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Moringa oleifera Seed Oil Formulation Physical Stability and Chemical Constituents for Enhancing Skin Hydration and Antioxidant Activity

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Abstract: *Moringa oleifera* seed oil has been recognized for its benefits in relation to the skin. The objective of this study was to evaluate the chemical composition and antioxidant activity of moringa seed oil, to formulate a moringa seed oil cream, and to determine the efficacy of moringa seed oil cream in vivo. The chemical components of moringa seed oil were analyzed by high-performance liquid chromatography and gas chromatography. The antioxidant activity of the oil was determined by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay. An oil-in-water cream containing moringa seed oil was developed and characterized for antioxidant activity. The moringa seed oil cream was further subjected to the accelerated stability test of heating–cooling cycles for six cycles and stored isothermally at 4, 30, and 45 °C for 28 days. The efficacy of moringa seed oil cream was investigated in 32 participants by measuring their skin hydration, erythema, melanin values, and visco-elasticity. The results showed that moringa seed oil contained α-tocopherol, plant sterols, and fatty acids. The oil had antioxidant activity with a 50% of initial concentration (IC_{50}) value of 121.9 mg/mL. The stability study indicated that the pH, viscosity, and rheological behavior of the cream containing moringa seed oil were not significantly changed after storage at 4, 30, and 45 °C for 28 days and six heating–cooling cycles. The moringa seed oil cream exhibited in vitro antioxidant activity and increased the in vivo skin hydration level compared with the cream base. There was no report of skin irritation from moringa seed oil cream application, suggesting that the moringa seed oil cream developed in this study was appropriate for pharmaceutical and cosmetic uses. A *M. oleifera* seed oil cream was successfully developed. The moringa seed oil cream possessed antioxidant activity, enhanced the skin hydration level, and reduced skin erythema, but did not affect the melanin content and skin visco-elasticity. The moringa seed oil cream did not induce skin irritation and, thus, was safe to use.

Keywords: *Drumstick tree*; free-radical scavenging; patch test; gas chromatography; high-performance liquid chromatography

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1. Introduction

Ultraviolet radiation, air pollutants, psychological stress, alcohol assumption, smoking, and chemical exposure are capable of inducing free radicals and reactive oxygen

species on the skin. Free radicals are defined as atomic, molecular, or ionic species containing an unpaired valence electron in an atomic orbital, resulting in the highly chemical reactive properties of free radicals. Free radicals are highly unstable and have electrons available to react with various biological substances, including lipid molecules, proteins, and DNA, causing cell damage and homeostatic disruption [1]. An excess of free radicals generates oxidative stress and damages cell membrane and lipoproteins through lipid oxidation processes.

Skin has endogenous antioxidants, such as glutathione, melanin, and enzymatic antioxidants [2]. However, the excess formation of free radicals requires exogenous antioxidant topical application in preventing oxidative stress and enhancing DNA repair. Several studies have shown that the oxidation could be prevented by prior antioxidant treatment. Antioxidants protect the skin by reducing free-radical production. Scavenging free radicals by antioxidants can prevent skin aging. Antioxidants also have anti-inflammatory properties in preventing sunburn and protecting the skin from sun damage and photoaging. By reducing inflammation, antioxidants stimulate skin repair and correct skin damage. Free radicals can trigger the skin's melanin production, causing skin color changes. Antioxidants prevent skin pigment generation by reducing photodamage. In addition, some antioxidants were shown to increase skin hydration to revitalize the skin [3].

Natural oils containing unsaturated fatty acids are widely used as natural antioxidants and moisturizers to prevent skin dryness and aging. However, the topical delivery of natural oil requires a formulation acting as an oil carrier and diluent to minimize the skin irritation caused by direct contact with the oils. Therefore, further research regarding natural oil formulation and its safety and efficacy for prevention and treatment of skin diseases is required.

Moringa oleifera seed oil has a light yellow color with a mild nutty odor. Research suggested that *M. oleifera* seed oil possesses a skin protecting effect. *M. oleifera* seed oil was suggested to maintain the natural skin pigmentation as it possesses a mild sun protective activity [4]. The anti-fungal activity of *M. oleifera* seed oil has been reported [5]. *M. oleifera* see oil has been used to alleviate earache and prevent mosquito bites. In some African countries, moringa seed oil is used in soap formulation to improve the stability of the lather and the cleaning efficiency (4).

The benefits of *M. oleifera* seed oil for the skin have been widely recognized. However, the research supporting its use is insufficient. The antioxidant activities and effects of skin hydration, skin color, and skin visco-elasticity of *M. oleifera* seed oil formulations have not been investigated. In addition, there are very limited data regarding the safe and effective dose of *M. oleifera* seed oil incorporated in formulations. In this study, we characterized the antioxidant activity of moringa seed oil. The chemical compositions of moringa seed oil were analyzed to validate its biological activities. A cream containing *M. oleifera* seed oil was formulated. The physical stability and antioxidant activity of the cream were tested. The safety and efficacy of the formulation were also reported.

2. Materials

Purified (100%) cold-pressed *M. oleifera* seed oil originating from India was purchased from Neo Moringa, Thailand. The 2,2-diphenyl-1-picrylhydrazyl and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Cetyl alcohol, stearic acid, stearyl alcohol, polysorbate 80, propylene glycol, sorbitan ester 80, propyl paraben, methyl paraben, and α-tocopherol were obtained from the P.C. Drug Center (Bangkok, Thailand).

3. Methods

3.1. Characterization of M. oleifera Seed Oil

3.1.1. Determination of α-Tocopherol in *M. oleifera* Seed Oil

The amount of α-tocopherol in *M. oleifera* seed oil was determined using high-performance liquid chromatography (HPLC) [6]. *M. oleifera* seed oil (1 g) was mixed

with deionized water and ascorbic acid (1 g) was added as an antioxidant. Potassium hydroxide (3 mL) and ethanol (3 mL) were added, and the saponification was performed under reflux at 85 °C for 30 min. A hexane and ethyl acetate (8:2) mixture was added and vortexed. The upper hexane layer was collected, washed with deionized water to remove excess potassium hydroxide and evaporated to dryness.

The sample was then dissolved in 1 mL ethanol. The amount of α-tocopherol was determined using HPLC (Agilent 1200 Series, Santa Clara, CA, USA). The separation was achieved on Platinum (C_{18}), 5 µm, 250 × 4.6 mm. The injection volume was 20 µL. The column temperature was 40 °C. The chromatography was performed using an isocratic elution mode in which 100% methanol was used as a mobile phase. The flow rate and detection wavelength was kept constant at 1 mL/min and 290 nm, respectively.

3.1.2. Quantification of Plant Sterols in *M. oleifera* Seed Oil by Gas Chromatography

Quantification of the plant sterols in *M. oleifera* seed oil was performed using gas chromatography [7]. Stock solutions of brassicasterol, campesterol, and stigmasterol at 500 ppm were prepared by dissolving each compound at 0.5 mg/mL in n-hexane. Working solutions with concentrations of 10–200 ppm were prepared by diluting the stock solutions of brassicasterol, campesterol, and stigmasterol with n-hexane and were kept at −20 °C. The plant sterol standards were derivatized by mixing 9 mL trimethylsilyl (TMS) pyridine, 3 mL hexane, and 1 mL chloroform with 100 µL of the sample. The derivatives were dried by dry nitrogen and filtered before injection.

The samples were prepared by liquid extraction and saponification. Moringa seed oil was weighed at 2.5 g. The 5-α-cholestan 50 ppm internal standard (1 mL) and ethanolic potassium hydroxide (1 mL) were added and mixed for 1 min and heated at 60 °C for 30 min. Deionized water (5 mL) and chloroform (10 mL) were added to the sample and mixed for 2 min. The upper layer was collected. The extraction was repeated by adding hexane into the lower part and mixing for 2 min. The upper layer was collected and evaporated at 50 °C to dryness.

Derivatization of the plant sterols in the moringa seed oil sample was performed by the above-mentioned method. Gas chromatography (GC) analysis was carried out by using a 6850 Series GC System (Agilent Technologies, Santa Clara, CA, USA) and a chemically bonded fused silica capillary column of methylsiloxane (HP1, 30 m × 0.32 mm i.d., 0.25 µm film thickness). The inlet temperature was 270 °C, and the detector temperature was 200 °C (1 min) with an increment of 5 °C/min to the final temperature 290 °C (14 min), at a flow rate of 0.5 mL/min, and the total run time was 33 min.

3.1.3. Determination of the Fatty Acid Composition in Moringa Seed Oil

The analysis of the fatty acid composition in *M. oleifera* seed oil followed the Compendium of methods for food analysis [8]. Moringa seed oil (1.0 g) was weighed in an Erlenmeyer flask and mixed with 30 mL of hexane:acetone (4:1 v/v) vigorously for 1 min. The upper layer was then filtered through Whatman No. 1 filter paper containing sodium sulfate anhydrous. The extraction of fatty acid in moringa seed oil was repeated two more times. The solvent was rotary evaporated to dryness. The residue was weighed and dissolved in 5 mL of 0.5 N potassium hydroxide in methanol. Tricosanoic acid methyl ester (800 µg/mL, 1 mL), as an internal standard, was added.

The mixture was placed in a water bath shaker at 100 °C for 5 min. After the mixture was cooled, 14% borontrifluoride (BF3) in methanol (2 mL) was added. The mixture was then placed in a water bath shaker at 100 °C for 15 min. After adding saturated sodium chloride solution (10 mL) to the mixture, the supernatant was further extracted with petroleum ether (4 mL/time) until a clear solution was obtained. All extracts were collected and rotary evaporated under controlled temperature and pressure until dry. The mixture was mixed with n-heptane (3 mL) and filtered through a nylon syringe filter 13 mm diameter, 0.45 µm before analysis using Varian CP-3800 GC. Each fatty acid content was calculated by using a standard fatty acid methyl ester mixture.

3.1.4. Peroxide Value

The peroxide value of moringa seed oil was determined by the iodometric titration method 965.33 [9]. We weighed 5 g of moringa seed oil and added 30 mL of acetic acid and chloroform (3:2). Potassium iodide (0.5 mL) was added and left for 1 min before adding 30 mL of water. A starch solution (0.5 mL) was added, and the sample was then titrated with 0.01 N sodium thiosulfate.

3.1.5. Thiobarbituric Acid Reactive Substances (TBARS) Assay

The lipid peroxidation of *M. oleifera* seed oil was analyzed by the reaction with thiobarbituric acid (TBA) [10]. Ten grams of moringa seed oil were weighed and mixed with 50 mL of deionized water. Hydrochloric acid (4 M, 2.5 mL) was added into the mixture. The sample was boiled until 50 mL of total volume was reached. The boiled sample was pipetted (5 mL), mixed with 5 mL of TBA reagent, and immersed in a water bath at 100 °C for 35 min. A blank sample was prepared by replacing the sample with deionized water. The absorbance was measured at 538 nm using a UV-visible spectrophotometer.

3.1.6. Color Measurement

Moringa seed oil color assessment was carried out by a colorimetric measurement using a spectrophotometer (Spectraflash SF600 Plus by Data Color International, Lawrenceville, NJ, USA), and the L*, a*, b*, C, h*, and ΔE coordinates, indicating the lightness, red/green coordinate, yellow/blue coordinate, chroma, hue angle, and the change in visual perception of two given colors, respectively, were analyzed.

3.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) Free-Radical Scavenging Assay

The antioxidant activity of *M. oleifera* seed oil was determined in terms of hydrogen donating or radical scavenging ability using the DPPH method. Moringa seed oil was diluted into 86–860 mg/mL. Moringa seed oil (500 µL) was mixed with 500 µL of 500 µM DPPH in absolute ethanol and incubated in the dark at room temperature for 30 min. After incubation, the mixture was pipetted into a 96-well plate (200 µL/well). The absorbance was measured at 517 nm using a UV-visible microplate reader (Anthos Zenyth 340, Cambridge, UK), and the mean values were obtained from triplicate experiments. Gallic acid in absolute ethanol and vitamin E were used as positive controls, and absolute ethanol was used as a negative control. In addition, vitamin E at the same concentration range was prepared and tested with a DPPH assay using the same protocol. The percentage of radical scavenging activity was calculated by the following equation.

$$radical\ scavenging\ activity\ (\%) = \frac{\left[\left(A_{517\ control}\right) - \left(A_{517\ samples}\right)\right] \times 100}{\left(A_{517\ control}\right)}. \quad (1)$$

The percentage of radical scavenging activity was plotted against the sample concentrations. The concentration of *M. oleifera* seed oil that decreased the DPPH to 50% of initial concentration (IC_{50}) was calculated using GraphPad Prism 7.

3.3. Formulation of Cream Containing M. oleifera Oil

An oil-in-water cream base containing *M. oleifera* seed oil was prepared using the formulation shown in Table 1. To prepare the cream, stearic acid, stearyl alcohol, cetyl alcohol, and sorbitan ester 80 were heated to 70 °C and were then incorporated into the heated water phase (75 °C) containing propylene glycol, polysorbate 80, and deionized water. The cream base was homogenized for 3 min, and concentrated paraben was added. The pH of the cream base was adjusted by using triethanolamine. For cream containing *M. oleifera* seed oil, the cream base was cooled to 50 °C and 75 g of *M. oleifera* seed oil (25 g) was gradually added and mixed with the cream base under homogenization for 3 min. For the cream base, mineral oil (25 g) was mixed with the other ingredients as a control. Concentrated paraben and triethanolamine was added. The pH of the cream base was adjusted using triethanolamine.

Table 1. Formula of Cream Containing *Moringa oleifera* Seed Oil.

Phase	Components	%*w*/*w*	Function
Oil	Stearyl alcohol	4.00	Stiffening agent
	Cetyl alcohol	5.00	Stiffening agent
	Stearic acid	3.00	Stiffening agent
	M. oleifera seed oil	25.00	Active ingredient
	Sorbitan ester 80	0.60	Emulsifier
Water	Propylene glycol	5.00	Humectant
	Polysorbate 80	4.40	Emulsifier
	Concentrated parabens	1.02	Preservative
	Triethanolamine	0.10	pH adjusting agent
	Purified water	51.88	Vehicle

3.4. Evaluation of the Physical Characteristics and Stability Testing of Cream Containing M. oleifera Seed Oil

The color, odor, and homogeneity of cream containing *M. oleifera* seed oil were observed visually. The pH of the cream was measured using a Mettler Toledo Electrode. The viscosity of the cream was measured using a Brookfield viscometer (Model DV-III, Brokkfield, CA, USA). The physical stability of the *M. oleifera* oil cream was studied with the heating–cooling cycle method for six cycles. Samples of the cream containing *M. oleifera* oil were stored in glass containers for 24 h in a refrigerator and were placed in an incubator at 45 °C for another 24 h, accounting for 1 cycle [11].

The long-term stability of *M. oleifera* seed oil cream was investigated by storage of the cream at 4, 30, and 45 °C for 28 days [12]. The appearance, color, odor, and homogeneity of *M. oleifera* oil cream after the stability studies were observed by visualization. The pH and viscosity were measured at the end of each cycle and long-term storage. The rheological characteristics of the moringa seed oil cream were characterized by a rheometer (HAAKE™, Thermo Scientific, Dreieich, Germany).

3.5. Antioxidant Activity of Cream Containing M. oleifera Seed Oil

Cream containing *M. oleifera* seed oil (1 g) or cream base (1 g) were extracted with 1.5 mL of absolute ethanol in a centrifuge tube by mixing vigorously for 5 min using a vortex mixer followed by 10 min-sonication. The extract was centrifuged at 7000 rpm at 25 °C for 10 min. The supernatant was collected and analyzed for antioxidant activity using a DPPH assay. The DPPH assay was carried out by mixing 500 µL of the cream extract with 500 µL of the DPPH solution. The mixture was kept in the dark at room temperature for 30 min. The absorbance was measured at 517 and 600 nm (as a reference wavelength) using a UV-visible microplate reader (Anthos Zenyth 340, Cambridge, UK), and the mean values were obtained from triplicate experiments. The percentage of radical scavenging activity of the cream was calculated by the following equation.

$$radical\ scavenging\ activity\ (\%) = \frac{\left[\left(A_{517\ control} - A_{600\ control}\right) - \left(A_{517\ samples} - A_{600\ control}\right)\right] \times 100}{\left(A_{517control} - A_{600\ control}\right)}. \quad (2)$$

3.6. Skin Irritation Test

All human research studies were approved by the Human Research Ethics Committee of Srinakharinwirot University (013/2557). Thirty-two volunteers aged 18 to 65 years old (averaged 31.9 ± 12.3 years old) were recruited into the skin irritation study. Subjects were excluded if they had contact dermatitis, or any allergic reactions on the tested region that may interfere with the test. Subjects who had received any anti-allergic medications

for treating allergic reactions caused by sunscreen cream, moisturizing cream, and antiaging cream were also excluded. All participants were required to sign an informed consent agreement.

The skin irritation test used in this study was modified from a patch test [13]. After recruitment, the subjects were informed that they were allowed to shower as normal, but they were not allowed to apply any other cosmetics or drugs at the site of the sample applications prior to the test for 24 h. The test products including cream containing moringa seed oil and cream base were applied at different areas at the outer side of left arms under an occlusive patch for 48 h. The patch sites were graded using a skin irritation grading scale according to the International Contact Dermatitis Research Group (ICDRG) [13].

The grading scale included doubtful (?+); a mild reaction, possible erythema, infiltration, and papules (+); a strong reaction, erythema, infiltration, papules, and vesicles (++); and a very strong reaction, intense erythema, infiltration, and coalescing vesicles (+++). The occlusion was removed after 48 h of application and the affected areas were observed and evaluated immediately according to the criteria. The next readings were done again 72, 96, 120, 144, 168, 192, 216, and 240 h after application. The further investigation of the moringa seed oil cream efficacy was continued only after less than 10% of all subjects had positive readings.

3.7. Skin Hydration, Erythema, Melanin Value, and Elasticity

The effects of moringa seed oil cream on the skin hydration, erythema, melanin content, and elasticity were investigated and compared to those of the cream base on its own. Prior to the study, the subjects were asked to wash their arms and wait for 15 min under controlled temperature (25 °C) and humidity (40–60%RH). The skin hydration, erythema, melanin contents, and elasticity of all 32 subjects were determined using a Corneometer®, Mexameter® MX 18, and Cutometer®. The measurements were performed at the marked area, 8 cm away from the front of the elbow and had an area of 3 × 3 cm. The subjects were informed to apply 0.2 g of Moringa seed oil cream and cream base twice a day, at 7–9 a.m. and 7–9 p.m. routinely for 4 weeks. During the experimental period, the subjects were asked to avoid sunlight and the application of any other moisturizers. The skin analysis was conducted after sample application to skin for 1, 2, 3, and 4 weeks.

After washing the arm at the sample application sites, the subjects were kept in a room with controlled temperature (25 °C) and relative humidity of 40–60%. The skin hydration test was performed using a Corneometer®, which indicated the hydration level of the superficial layers of the skin (stratum corneum) via measurement of the skin's dielectric properties. The skin hydration effects of moringa seed oil cream and cream base were evaluated in terms of the difference in skin hydration (%) compared with the baseline and the number of participants with sufficiently hydrated skin. Mexameter® MX 18 was used to measure the hemoglobin and melanin, which are responsible for the redness and color of the skin, respectively.

The absorption and reflection intensity of light at wavelengths of 568 and 660 nm, respectively, were determined and computed as erythema values. A melanin value was computed from the intensities of the absorbed and reflected light at 660 and 880 nm, respectively. The skin elasticity was measured using a Cutometer®. The skin elasticity was reported as the final distension (U_f) and immediate retraction (U_r). The Ur/Uf parameter was used to characterize the elastic property of the skin. The curves from the skin viscoelasticity measurement were analyzed using the Software Cutometer®MPA 580 to obtain the final distension of the first curve (U_f) and the ratio of elastic recovery to the total deformation (U_r/U_f) parameters [14]. All measurements were performed in triplicate.

3.8. Satisfaction Regarding Cream Containing M. oleifera Seed Oil

The satisfaction regarding the cream containing *M. oleifera* seed oil was measured by means of a questionnaire. Healthy volunteers reported the consumer acceptability scores on a five-point hedonic scale (scale 1: extremely dislike, scale 2: slightly dislike, scale 3:

neither like nor dislike, scale 4: slightly like, and scale 5: extremely like) after using blinded cream base or *M. oleifera* seed oil cream. Participants evaluated their satisfaction regarding the color, smoothness, skin hydration feeling, skin absorption, spreadability, and odor.

3.9. Statistical Analysis

Statistical analysis of the data was performed using a one-way analysis of variance (ANOVA), followed by Newman–Keuls as a post-hoc test to assess the significance of differences (GraphPad Prism, La Jolla, CA, USA). To determine the significance of the difference between the means of two groups, a t-test was performed. In all cases, a value of $p < 0.05$ was considered statistically significant.

4. Results

4.1. Physical and Chemical Characterizations of M. oleifera Seed Oil

The amount of α-tocopherol in the *M. oleifera* seed oil determined by HPLC was 15.22 ± 2.99 mg/100 g of *M. oleifera* seed oil. The plant sterols analyzed by GC included 58.12 mg brassicasterol, 47.38 mg campesterol, and 28.94 mg stigmasterol/100 g of *M. oleifera* seed oil. The fatty acid composition of *M. oleifera* seed oil was reported in Table 2. The results suggest that the predominant fatty acid in moringa seed oil was oleic acid (71.57%), followed by palmitic acid (8.22%), stearic acid (5.25%), and behenic acid (4.15%).

Table 2. Fatty AcidC of *M. oleifera* Seed Oil.

Fatty Acid	Amount (g/100 g of *M. oleifera* Seed Oil)
Saturated fatty acid	
Palmitic acid ($C_{16:0}$)	8.22 ± 2.16
Stearic acid ($C_{18:0}$)	5.25 ± 0.10
Behenic acid ($C_{22:0}$)	4.15 ± 2.35
Arachidic acid ($C_{20:0}$)	2.92 ± 1.18
Unsaturated fatty acid	
Oleic acid ($C_{18:1,\ cis\text{-}9}$)	71.57 ± 0.23
α-Linolenic acid ($C_{18:3\ n\text{-}3}$)	1.07 ± 1.31
Palmitoleic acid ($C_{16:\ 1}$)	1.57 ± 0.30
Linoleic acid ($C_{18:2,\ cis}$)	0.97 ± 0.40

The rancidity and lipid peroxidation of *M. oleifera* seed oil were characterized by the peroxide values and TBARS, respectively. HPLC analysis revealed that peroxide value of *M. oleifera* seed oil was 5.36 ± 3.05 mEq/kg, which was lower than the criterion of the Joint Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). Food Standard Programme Codex Alimentarius Commission [15]. The peroxide value of the cold-pressed oil was less than 15 mEq/kg, which was considered acceptable and safe for use. The TBARS value of *M. oleifera* seed oil reported in this study was 0.17 ± 0.00 mg malondialdehyde (MDA)/kg oil. The results of the color measurement of *M. oleifera* seed oil are reported in Table 3.

The color of *M. oleifera* seed oil was specified by the International Commission on Illumination (CIE) L*a*b* coordinates. L*, a*, b*, c*, H*, and ΔE indicate the lightness, red/green coordinate, yellow/blue coordinate, chroma, hue angle, and the change in visual perception of two given colors, respectively. The results of color measurement showed that L* and b* were positive values suggesting that moringa seed oil cream had a light yellow color without a red color. The c* (chroma) and H* (hue) were calculated from the a* and b* coordinates indicating the strength or dominance of the hue. Hue is a color description. ΔE is a parameter, created by Commission Internationale de l'Eclairage to represent the difference between two appearing colors that human eye can see. The ΔE

less than 1.0 indicated an unnoticeable difference of two colors. This result suggests that moringa seed oil cream has a consistent color, and the difference of two colors in the product was unnoticeable by consumers.

Table 3. Color Analysis Results of *M. oleifera* Seed Oil.

Color	Value
L*	61.94 ± 27.11
a*	1.42 ± 5.77
b*	64.17 ± 2.04
c*	64.55 ± 1.58
H*	90.30 ± 2.45
ΔE	0.03 ± 0.00

L*, a*, b*, c*, H*, and ΔE indicate the lightness, red/green coordinate, yellow/blue coordinate, chroma, hue angle, and the change in visual perception of two given colors, respectively.

4.2. Antioxidant Activities of M. oleifera Seed Oil

The free-radical scavenging potential of *Moring oleifera* seed oil was investigated using the DPPH method. DPPH is a reagent composing of free-radical molecules. It is commonly used for detecting the radical scavenging activities of compounds. The reduced absorption of DPPH indicates the capacity of compound to scavenge free radicals. The scavenging effect of *M. oleifera* seed oil was observed as shown in Figure 1. *M. oleifera* seed oil was found to promote the reduction of DPPH. The free-radical scavenging activity increased with the concentration of moringa seed oil and vitamin E. The concentration of moringa seed oil and vitamin E that inhibited 50% of free radicals were 121.9 mg/mL and 110.4 mg/mL, respectively. Gallic acid (0.5 mg/mL) and vitamin E (435 mg/mL), used as positive controls, could inhibit DPPH free radicals by 93.37 ± 0.15% and 90.61 ± 0.51%, respectively.

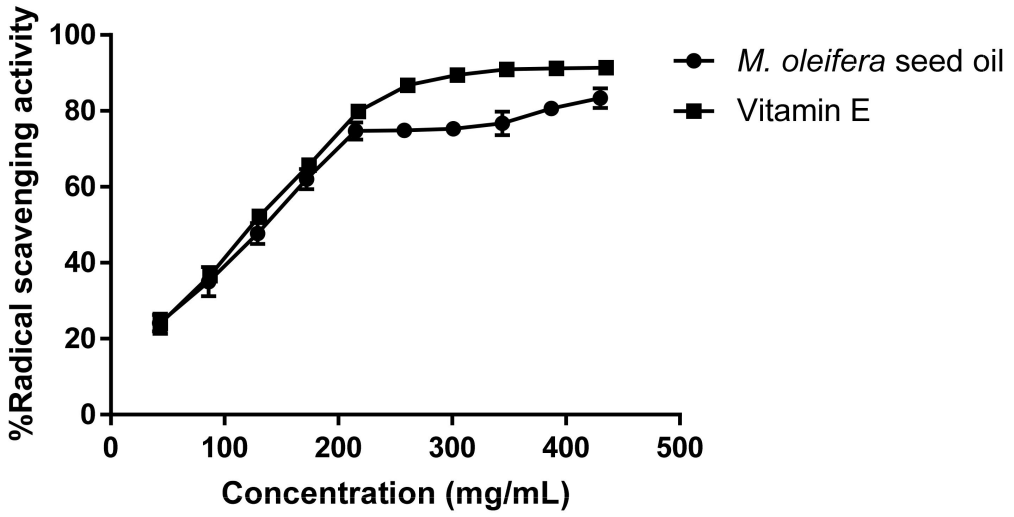

Figure 1. Percentage of the radical scavenging activity of *M. oleifera* seed oil and Vitamin E measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The results are the mean ± S.D. of three experiments. Moringa seed oil was mixed with 500 μM DPPH in absolute ethanol and incubated in the dark at room temperature for 30 min. After incubation, the absorbance was measured at 517 nm using a UV-visible microplate reader. Gallic acid in absolute ethanol and vitamin E were used as positive controls, and absolute ethanol was used as a negative control.

4.3. Evaluation of Physical Characteristics and Stability of Cream Containing M. oleifera Seed Oil

The freshly prepared formulation was characterized as a light glossy yellow cream with a smooth texture and the smell of *M. oleifera* seed oil (Figure 2). The cream base and cream containing moringa seed oil had pH values of 5.07 ± 0.03 and 5.43 ± 0.04,

respectively. The viscosity of the moringa seed oil cream determined by Brookfield viscometer was 17,786 ± 1,442 cP. A plot of viscosity versus shear rate for the cream containing *M. oleifera* seed oil clearly indicated that the apparent viscosity of the moringa seed oil cream decreased significantly with the increasing shear rate suggesting a pseudoplastic flow character.

Figure 2. Appearance of the *M. oleifera* seed oil cream. *M. oleifera* seed oil cream was prepared by heating stearic acid, stearyl alcohol, cetyl alcohol, and sorbitan ester 80 to 70 °C and incorporating the melted oil phase into the heated water phase (75 °C) containing propylene glycol, polysorbate 80, and deionized water. *M. oleifera* seed oil (25 g) was gradually added and mixed with the cream base (75 g) under homogenization for 3 min. Concentrated paraben and triethanolamine was added. The pH of the cream base was adjusted using triethanolamine.

The physical stability of the cream was characterized by the absence of creaming or coalescence of the internal phase, and the cream maintained its appearance, odor, color, and other physical properties. After stability tests at all cycles and 28 day-storage at 4, 30, and 45 °C, the appearance, color, odor, and texture of the moringa cream were not changed. The pH values of the formulation after being freshly prepared and after the heating–cooling cycles and 28-day stability testing were not significantly different, indicating the physical stability of this formulation (Figure 3).

The viscosity of *M. oleifera* cream was not changed after passing each heating–cooling cycle of stability testing and 28 day-storage at 4, 30, and 45 °C as shown in Figure 4. The rheograms of the *M. oleifera* cream after the heating–cooling stability study (Figure 5A) and 28-day stability study (Figure 5B) suggests a pseudoplastic flow of the cream. The rheological behaviors of the cream after fresh preparation and stability testing showed a pseudoplastic flow indicating the physical stability of the cream.

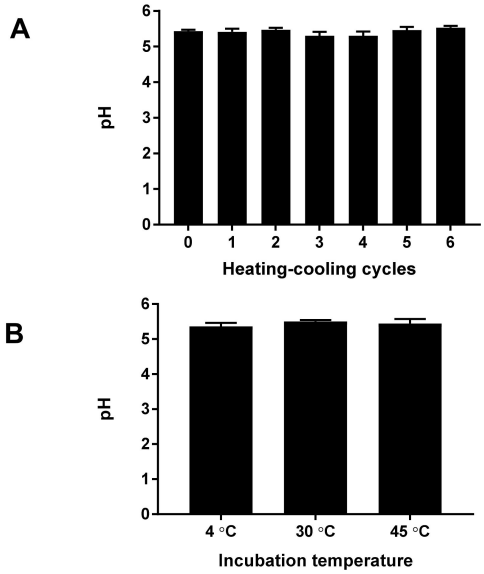

Figure 3. (**A**) Effect of thermal cycling on the pH of cream containing *M. oleifera* seed oil. (**B**) Effect of storage temperature on the pH of the cream containing *M. oleifera* seed oil. The results are the mean ± SD of three experiments. The physical stability of the *M. oleifera* oil cream was studied with a heating–cooling cycle method for six cycles. Samples of cream containing *M. oleifera* oil were stored in glass containers for 24 h in a refrigerator and were placed in an incubator at 45 °C for another 24 h, accounting for one cycle. The long-term stability of the *M. oleifera* seed oil cream was investigated by storage of the cream at 4, 30, and 45 °C for 28 days. The pH of the cream was measured using a Mettler Toledo Electrode.

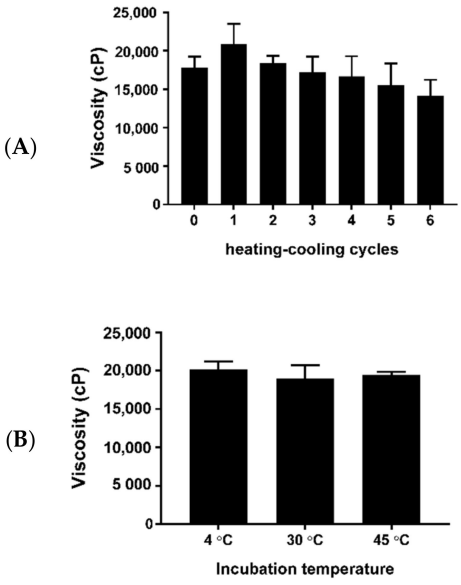

Figure 4. Viscosity of cream containing *M. oleifera* seed oil after (**A**) fresh preparation and the six heating–cooling cycle stability test and (**B**) 28-day stability test at 4, 30, and 45 °C. The results are the mean ± SD of three experiments. The physical stability of the *M. oleifera* oil cream was studied by the heating–cooling cycle method for six cycles. Samples of cream containing *M. oleifera* oil were stored in glass containers for 24 h in a refrigerator and were placed in an incubator at 45 °C for another 24 h, accounting for one cycle. The long-term stability of *M. oleifera* seed oil cream was investigated by storage of the cream at 4, 30, and 45 °C for 28 days. The viscosity of the cream was measured using a Brookfield viscometer.

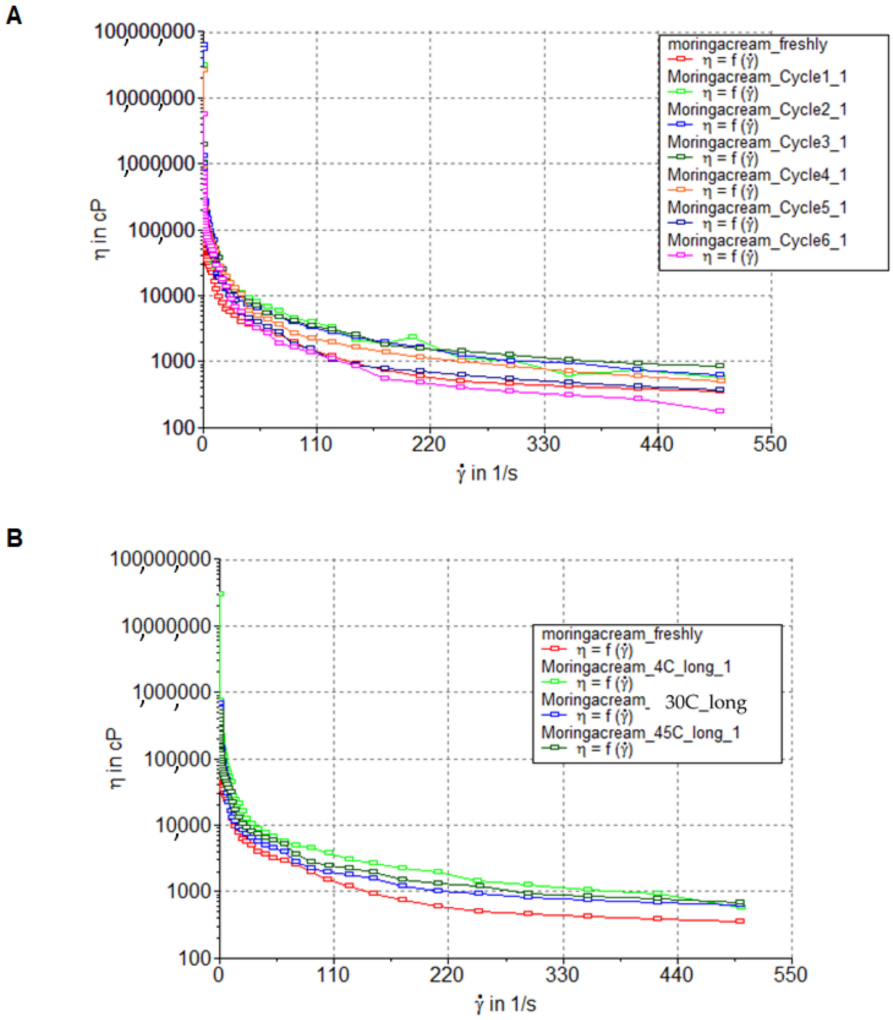

Figure 5. (**A**) Effect of thermal cycling on the rheology of cream containing *M. oleifera* seed oil as a function of the shear rate. (**B**) Effect of storage temperature on the rheology of cream containing *M. oleifera* seed oil as a function of the shear rate. The rheological characteristics of the moringa seed oil cream were characterized by a rheometer. Samples of cream containing *M. oleifera* oil were stored in glass containers for 24 h in a refrigerator and were placed in an incubator at 45 °C for another 24 h, accounting for one cycle. The long-term stability of M. oleifera seed oil cream was investigated by storage of the cream at 4, 30, and 45 °C for 28 days.

4.4. M. oleifera Seed Oil Cream Exhibited DPPH Free-Radical Scavenging Capacity

The DPPH free-radical scavenging assay of *M. oleifera* seed oil cream showed that cream containing *M. oleifera* seed oil presented free-radical scavenging activity. The percentage of inhibition of scavenging activity for moringa seed oil cream containing 83.33 mg/mL of *M. oleifera* seed oil showed 35.97 ± 0.01% DPPH inhibition. The cream base without *M. oleifera* seed oil showed a percent inhibition of scavenging activity of only 4.22 ± 0.21%. The results suggest that the antioxidant activity of *M. oleifera* seed oil cream depended on the presence of *M. oleifera* seed oil.

4.5. Human Skin Irritation of M. oleifera Seed Oil Cream

The human skin irritation test showed that there were no significant adverse effects on the participants' skin after applying *M. oleifera* cream. The results demonstrated completely negative readings at all time points of observation, i.e., day 2, 3, 4, 5, 6, 7, 8, 9, and 10 in all participants. The results suggest that *M. oleifera* was safe to use for human skin according to the International Contact Dermatitis Research Group (ICDGR) standard.

4.6. Skin Moisturizing Effect of M. oleifera Seed Oil Cream

The skin hydration level significantly increased up to 16%, 76%, 77%, and 85% after application of *M. oleifera* seed oil cream every day twice daily for 1, 2, 3, and 4 weeks, respectively (Figure 6A). Application of the cream base increased the skin hydration level but to a significantly lower extent. The increases in the skin hydration level using the cream base were 3%, 58%, 51%, and 48% after the cream base was applied for 1, 2, 3, and 4 weeks, respectively. These results suggest that *M. oleifera* seed oil enhanced the skin hydration compared with the cream base. At baseline, the minimum and maximum skin hydration values were 18.37 and 51.67, respectively.

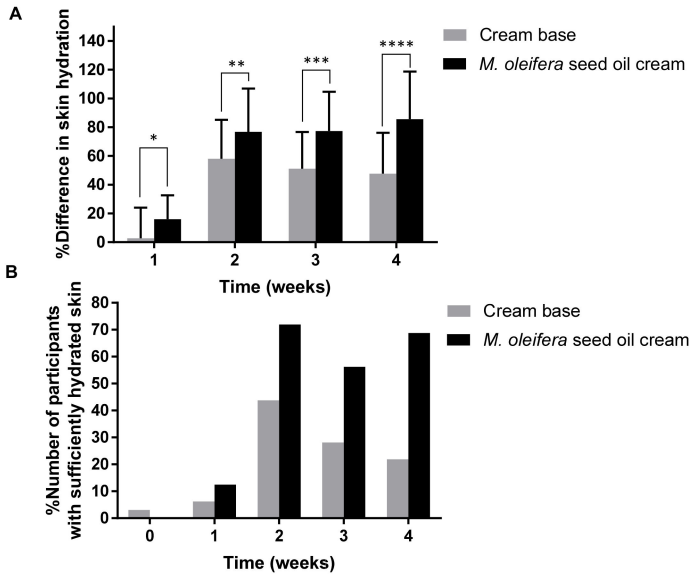

Figure 6. (**A**) Percentage of difference in the skin hydration of subjects who applied *M. oleifera* seed oil cream and the cream base. (**B**) Percentage of the number of subjects with sufficiently hydrated skin after application of the *M. oleifera* seed oil cream and cream base. The results are the mean ± SD of three measurements (n = 32). The skin hydration test was performed using a Corneometer®. After washing the arm at the sample application sites, the subjects were kept in a room with controlled temperature (25 °C) and relative humidity (40–60%). All measurements were performed in triplicate. * p<0.05, ** p<0.01, *** p <0.001, and **** p <0.0001 vs. cream base.

The application of moringa seed oil cream for 4 weeks increased the minimum and maximum skin hydration levels to 33.83 and 77.87, respectively. The cream base increased the minimum and maximum skin hydration levels to 26.43 and 64.80, respectively, which were lower than those of moringa seed oil cream. The skin hydration level was classified as very dry, dry, and sufficiently hydrated at values of < 30, 30–45, and >45, respectively. The result showed that the % of participants who applied moringa seed oil cream and had sufficiently hydrated skin was significantly greater than that of participants who applied only the cream base (Figure 6B).

4.7. Effect of M. oleifera Seed Oil Cream and Cream Base on Skin Erythema and Melanin Values in Subject's Skin

The average erythema value measured by Mexameter® was statistically reduced after 2, 3, and 4 weeks of moringa seed oil cream application (Figure 7A). The melanin values after the application of moringa seed oil cream and the cream base were not significantly different from the baseline, suggesting that moringa seed oil cream and the cream base did not have whitening/brightening effects (Figure 7B).

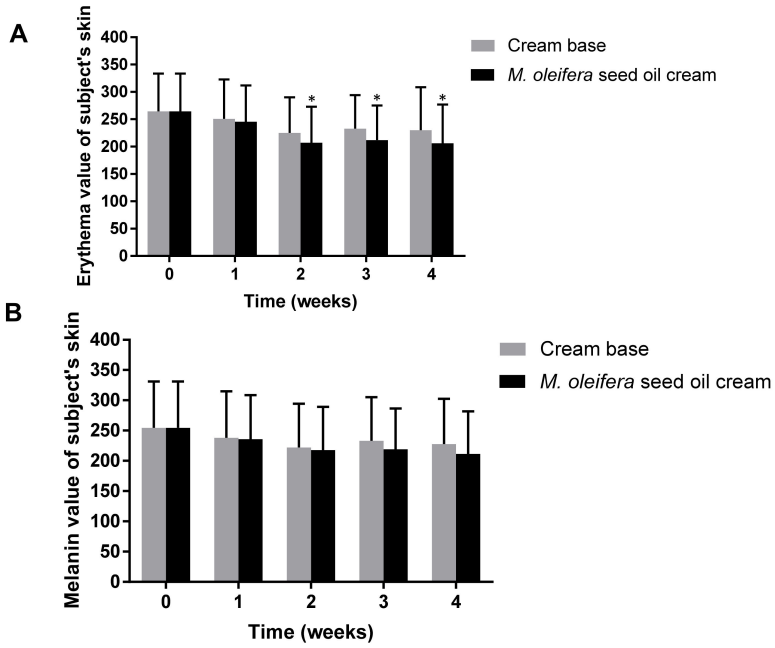

Figure 7. (**A**) Erythema value of the subject's skin after the application of M. oleifera seed oil cream and the cream base (**B**) Melanin value of the subject's skin after the application of M. oleifera seed oil cream and the cream base. The results are the mean ± SD of three measurements (n = 32). A Mexameter® MX 18 was used to measure the hemoglobin and melanin. The absorption and reflection intensity of the light at wavelengths of 568 and 660 nm, respectively, were determined and computed as the erythema value. The melanin value was computed from the intensity of the absorbed and reflected light at 660 and 880 nm, respectively. Subjects washed their arms and rested for 15 min. The erythema and melanin parameters were measured using a Mexameter® probe by providing a constant pressure on the measurement site. All measurements were performed in triplicate.

4.8. Effect of M. oleifera Seed Oil Cream and Cream Base on Skin Elasticity

The value of U_r/U_f represented the immediate recovery of the skin with normal pressure after skin deformation [16]. The resistance of the skin to the negative pressure represented the firmness, and the skin's ability to return into the original state represented elasticity. U_r/U_f represents the elasticity of the skin and its ability to recover its original position. The results showed that there was no significant change in the U_r/U_f values after applying either moringa seed oil cream or the cream base when compared with the baseline (Figure 8). We concluded that the application of moringa seed oil and cream base for 4 weeks did not alter the skin elasticity.

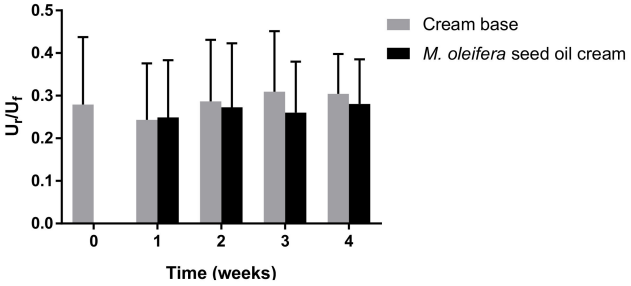

Figure 8. Visco-elasticity of the subject's skin after the application of moringa seed oil cream and the cream base. The results are the mean ± SD of three measurements (n = 32). The skin elasticity was measured using a Cutometer®. The subjects were asked to wash their arms and rest at constant temperature (25 °C) and humidity (40–60%RH) for 15 min. The skin elasticity was reported as the final distension (Uf) and immediate retraction (Ur). The Ur/Uf parameter was used to characterize the elastic properties of the skin.

4.9. Satisfaction of Subjects After Application of M. oleifera Seed Oil Cream and Cream Base

The results of the participant's satisfaction showed that participants preferred the color, smoothness, and skin hydration feeling of the moringa seed oil cream to the cream base (Figure 9). The scores of satisfaction in the skin absorption, spreadability, and odor of the two products were not significantly different.

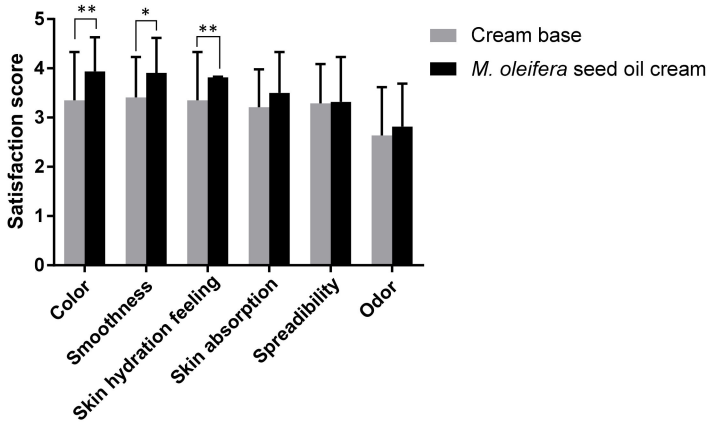

Figure 9. The subjects' satisfaction regarding various attributes of the moringa seed oil cream and the cream base. The results are the mean ± SD of three measurements (n = 32). The satisfaction regarding the cream containing *M. oleifera* seed oil was measured by means of a questionnaire. Healthy volunteers reported the consumer acceptability scores on a 5-point hedonic scale (scale 1: extremely dislike, scale 2: slightly dislike, scale 3: neither like nor dislike, scale 4: slightly like, and scale 5: extremely like) after using a blinded cream base or *M. oleifera* seed oil cream. Participants evaluated their satisfaction with the color, smoothness, skin hydration feeling, skin absorption, spreadability, and odor. * p<0.05, ** p<0.01 vs. cream base.

5. Discussion

M. oleifera seed oil obtained from cold-pressed extraction contains several bioactive compounds that have antioxidant and moisturizing properties in topical use. The amount of α-tocopherol in cold-pressed *M. oleifera* seed oil was found to be slightly higher than the average amount of α-tocopherol previously reported. Anwar et al. reported that Soxhlet extraction of *M. oleifera* seed oil from Pakistan using hexane as a solvent yielded 134.42 mg/kg of α-tocopherol [17]. Ogunsina et al. showed that cold-pressed moringa seed oil exhibited higher total tocopherol contents compared with hexane-extracted moringa seed oil, suggesting that the extraction method of moringa seed oil influenced the α-tocopherol yield [18]. α-Tocopherol is a form of vitamin E, which is a major naturally antioxidant used for protecting skin from the effects of oxidative stress, including photoaging [19].

The plant sterol components contained in cold-pressed moringa seed oil were found to be brassicasterol, campesterol, and stigmasterol. Campesterol and stigmasterol were also major sterol components reported by many researchers [20–23]. Bezerra and Filho revealed that the major plant sterols characterized by GC were β-sitosterol, campesterol, stigmasterol, and brassicasterol. The concentrations of plant sterols, except for brassicasterol, found in this study were lower compared with in the moringa seed oil reported in other studies [24]. The reported content of each plant sterol were different depending on the temperature and plant location, harvest time, fertilization, genetic characters, and growing conditions [25].

The fatty acid profiles of *M. oleifera* seed oil consisted of a high level of oleic acid followed by palmitic acid, and stearic acid. These results were consistent with the fatty acid profiles of moringa seed oil in other regions showing that oleic acid was the most abundant unsaturated fatty acid found in moringa seed oil [26]. Oleic acid is monounsaturated fatty acid shown to increase the skin permeability. Correa et al. reported that trans-epidermal water loss and fluorescein penetration increased with increased doses of oleic acid.

Oleic acid penetrated into the skin and disordered stratum corneum lipids and disrupted the skin barrier function by having an affinity between oleic acid and stratum corneum lipids [27]. In addition to α-tocopherol, several studies have reported that unsaturated fatty acids, including oleic acid and α-linoleic acid, were shown to have antioxidant activity [28,29]. According to the chemical compositions of *M. oleifera* seed oil, the oil is recommended for use in pharmaceutical preparations, particularly for skin hydration.

M. oleifera seed oil contains fatty acids and plant sterols, which are prone to lipid peroxidation. Therefore, it was necessary to determine the peroxide value to indicate the rancidity of the oil. The TBARS value, defined as the amount of malondialdehyde (MDA) in 1 kg of *M. oleifera* seed oil, was used to analyze the lipid peroxidation of the oil. The TBARS value of *M. oleifera* seed oil reported in this study was 0.17 mg MDA/kg oil, which was lower than 1 mg MDA/kg oil, indicating a low extent of lipid peroxidation according to the Association of Official Agricultural Chemists (AOAC) standard criteria [30]. The results suggest that *M. oleifera* seed oil is qualified for use as a pharmaceutical or cosmetic active ingredient.

A DPPH assay was used to test the free-radical scavenging activity of *M. oleifera* seed oil, which donated electrons or hydrogens to DPPH to neutralize the free radicals. In the present study, the IC_{50} value of moringa seed oil was 121.9 mg/mL, whereas vitamin E had an IC_{50} value of 110.4 mg/mL. The antioxidant activity of moringa seed oil likely resulted from the presence of α-tocopherol and different types of unsaturated fatty acids in the oil. The antioxidant activity may prevent lipid peroxidation and protect moringa seed oil from rancidity.

The application of pure moringa seed oil directly to the skin may cause skin irritation and/or sensitization in normal individuals especially for those with sensitive skin. Therefore, the inclusion of moringa seed oil in the cream was an alternative method of using an essential oil for therapeutic or cosmetic uses. In this study, cream containing an effective concentration of *M. olerifera* seed oil was formulated. The moringa seed oil cream had a light yellow color, nutty odor, and homogeneous texture. The pH of the moringa seed oil cream was close to that of human skin, and the viscosity yielded an easily spreadable cream formulation.

The rheology of the cream after the heating–cooling cycle stability study and long-term stability study (4 weeks) showed pseudoplastic flow. The cream flew instantaneously upon application of stress and displayed shear thinning behavior without a yield stress. Therefore, when the cream was applied to the skin, the increase in the shear rate may result in less resistance to flow and the release of moringa seed oil to the skin [31]. Normally, emulsion is a thermodynamically unstable system consisting of immiscible oil and water mixed together. The oil-in-water cream contains oil droplets dispersed in an aqueous phase.

Coalescence, flocculation, creaming, and breaking are unstable states of the common cream/emulsion. The stability of moringa seed oil cream was previously studied. The results showed that excipients, such as polysaccharides and emulsifiers influenced the physicochemical properties and stability of the emulsion. In addition, the viscosity of the emulsion also affected its stability. This study demonstrated a new cream/emulsion formulation containing M. oleifera seed oil as the active ingredient. The 25% of moringa seed oil was found to be the high concentration of moringa seed oil incorporated into the cream/emulsion formulation [32,33]. The high concentration of moringa seed oil could result in an unstable emulsion. However, the cream containing moringa seed oil developed in this study had an unchanged appearance, pH, viscosity, and rheology throughout the stability studies.

According to the antioxidant activity of moringa seed oil, moringa seed oil cream may protect the skin by limiting the free-radical production, preventing oxidative stress, and enhancing DNA repair upon exposure to the air, UV radiation, pollutants, and chemicals that cause skin damage [34,35]. In addition, moringa seed oil cream may provide skin with hydration and increase the moisture retention to aid in revitalizing the skin.

The moringa seed oil cream developed in this study passed the standard of a patch test suggesting that moringa seed oil cream is safe for use on human skin. A cream formulation was selected as the drug delivery system for the moringa seed oil. The cream base itself also had a moisturizing effect for the skin as shown in Figure 6. Therefore, cream is a preferred vehicle because it provides a good delivery of the active ingredient, and, at the same time, the vehicle itself also promotes better skin hydration [36]. In this study, an oil-in-water cream was developed because it provides a more pleasant application compared to a water in oil cream [37]. The oil-in-water cream was therefore more acceptable to the consumers to apply the product more frequently. In addition, an oil-in-water skin cream spreads over the skin more easily, which could result in enhanced absorption of the active moringa seed oil.

The skin hydration level of the subjects applying moringa seed oil cream was significantly increased compared with those using the cream base. Moringa seed oil increased the skin hydration by creating a hydrophobic barrier over the skin and blocked dehydration. Oleic acid in the moringa seed oil disrupted the skin barrier, increased the molecular interactions of the moringa seed oil with stratum corneum lipids and enhanced the oil penetration efficiency into the epidermis [38]. The skin erythema was significantly reduced after the application of moringa seed oil. Erythema was a result of skin inflammation and irritation. The level of erythema values was directly related to the hemoglobin content in the skin.

The reduction in erythema values after the application of moringa seed oil cream suggests that the cream is safe for use in cosmetic and pharmaceutical applications without causing any significant skin irritation [39]. These results were confirmed by the results of the patch test showing no irritation to the skin and that the formulation was appropriate for human use [40]. The melanin value and visco-elasticity were not increased with the use of moringa seed oil cream, indicating that moringa seed oil cream did not affect the melanin content and elasticity of the skin in a 4-week application. The satisfaction score of participants revealed that the consumers were satisfied with the color, smoothness, and skin hydration feeling of the moringa seed oil cream. Therefore, it might not be necessary to add any coloring agents to the cream. However, a flavoring agent was recommended to be included in the cream formulation.

6. Conclusions

We successfully developed an *M. oleifera* seed oil cream. Moringa seed oil cream possessed antioxidant activity, enhanced the skin hydration level, and reduced skin erythema, but did not affect the melanin content and skin visco-elasticity. There was no report of skin irritation from the application of the cream, suggesting that the moringa seed oil cream developed in this study is appropriate for pharmaceutical and cosmetic uses.

Author Contributions: Conceptualization, S.A., S.T. and C.C.; Methodology, S.A., S.T. and C.C.; Investigation, P.T.; Writing—Review & Editing, S.A., P.T., S.T., P.J., S.R.S., and C.C.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Informed consent was obtained from all subjects involved in the study.

Informed Consent Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Srinakharinwirot University. This is approved on 29th August, 2014 (Approval number 013/2557).

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the confidentiality and anonymity of participants.

Conflicts of Interest: The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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