract contained higher concentrations of phenolics and tannins, while the leaf one contained a higher concentration of flavonoids. The free radical scavenging activity of the extracts revealed that the bark extract was more active than the leaf equivalent, confirming a correlation between the scavenging activity and phenolic as well as tannin contents of the extracts. Furthermore, the extracts stabilized oil-in-water emulsions for 60 days at various temperatures. The results obtained from this study revealed that these extracts can be used as active ingredients in photoprotective personal care products to counter the effect of ultraviolet (UV) light on the skin. Antioxidants protect cells against the damaging effects of reactive oxygen species, otherwise known as free radicals, which cause oxidative stress leading to cellular damage.

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