FORMULATION AND EVALUATION OF POTENTIAL ANTIBACTERIAL DAY CREAM USING COCOA ANTIBACTERIAL EXTRACT (COCOA-ABE) FROM *Theobroma cacao* FOR SKINCARE

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ABSTRACT – *In this modern era, the cosmetics industry is committed to providing advanced skincare products by using a combination of scientific ingredients and plants to develop a safe and effective product. Theobroma cacao (T. cacao) is one of the plants that contain chemical compounds with antibacterial characteristics. This study aims to formulate and evaluate a cocoa antibacterial day cream containing cocoa shell extract of T. cacao for skin care. The methodology started with the preparation of cocoa-ABE, which was extracted with water. The cocoa-ABE extract powder of T. cacao was incorporated at varying amounts into four different oil-in-water (O/W) emulsion bases. The developed formulations (F1, F2, F3, and F4) were evaluated for physicochemical evaluation. Then, the selected formulation will be further tested for stability, antibacterial, antioxidant, anti-inflammatory, and heavy metal content. Based on the results, F4 was selected for the stability test after demonstrating a smooth consistency with an appropriate pH of 6.8 compared to other formulations. F4 remained stable in terms of pH with slight color changes during three months of storage at room temperature and 40°C. F4 showed potent antibacterial activity against Staphylococcus aureus and Staphylococcus epidermidis. Besides, F4 exhibited potential antioxidant activity by inhibiting reactive oxygen species production but no anti-inflammatory activity against nitric oxide production. The heavy metal content of F4 was found to be in the safe permissible range. This study shows that the cocoa shell extract of T. cacao is a promising candidate for the development of topical antibacterial skincare products.*

Keywords: Antibacterial, cocoa shell extract, day cream, formulation, skincare, Theobroma cacao

INTRODUCTION

Aside from its primary function as a barrier between the body and the external environment, skin is also known as the cutaneous membrane or integumentary system, which is made up of accessory organs with varying surface areas and weights depending on the body. In addition, the epidermal layer's presence on the skin helps to regulate body temperature, fluid balance, and excretion (Tan *et al*., 2022). However, microorganisms such as bacteria could interrupt the barrier efficiency, which leads to various skin-related diseases such as acne (Sultan & Parumpu, 2021). Therefore, discoveries about microorganisms or bacteria-related skin diseases have resulted in the development of a wide range of organic and inorganic skin care products that are widely available on the market.

Natural ingredients have been used for decades in home skin care remedies. However, certain users tend to buy ready-made skincare due to limited access to natural ingredients, unpleasant scents produced by the ingredients themselves, and time-consuming ingredient preparation. On the other hand, due to consumers' demand and concerns towards synthetic or chemical substances applied to their skin, natural ingredients are becoming part of modern formulations in producing convenient organic skincare (Tan *et al*., 2022), which can be obtained from various fruits, leaves, flowers, herbs, minerals, water, and land.

Originating from ancient Central America, *T. cacao*, known as cocoa, belongs to the family *Sterculiaceae* (Zainal *et al*., 2016). Consisting of approximately 20 types, cocoa could be considered as an essential perennial crop on earth and is commercially cultivated and exploited not only for seed output, which is destined for chocolate production, but also for the by-products (cocoa pod husk, cocoa bean shells, and cocoa mucilage), which are processed into end-products such as fuel or boilers, animal feed, fertilizer, beverages, ice creams, and cosmetics (Vásquez *et al*., 2019). Compounds that occur naturally in cocoa, such as the procyanidins and catechins, as well as smaller amounts of gallocatechin and epigallocatechin, have also been implicated as being responsible for the antioxidant activity of the

cocoa component (Scapagnini *et al*., 2014). Containing more phenolic antioxidants than other foods, cocoa can avoid nerve injuries, delay the start of premature aging, and serve as skin protection against oxidative damage caused by UV radiation (Oracz & Żyżelewicz, 2020). The butter derived from cocoa seeds contain significant amounts of fatty acids and phytosterols that can restore the elasticity of the skin and heal infectious skin conditions like eczema and dermatitis (Singh *et al*., 2020). Besides, enhanced usage of cocoa by-products could overcome environmental problems due to their inability to decompose and be left to rot in cocoa cultivation areas. Several reports have shown that the cocoa shell contains many beneficial bioactivities, and its extract has great potential to be used in developing commercial products.

Creams are semi-solid dosage forms of emulsions of oil and water. In the cosmetic field, there are two types of cream preparation. They are classified as oil-in-water (O/W) creams and water-in-oil (W/O) creams. Oil-in-water (O/W) creams are composed of small droplets of oil dispersed in a continuous phase, and water-in-oil (W/O) creams are composed of small droplets of water dispersed in a continuous oily phase (Mohiudin, 2019). Nowadays, the cosmetic industry releases a day cream that includes sunscreen for skin protection against harmful ultraviolet (UV) radiation (Rodrigues & Jose, 2020). Oil-in-water (O/W) cream preparations are widely used for cosmetics and aesthetic products because they are easier to apply to any part of the body and more comfortable to use on the face as part of an everyday skincare routine. Besides that, they are not sticky and are easier to wash with water than water-in-oil cream preparations (Sultan & Parumpu, 2021). In this study, oil-in-water (o/w) emulsions were prepared and physicochemically characterized. Thus, this research was conducted to develop a formula for antibacterial day cream using cocoa shell antibacterial extract (cocoa-ABE) as an active ingredient.

MATERIALS AND METHODS

Materials and tools

The materials used were sunscreen (titanium dioxide, zinc oxide, Bisabolol (and) Titanium Dioxide (and) Polyhydroxystearic Acid (and) HydrogenDimethicone (and) Tocopheryl Acetate (and) C12-15 Alkyl Benzoate (and) Argania Spinosa Kernel Oil (and) Alumina), emulsifying agents (Polysorbate 20), moisturizer (Caprylic/Capric Tryglyceride), emulsifier (natural emulsifier, Ceteth-20 (and) Cetyl Alco-hol (and) Glyceryl Stearate (and) PEG-75 Stearate, Steareth-20), cold process emulsifier (Cetyl Alcohol (and) Glyceryl Stearate (and) Glycol Stea-rate (and) Caprylic/Capric Triglyceride (and) Sodium Acrylate/Sodium Acryloyl Dimethyl Taurate Copolymer), film former (Hydrogenated Polycyclopen-tadiene (and) Isododecane), emollient (C13-14 Alkane), preservative (Caprylyl Glycol (and) Glyceryl Laurate (and) Glyceryl Undecylenate), Silica, O/W emulsifier & thickener (Glyceryl Stearate (and) PEG-100 Stearate), thickening/conditioning agent (Stearyl stearate), (Hydrogenated Polycyclopen-tadiene (and) Caprylic/Capric Triglyceride) were purchased from Chemie Alliance (Malaysia). Other ingredients used in the cream formulations such as *Theobroma cacao Seed Oil*, Disodium EDTA, Glycerin, Carbomer, Rose Extract, Sodium Hydroxide 10% Solution, and preservative (Phenoxyethanol (and) Ethylhexylglycerin). The tools used were Eutech pH 700 meter (Thermo Fisher Scientific, United States), homogenizer (Silverson, United Kingdom), centrifuge (Eppendorf centrifuge 5430 R, Germany) and zetasizer (Malvern® ZETASIZER NANO Instrument, United Kingdom).

Plant Materials and Preparation

Unfermented cocoa beans were collected from Cocoa Research and Development Centre Malaysian Cocoa Board, Jengka Pahang, Malaysia. Cocoa shells that represent 12-15% of the cocoa bean (Öztürk & Ova, 2018) were chosen due to containing antibacterial properties against Gram-positive and Gram-negative bacterial pathogens (Zainal *et al*., 2006; Zainal *et al*., 2019) for extraction process. The unfermented cocoa shell (UCS) was obtained after a deshelling process that included heating at 90°C for 2 minutes at a rotation speed of 600 rpm by an infrared micronizer, a breaking process with a bean-breaker, and shells separation from nibs using a winnower. The UCS was then stored at room temperature for further usage and analysis conducted in the Biotechnology Laboratory of Malaysian Cocoa Board, Nilai Malaysia.

Extraction of Cocoa-ABE

The extraction of Cocoa-ABE was conducted at the Bio Aromatic Research Centre of Excellence, Universiti Malaysia Pahang. The extraction yield is 20%. The extraction process was initiated by placing a 20 kg sample in the drying oven which was subsequently ground into a powder form. Readily ground samples were sieved before proceeding with multipurpose extraction (water distillation) at 100°C for 2 hours. The sample was then filtered with muslin cloth followed by filtration using 100-micron filter paper which resulted in a more refined extract. The extraction process

continued with sample concentration by vacuum concentrator before spray drying at 13 kg/h for 6 hours. Lastly, cocoa-ABE obtained (4 kg) was stored at 4°C for further usage or analysis.

Formulation of The Cream

The basic formulations of the cream were summarized in Tables 1-4. The four formulations with different ingredients were prepared for comparison purposes. The comparison was made with the commercial brand, namely Wardah.

The antibacterial day protection ream was made by the following method. Firstly, the oil phase was heated at 70°C until all parts melted. Then, the water phase was heated in another glass beaker on a hot plate at 70°C until melted. The oil phase was slowly added to the water phase while constantly stirring with a 3000-rpm homogenizer (Silverson, United Kingdom) to form a white emulsion. After the base was formed, the Cocoa-ABE was added to the cream base, and the preparation was stirred again until homogeneous. A few drops of rose perfume were added during stirring for the aroma effect. In making cream preparations, the water and oil phases should be at the same temperature so that the cream base can be appropriately formed and there was no phase separation. The cream was formed when the mixture reached a viscous consistency.

Table 1: The Composition of Antibacterial Day Protection Cream for Formulation 1

Phase	Ingredients	Function	Weight percentage (wt %)
A			
Oil Phase	*Theobroma cacao* seed oil	Moisturiser	7.3 – 7.5
	Natural Emulsifier	Natural Emollient	5.8 – 6.0
B			
Water Phase	Distilled Water	Water	72.0 – 74.0
	Carbomer	Thickener	1.1 – 1.3
	Theobroma cacao Bean Shell Extract (Cocoa-ABE)	Active ingredient/antibacterial	1.5 – 1.7
	Titanium Dioxide	Sunscreen	2.0 – 2.2
	Zinc Oxide	Sunscreen	2.2 – 2.5
	Rose Extract	Fragrance	1.2 – 1.5
	Polysorbate 20	Emulsifying Agents	3.0 – 3.4
	Phenoxyethanol (and) Ethylhexylglycerin	Preservative	1.2 – 1.5
	Sodium Hydroxide 10% Solution	pH Balancer	Few drops

Table 2: The Composition of Antibacterial Day Protection Cream for Formulation 2

Phase	Ingredients	Function	Weight percentage (wt %)
A			
Water Phase	Distilled water	Water	65.0 – 70.0
	Carbomer	Cool process emulsifier	1.0 – 1.5
B			
Oil Phase	*Theobroma cacao* Seed Oil	Moisturiser	7.0 – 7.5
	Caprylic/Capric Tryglyceride	Moisturiser	7.0 – 7.5
	Ceteth-20 (and) Cetyl Alcohol (and) Glyceryl Stearate (and) PEG-75 Stearate, Steareth-20	Emulsifier	6.0 – 6.2
	Polysorbate 20	Emulsifying Agents	3.0 – 3.3
	Titanium Dioxide	Sunscreen	1.6 – 1.8
	Zinc Oxide	Sunscreen	0.5 – 0.8
	Phenoxyethanol (and) Ethylhexylglycerin	Preservatives	1.6 – 1.8
	Theobroma cacao Bean Shell Extract (Cocoa-ABE)	Active ingredient/antibacterial	2.0 – 2.3
	Rose Extract	Fragrance	2.0 – 2.3

Table 3: The Composition of Antibacterial Day Protection Cream for Formulation 3

Phase	Ingredients	Function	Weight percentage (wt %)
A			
Water Phase	Distilled Water	Water	71.0 – 73.0
	Disodium EDTA	Chelating agent/Stabilizer	0.1 – 0.2
	Glycerin	Moisturiser	3.0 – 3.3
B			
	Cetyl Alcohol (and) Glyceryl Stearate (and) Glycol Stearate (and) Caprylic/Capric Triglyceride (and) Sodium Acrylate/Sodium Acryloyl Dimethyl Taurate Copolymer	Cold Process Emulsifier	5.0 – 7.0
C			
Oil Phase	Bisabolol (and) Titanium Dioxide (and) Polyhydroxys-tearic Acid (and) Hydrogen Dimethicone (and) Tocopheryl Acetate (and) C12-15 Alkyl Benzoate (and) Argania Spinosa Kernel Oil (and) Alumina	Sunscreen	8.0 – 11.0
	Theobroma cacao Seed Oil	Moisturiser	1.0 – 3.0
	Hydrogenated Polycyclopen-tadiene (and) Isododecane	Film Former	5.0 – 6.0
	C13-14 Alkane	Emollient	1.0 – 3.0
D			
	Rose Extract	Fragrance	0.5 – 1.0
	Caprylyl Glycol (and) Glyceryl Laurate (and) Glyceryl Undecylenate	Preservative	0.5 – 1.0
	Silica	Silica	0.6 – 0.8
	Theobroma cacao Bean Shell Extract (Cocoa-ABE)	Active ingredient/antibacterial	0.4 – 0.6

Table 4: The Composition of Antibacterial Day Protection Cream for Formulation 4

Phase	Ingredients	Function	Weight percentage (wt %)
A			
Water Phase	Distilled Water		65.0 – 66.0
	Disodium EDTA	Chelating agent/Stabilizer	0.1 – 0.2
	Cetyl Alcohol (and) Glyceryl Stearate (and) Glycol Stearate (and) Caprylic/Capric Triglyceride (and) Sodium Acry-late/Sodium Acryloyl Dimethyl Taurate Copolymer	Cold Process Emulsifier	5.0 – 6.0 2.0 – 4.0
	Glycerin	Moisturiser	
B			
Oil Phase	Glyceryl Stearate (and) PEG-100 Stearate	O/W emulsifier & thickener	3.4 – 3.6
	Stearyl stearate	Thickening/conditioning agent	2.2 – 2.5
	Bisabolol (and) Titanium Dioxide (and) Polyhydroxys-tearic Acid (and) Hydrogen Dimethicone (and) Tocopheryl Acetate (and)	Sunscreen	8.0 – 10.0

	C12-15 Alkyl Benzoate (and) Argania Spinosa Kernel Oil (and) Alumina Hydrogenated Polycyclopen-tadiene (and) Caprylic/Capric Triglyceride		1.0 – 1.2
	C13-14 Alkane	Natural emollient	2.5 -3.0
	Theobroma cacao seed oil	Moisturiser	2.5 – 3.0
C			
	Rose Extract	Fragrance	0.3 – 0.5
	Caprylyl Glycol (and) Glyceryl Laurate (and) Glyceryl Undecylenate	Preservative	1.0 – 1.2
	Silica	Silica	0.8 – 1.0
	Theobroma cacao Bean Shell extract (Cocoa-ABE)	Active ingredient/antibacterial	0.3 – 0.5

Physicochemical Evaluation

The prepared antibacterial day protection cream of F1, F2, F3, and F4 containing cocoa-ABE and the commercial cream were evaluated using standard methods. The methods include pH determination, cream centrifugation, a spreadability test, and visual observation for appearance, cream removal, homogeneity, and smear test.

pH

One (1) g of the cream sample was weighed in a suitable beaker, which was then diluted with 9 mL of distilled water before being measured with a calibrated pH meter. The pH value of the cream should be in the range of 4 to 6, referring to previous research by Lukić et al. (2021), who stated that topical products need to be acidified for safe and effective cosmetic products.

Appearance and Cream Removal

The appearance of the cream was examined based on its color and odor (Tan et al., 2022). The removal of the cream was checked by washing the applied part with tap water (Tan et al., 2022).

Homogeneity and Smear Test

The formulated cream was tested for homogeneity by visual appearance and touch. A small amount of the cream was applied to the skin in order to examine whether the cream was homogenously mixed or not (Phetmung & Sawatdee, 2019). After application of the cream, smear test was checked to observe the after-feel effect on the skin (Ilomuanya et al., 2018). The smear test determined whether the cream was greasy or non-greasy.

Cream Centrifugation

Centrifugation is a process of applying centrifugal force for the sedimentation of heterogeneous mixtures with a centrifuge (Esoje et al., 2016; Mawazi et al., 2019), which made it necessary to conduct a centrifugation stability test to avoid having an end product with undesired phase separation. The centrifuging test was conducted by weighing 20 g of each of the formulations in separate centrifuge tubes, which were then placed in an Eppendorf centrifuge 5430 R (3000 rpm in 15 minutes).

Spreadability Test

The spreadability test was evaluated by sandwiching the cream between two glass slides (parallel plate method). Two glass slides with standard dimensions (7.5 x 2.5 cm) were selected. About 0.5 g of the cream was measured and placed in a previously marked 1 cm diameter in the center of the slide. Another slide was placed on top, and then a 100 g weight was placed on the upper slide so that the cream between the slides was uniformly pressed to form a thin layer (Phetmung & Sawatdee, 2019). The weight was removed and the excess cream that had adhered to the slide was wiped off. A 200 g weight was placed on top of the slide for 2 minutes at room temperature. The diameter of the cream spread (cm) was measured in triplicate (Tan et al., 2022).

Stability Test

The stability test was conducted for the formula F4 at the Industrial Biotechnology Research Centre, SIRIM Berhad. The pH, color measurement, and viscosity were all monitored for three months at three different temperatures: room temperature (RT), 40°C, and 50°C. The in-house method was used to carry out the test by referring to Guidelines on Stability Testing of Cosmetic Products (Cosmetics Europe, 2004). In addition, a microbial limit test was performed on the formula F4.

pH

The pH of the antibacterial day protection cream formulation F4 stored at different storage conditions was determined using a calibrated pH meter. The pH was determined at 25°C by diluting an appropriate quantity of cream with distilled water in a beaker.

Color Measurement

The color changes of the formula F4 were assessed using a Minolta CM 3500d color spectrophotometer at room temperature of ±25°C. The measurements were done in the CIE parameters of L*a*b* color space system and the results expressed in ΔE indicate a stability test. The L* component describes brightness, and the a* and b* components describe the green-to-red ratio and yellow-to-blue ratio, respectively. Meanwhile, ΔE defines the ratio of color changes, which determines the color changes in the product when compared to the standard sample in the initial month.

Viscosity

The Brookfield Digital Viscometer DV-II was used to measure the viscosity of the fomula F4 at temperatures between 24°C and 26°C. Four speeds of 6, 12, 30, and 60 revolutions per minute (rpm) were used with spindle No. 29.

Microbiology Limit Test

The total viable microbial count in the F4 was conducted using the colony count technique. The reference standards used were Cosmetics-Microbiology-Enumeration and Detection of Aerobic Mesophilic Bacteria (ISO 21149:2017) and Cosmetics-Microbiology-Enumeration of Yeast and Mould (ISO 16212:2017).

Antibacterial Activity Test

The antibacterial activity test of the formula F4 was carried out at the Industrial Biotechnology Research Centre, SIRIM Berhad. The method used for the test was cylinder plate assay with the reference standard of In-house qualitative *in-vitro* antibacterial test. The bacteria used were *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (ATCC 12228) to evaluate the bactericidal activity of the day cream at contact times of 24 hours. About 50 mL of molten tryptic soy agar (TSA) was inoculated with 0.1 mL of bacterial 10^8 CFU/mL suspension and mixed evenly. Five mililitre of the inoculated molten TSA agar was used to lawn a solidified 10 mL TSA plate in a 90 mm sterile, disposable petri dish and was let to solidify for 15 minutes. A metal cylinder (10 mm height, 8 mm outer diameter, 6mm inner diameter) filled with day cream sample was placed onto the lawned TSA plate. The plate was then incubated at 35°C for 24 hours before being examined for inhibition zone. The size of the inhibition zone (mm) was measured using calipers.

Antioxidant

Sample Preparation

The antioxidant properties of the formula F4 were performed at the Industrial Biotechnology Research Centre, SIRIM Berhad. The F4 was evaluated using the Cellular Antioxidant Activity (CAA) assay. The sample was diluted in an aqueous solution with a final concentration that ranged from 20 to 1.25 mg/mL. These non-toxic concentrations were selected due to limitation of sample solubility and sterile techniques practice.

Measurement of Reactive Oxygen Species (ROS)

The human keratinocyte cell line (HaCaT) was seeded into a 24-well plate and cultured until 90% confluence was reached. After reaching 90% confluence, cells were treated with F4 samples at selected concentrations for 24 hours. Cells were gently washed with phosphate buffer saline (PBS) after incubation. Then, a 2'-7'-dichlorodihydrofluorescin diacetate (DCFH-DA) probe solution was added to each well before incubated in the dark for 30 minutes. The solution was then removed from the cells and rinsed with PBS before subjected to a UVB dosage of 70 mJ/cm^2. The supernatant was collected immediately after irradiation and read using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The percentage of ROS inhibition calculation:

$$\frac{ROS_{ref} - ROS_{sample}}{ROS_{ref}} \times 100\% \quad (1)$$

where;

ROS_{ref} : Cells irradiated with UVB only (without F4 sample)

ROS_{sample} : Cells irradiated with UVB and treated with F4 sample/ standard

The half maximal inhibitory concentration (IC_{50}) calculation based on the log/linear equation derived from the graph of ROS Inhibition (%) vs sample concentration:

$$Y = mX + c \quad (2);\ with\ R^2 > 0.9$$

where;

Y: ROS Inhibition (%)

m: Slope

c: Intercept

X: Concentration of day cream sample

Cell Viability

An MTT assay was carried out to evaluate the viability of the cells. Each well was added with a 0.5

mg/mL MTT solution, and cells were incubated for 4 hours at 37°C in a humidified incubator with 5% CO_2. The cells were then centrifuged and dissolved in DMSO. A spectrophotometer was used to measure absorbance at 630 nm. The viability of cells (MTT assay) calculation:

$$\frac{B}{A} \times 100\% \quad (3)$$

where;
A: Cells irradiated with UVB only (without F4 sample)
B: Cells irradiated with UVB and treated with F4 sample/standard

Anti-inflammatory

Sample Preparation

The inflammatory properties of the formula F4 were determined using the Cellular Anti-Inflammatory Assay: Nitric Oxide (NO) Assay, at the Industrial Biotechnology Research Centre, SIRIM Berhad. The F4 samples with concentrations ranging from 0.625 to 10 mg/mL were prepared. A 0.2 µm membrane filter was used to filter samples before treatment step.

Measurement of Nitric Oxide (NO) Inhibition

Murine macrophage RAW 264.7 (ATCC TIB-71) cells were seeded in a 24-well plate and incubated for 24 hours at 37°C and 5% CO_2. The medium was removed and replaced with FBS-free DMEM media after 24 hours. Then the cells were treated with the F4 sample at selected concentrations, 50 µg/mL of Nitro-L-arginine methyl ester (L-NAME) as a positive control, and blank (medium without treatment). The cells were incubated for 1 hour before being stimulated with 0.1 µg/mL of lipopolysaccharide (LPS) derived from *E. coli* for 24 hours. The presence of nitrite in culture media was determined by adding 100 µL of Griess reagent to 100 µL of cultured media. A microplate reader (FLOUstar Omega, BGM Labtech, Germany) was used to measure the absorbance at 542 nm. The amount of nitrite content in the media was calculated based on sodium nitrite ($NaNO_2$) standard curve.

$$\frac{NO_{ref} - NO_{sample}}{NO_{ref}} \times 100\% \quad (4)$$

where;
NO_{ref} : Nitrite content in cells stimulated with LPS only
NO_{sample} : Nitrite content in cells stimulated with LPS and treated with F4 sample

The half maximal inhibitory concentration (IC_{50}) calculation based on the log/linear equation derived from the graph of NO Inhibition (%) vs sample concentration:

$$Y = mX + c \quad (5);\ with\ R^2 > 0.9$$

where;
Y: NO Inhibition (%)
m: Slope
c: Intercept
X: Concentration of F4 sample

Cell Viability

An MTT assay was used to assess the viability of the cells. Each well was added with 5 mg/mL of MTT solution and incubated for 4 hours at 37°C and 5% CO_2. The formazan precipitate was solubilized with DMSO after removing the medium. A microplate reader (FLOUstar Omega, BGM Labtech, Germany) was used to measure the absorbance at 630 nm. Viability of cells:

$$\frac{B}{A} \times 100\% \quad (6)$$

where;
A: Absorbance of reference (Cells stimulated with LPS only)
B: Absorbance of test (Cells stimulated with LPS and treated with F4 sample)

Heavy metal content

The heavy metals determination the formula F4 was conducted at the Industrial Biotechnology Research Centre, SIRIM Berhad. The method used for this test was CPCT/TP/MM/In-House 018 based on AOAC 2015.01: determination of heavy metals by inductively coupled plasma mass spectrometry (ICP-MS). The standard reference used was the Guidelines for Control of Cosmetic Products in Malaysia, 1st Revision – July 2017, National Pharmaceutical Regulatory Division, Ministry of Health, Malaysia. The formula F4 was tested for arsenic, cadmium, lead, and mercury.

RESULTS AND DISCUSSION

Physicochemical Evaluation

Table 5 showed the results of physicochemical evaluation of antibacterial day protection cream containing cocoa-ABE.

The pH of the antibacterial day protection cream formulations F1, F2, F3, and F4 recorded values of pH 6.7, 7.1, 8.5, and 6.8, respectively, after a day of preparation. The pH of F4 was more acceptable to be applied on skin because it was in the range of skin pH at 6.8. Hence, the antibacterial day protection cream formulation F4 was chosen as it has a similar pH to that of commercial cream, which complies with the standard guideline that it must be in the pH range between 4-6 to avoid any irritation to the skin (Lukić *et al*., 2021).

All formulated antibacterial day protection creams had a characteristically acceptable color and odor. The formulas F1, F2, and F3 had characteristics

of a flower scent after the application of the fragrance. The cream base was white, and the color changed to off-white after the addition of the cocoa-ABE. The example appearance of antibacterial day protection cream is shown in Figure 1. The description of the cream's color was compared to the standard colors such as pure white, white, signal white, slightly yellow, off white, light gray, and other from pantone paper (Phetmung & Sawatdee, 2019). The formula F4 and the commercial day cream were easily removed with tap water when applied to the skin. It indicates that F4 could be used easily without being sticky after use when compared to F1, F2, and F3.

The results of the homogeneity test on the cream, which was observed for a day after the preparation showed the formula F4 had a homogenous texture and was stable without phase separation. This was confirmed by visual examination and touch. Based on the observed cream consistency, developed formulation F4 produced a cream with a smooth consistency that was similar to the commercial cream rather than F1, F2, and F3. The smear test of the antibacterial day protection cream formulation F4 showed that it was easily absorbed and had a non-greasy texture when applied to the skin. The type of smear formed on the skin was not greasy after the application of F4, which indicates it had a good moisturizing effect (Ijaz *et al*., 2022).

The formulations F1, F2, F3, and F4 were examined for phase separation after the centrifugation process, which is an indicator of formulation instability. After centrifugation, the formula F4 remained stable with no obvious phase separation. This might be explained by the similar densities between the oil and water phases or the strong interfacial interaction between the ingredients (Tan *et al*., 2022). The efficacy of the cream depends on its spreadability (Ijaz *et al*., 2022). The spreadability of the cream is measured by the spread's diameter. The spreadability of F1, F2, F3, F4, and the commercial recorded 5.7, 7.8, 5.8, 4.9, and 5.5, respectively. F1, F2, F3, and F4 have different spreadability values because the cream base and ingredients are not the same. Based on the results, F4 formed a uniform distribution of cream compared to other formulations based on physical observation. An ideal cream should be easily spread over a larger area with a small force applied (Tan *et al*., 2022).

Four trial formulations were evaluated based on their pH, appearance, cream removal, homogeneity by visual and touch, smear test, cream centrifugation, and spreadability test. Based on the evaluation of these attributes, the best developed formulation was F4, which has similar characteristics to a commercial product, and it was chosen for further testing, which is the stability test.

Figure 1: Formulated Antibacterial Day Protection Cream Product

Table 5: Evaluation of Physical-chemical Parameters of Antibacterial Day Protection Cream F1, F2, F3, and F4

	Antibacterial Day Protection Cream Formulation				**Commercial**
	F1	**F2**	**F3**	**F4**	
pH	6.7	7.1	8.5	6.8	6.3
Color	Off-white	Off-white	Off-white	Off-white	White
Smell	Good	Good	Good	Good	Good
Homogeneity	Not homogenous	Not homogenous	Not homogenous	Homogenous	Homogenous
Smear test	Greasy	Non-greasy	Non-greasy	Non-greasy	Non-greasy
Cream centrifuge	2 layers of phase separation	2 layers of phase separation	No phase separation	No phase separation	No phase separation
Spreadability diameter (cm)	5.7	7.8	5.8	4.9	5.5

Stability Evaluation

The selected formula F4 was subjected to accelerated stability testing for a duration of three months at room temperature and 40 °C. The cream was kept at different temperatures to observe whether the product complies with the standard and to observe any changes occur initially and monthly thereafter once the product was completely prepared (Maha et al., 2018). The stability of the F4 was evaluated in terms of pH, changes in color, viscosity, and a microbiology limit test.

pH

As shown in Table 6, the pH of formula F4 was in the range of 4.25–4.81 throughout storage in different conditions for three months of observation. The results of the pH measurement were within the acceptable range for skin pH, making it safe for the application of the cream on the skin's surface. This is because, according to Maha et al. (2018), the pH of a topical preparation should be in a range of 4.5 to 6.5, which is the same as the pH of the skin. A highly alkaline cream may lead to scaly skin, whereas a highly acidic cream results in skin irritation (Mawazi et al., 2022).

Color

Based on the reading in Table 7, the amount of color change was between 0.16 and 2.31 throughout the three months of observation under different conditions. This color change value is within the passing limit set by the guideline, which is capped at 5% from the initial color reading (Cosmetics Europe, 2004). A slight change in the color of the formula F4 may occur from the chemical interaction among the formulated raw materials (Phetmung & Sawatdee, 2019). Color is considered as an important element in the cosmetics industry to meet quality standards, enhance the appearance of a product, and also evaluate how the human skin reacts to a makeup sample (Oliveira & Tescarollo, 2021).

Viscosity

The most important parameter in the evaluation is viscosity since it controls many properties of the formulation, including spreadability and pourability of the product from the container (Phetmung & Sawatdee, 2019). Table 8 presents the viscosity readings of the formula F4 at different speeds recorded for three months. The F4 samples kept at different storage conditions exhibited a high viscosity of 19,150–35,750 cPs at 6 rpm; however, when the rpm was gradually increased, F4 samples had a viscosity of 7,950–16,340 cPs at 60 rpm. The viscosity of formula F4 decreases as the speed increases, indicating that the cream shows a shear-thinning behavior, which represents the usage effect of the cream. This may be due to the molecular structure of the cream which makes it move with minimal friction. According to Xie and Jin (2016), the ideal viscosity range for the cream base is between 20,000 and 200,000 cP (20 and 200 Pa.s), which allows the cream to start flowing at 80% of the needed pressure. The evaluation of this parameter helps to evaluate whether a product has an appropriate consistency or fluidity and may indicate whether it is reliable enough to predict, thus indicating the behaviour of the product over a period of time (Kamaruzaman & Yusop, 2021).

Microbiology Limit Test

The developed formulation of the formula F4 was evaluated for its microbial stability. Microbial limit testing for cosmetic and pharmaceutical products is an important part of ensuring the microbial safety of the cream for the consumer, maintaining the quality of the product, and confirming hygienic as well as high-quality handling (Phetmung & Sawatdee, 2019). The growth of microbial organisms indicates contamination has occurred in the product. Table 9 shows the colony count results for the formula F4. The values obtained from a sample were in colony-forming units per gram (CFU/g). Colony-forming units of aerobic mesophilic bacteria in the F4 at the initial month (RT) and in the first month (50°C) were less than 1.00×10^1 CFU/g, while in the third month (RT), it was 3.00×10^1 CFU/g. The colony-forming unit of yeast and mould was recorded at below 1.00×10^1 CFU/g in the initial month and first month, whereas 1.00×10^1 CFU/g was recorded in the third month. The microbiology test performed on the formula F4 showed that the cream did not exceed the microbiological quality control limits with a value less than or equal to 1×10^3 CFU/g or CFU/mL (International Organization for Standardization, 2014; Association of Southeast Asian Nations, 2017). This indicates that the formula F4 is acceptable for topical cosmetic products. According to Jairoun et al. (2020), high levels of contamination of yeast and mould, and aerobic mesophilic bacteria in cosmetic creams can happen because of the cream's rich textures, which were made using growth factors, essential minerals, and high moisture levels that provide an ideal condition for microbial growth.

Table 6: pH of Antibacterial Day Protection Cream Formulation F4

Condition		pH
Week 0	RT	4.81
Week 4	RT	4.81
	40℃	4.81
Week 8	RT	4.74
	40℃	4.25
Week 12	RT	4.81
	40℃	4.54

Table 7: The Color Reading for Antibacterial Day Protection Cream Formulation F4

Condition		Color			
		L*	a*	b*	ΔE*
Week 0	RT	78.52	4.36	12.36	-
Week 4	RT	78.59	4.33	12.68	0.21
	40℃	78.61	4.38	12.86	0.22
Week 8	RT	78.77	4.25	12.41	0.37
	40℃	78.21	4.67	12.53	0.46
Week 12	RT	78.14	4.23	13.50	0.78
	40℃	76.53	4.78	13.42	2.31

L* = light to dark ratio, a* = green to red ratio, b* = yellow to blue ratio, ΔE* = changes of color ratio

Table 8: The Viscosity Reading for Antibacterial Day Protection Cream Formulation F4

Condition		Viscosity (spindle 29); cPs			
		6 rpm	12 rpm	30 rpm	60 rpm
Week 0	RT	29,480	23,540	12,601	11,935
Week 4	RT	27,500	20,200	9,400	7,950
	40℃	19,150	14,200	10,208	8,969
Week 8	RT	29,150	21,025	15,380	12,275
	40℃	27,700	21,550	16,315	13,805
Week 12	RT	24,400	22,745	17,780	14,050
	40℃	25,000	22,870	17,625	14,305

Table 9: Microbiological Test Results of Antibacterial Day Protection Cream Formulation F4

Condition		Aerobic Mesophilic Bacteria	Yeast & Mould
Week 0	RT	< 1.00 x 10^1 CFU/g	< 1.00 x 10^1 CFU/g
Week 4	50°C	< 1.00 x 10^1 CFU/g	< 1.00 x 10^1 CFU/g
Week 12	RT	3.00 x 10^1 CFU/g	1.00 x 10^1 CFU/g

Antibacterial Activity Test

The antibacterial activity of the formula F4 against two Gram-positive bacteria, *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*) was examined by the size of inhibition after exposing the agar to the concentration of F4 for 24 hours. The activity tested was repeated two times or in duplicate to get an average. As shown in Table 10, the antibacterial abilities of the formula F4 against clinical and standard strains of *S.aureus* and *S.epidermidis* were basically the same. The inhibition zones which are the clear zone that appeared around the F4 sample were both 10 mm on average. Ardhany *et al*. (2019) stated that antibacterial activities can be classified into three levels: weak activity (<12 mm), moderate activity (12-20 mm), and strong activity (>20 mm). The results revealed that the formula F4 has an antibacterial activity against *S.aureus* and

S.epidermidis as it has the ability to inhibit their growth. *Staphylococcus* is the most prevalent skin bacteria in humans which can lead to skin infections that could be transmitted from a person to another (Mohammed *et al*., 2020).

Table 10: Antibacterial Activity of Antibacterial Day Protection Cream Formulation F4 After 24 hours Treatment

Bacteria	Inhibition Zone (mm)
Staphylococcus aureus ATCC 6538	10 ± 0.00
Staphylococcus epidermidis ATCC 12228	10 ± 0.00

Antioxidant

Anti-wrinkle creams and lotions containing antioxidant compounds appear to be an effective way of protecting the skin against oxidative stress caused by numerous external sources while helping to reduce the effects of aging on the skin (Ilomuanya et al., 2018; Mahawar *et al*., 2019). The interaction of UV radiation with endogenous photosensitizers generates free radicals and ROS, which accumulate and cause structural and physiological changes in each layer of the skin as well as changes in the appearance of the skin. Table 11 shows the ROS inhibition and viability of the cells after being treated with the formula F4. The acceptance criteria for the CAA assay were done on epigallocatechin gallate (EGCG) as a positive control. The amount of 0.025 mg/mL of EGCG shall indicate at least 50% inhibition of ROS release with cell viability of more than 90% (Wolfe & Liu, 2007). According to the data in Table 11, F4 sample showed no cytotoxicity effect against human keratinocyte cells at a concentration of 1.25 mg/mL to a maximum of 20 mg/mL, with more than 50% cell viability. Furthermore, the formula F4 sample promotes 36.41 ± 1.23 % of ROS scavenging activity at the highest concentration of 20 mg/mL of sample, which indicates the formula F4 has potential antioxidant activity by inhibiting ROS production. However, further research is needed because the antioxidant activity of the formula F4 sample at highest concentration is still below the acceptable limit that should be more than 50% inhibition.

Table 11: The Effect of Antibacterial Day Protection Cream Formulation F4 on The ROS Release and Cell Viability

Concentration (mg/mL)	ROS Inhibition (%)	Cell Viability (%)
20	36.41 ± 1.23	93.09 ± 4.44
10	26.10 ± 2.60	96.17 ± 6.26
5	9.44 ± 0.97	98.09 ± 8.29
2.5	3.38 ± 2.90	103.83 ± 2.19
1.25	-7.10 ± 1.84	102.87 ± 2.99

Anti-inflammatory

Inflammation is the normal response of the immune system to various injuries caused by physical forces, irradiation, extreme temperatures, irritants, and mostly infectious pathogens. Pro-inflammatory cells are responsible for attacking pathogens by releasing pro-inflammatory molecules such as nitric oxide (NO), prostaglandins, and cytokines (Cheenpracha *et al*., 2010). However, chronic inflammation-related diseases may develop when these pro-inflammatory molecules are produced inappropriately over a long period of time. This study evaluated the anti-inflammatory effect of the antibacterial day protection cream formulation F4 using a murine macrophage cell line (RAW 264.7) stimulated with LPS. Nitrite accumulation in the culture medium was measured in order to examine its effect on NO production (Silva *et al*., 2021). Acceptance criteria for the NO inhibition assay were performed on L-NAME as a positive control. The amount of 50 µg/mL of L-NAME showed at least 50% inhibition of NO production with cell viability greater than 50%. Based on the data in Table 12, The formula F4 samples consistently inhibited NO production while maintaining cell viability greater than 50% at concentrations of 0.625 mg/mL up to a maximum of 10 mg/mL. This demonstrates that formula F4 does not have any cytotoxicity effect at the highest tested concentration of the sample, with cell viability of 92.3±9.1%. However, Table 12 shows that the consistent inhibition of NO production is less than 0% and not more than 50%, indicating that the formula F4 has no anti-inflammatory activity.

Table 12: The Effect of Antibacterial Day Protection Cream Formulation F4 on The Inhibition of NO Production and Cell Viability

Concentration (mg/mL)	NO Inhibition (%)	Cell Viability (%)
0.625	-11.1 ± 11.4	86.3 ± 9.2
1.25	-8.8 ± 16.6	86.2 ± 5.3
2.5	-17.2 ± 7.8	94.7 ± 8.4
5.0	-31.7 ± 11.5	89.5 ± 4.2
10.0	-24.1 ± 7.6	92.3 ± 9.1

Heavy Metal Content

Heavy metals are any chemical elements with a specified gravity of at least five times that of water (Selvaraju *et al*., 2020). Heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr), arsenic (As), mercury (Hg), cobalt (Co), and nickel (Ni) can be found as ingredients or impurities in cosmetic raw materials and finished cosmetic products. These metals can be found in cosmetic products due to the raw materials used in their production, particularly when additives and color minerals are used. In addition, the water used for their production could have metallic contaminants (Arshad *et al*., 2020). Based on the results shown in Table 13, the heavy metals levels for the formula F4 are within the permissible limit established by guidelines for the control of cosmetic products (National Pharmaceutical Regulatory, 2017). As was discovered to be present in less than 0.03 mg/kg, which is within the permitted range of less than 5 mg/kg of the standard. According to Selvaraju et al. (2020), prolonged exposure to As can lead to a number of chronic side effects including diarrhea and vomiting, as well as skin disorders, diabetes, and cancers. The level of Pb content measured in the F4 was found to be 0.17 mg/kg, which is within the guideline range of less than 20 mg/kg. Pb is a toxic metal in the environment, not to mention in the body. A high content of Pb in the human body can cause anemia, kidney disease, osteotoxicity, and cancer in several body parts (Afridi *et al*., 2023). The amount of Cd in the F4 was below 0.01 mg/kg and within the permissible limit of 5 mg/kg. According to Attard & Attard (2022), the presence of Cd in cosmetics is due to its colored salts, which range in color from deep yellow to orange. Although some cosmetic products have a very low level, Cd has been linked to a number of toxicities in humans, primarily because it is absorbed after being applied topically. Table 13 showed that the F4 had a Hg level of 0.02 mg/kg, which is less than the permitted limit of 1 mg/kg. A study by Irfan *et al*. (2022) showed the presence of Hg in six samples of cosmetic creams. Some of the samples contained up to 3.4 mg/kg of Hg, and a few samples even exceeded 100 mg/kg. Hg is one of the most dangerous heavy metals, causing negative impacts on the human body. Some face cream products might incorporate Hg in order to achieve a whitening effect. Continuous Hg exposure will inhibit the formation of melanin (melanogenesis), which is a pigment used for light absorption and responsible for producing the color of human skin and hair (Sulistyarti *et al*., 2022). The low content of As, Pb, Cd, and Hg in the formula F4 product results in an effective and safe topical product to use on the skin.

Table 13: Heavy Metal Contents of Antibacterial Day Protection Cream Formulation F4

Heavy metal	Measurement techniques	Requirements of Guidelines for Control of Cosmetic Products	Results (mg/kg)	Remarks
Arsenic (As)	ICP-MS	5 max	< 0.03	Pass
Lead (Pb)		20 max	0.17	Pass
Cadmium (Cd)		5 max	<0.01	Pass
Mercury (Hg)		1 max	0.02	Pass

CONCLUSIONS

The best formulation of the antibacterial day protection cream was chosen according to the results of physicochemical evaluation. The antibacterial day protection cream formulation F4 was successfully developed that met the relevant pharmaceutical characteristics. The prepared formulations showed good spreadability, no evidence of phase separation and good consistency during the study period. Stability parameters like pH, color changes and viscosity of the formulation F4 showed that there were no obvious changes during the study period. This study demonstrated that the antibacterial day protection cream formulation F4 shows anti-microbial, potential antibacterial, potential antioxidant activity, but not for anti-inflammatory activity. However, further study is required since the formula F4 has weak antibacterial properties. Also, the heavy metal content of the formula F4 was found to be below the permissible limit. Therefore, the antibacterial day protection cream formulation F4 was chosen as a development product because of their practical use for daily skincare. Further improvement of the formula will be applied to enhance the potential effect of cocoa-ABE for daily usage. From the present study it can be concluded that it is possible to develop face creams containing cocoa shell extracts and can be used as daily skincare to protect skin.

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