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Research Article

Formulation and The Hen's Egg Chorioallantoic Membrane (HET-CAM) Test to Predict Irritation Potential of FCEE and FCEA Facial Cleansing Gel

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Abstract

Limau citrus (*Citrus amblycarpa* (Hassk.) Ochse) peel contains vitamin C, vitamin A, flavonoids, essential oils, coumarin, and rosmarinic acid derivatives. Due to its antioxidant and antibacterial properties, it can be used as an active ingredient in facial cleansers. This study examined the quality of the ethanol fraction (FCEE) and ethyl acetate fraction (FCEA) formulas derived from *C. amblycarpa* peels and their irritation potential. *Citrus amblycarpa* peel was macerated with 96% ethanol and partitioned successively with *n*-hexane and ethyl acetate. The facial cleansing gel FCEE and FCEA, with concentrations of 0.20%, 0.35%, and 0.50%, respectively. The Hen's Egg Chorioallantoic Membrane (HET-CAM) method was used to determine the potential irritation. The evaluation of the FCEE and FCEA formulas showed a greenish-brownish yellow color, a distinctive aroma of *C. amblycarpa*, and a soft texture. The pH values ranged from 5.66 to 6.27. All formulas were homogeneous, and the viscosity values were recorded in the range of 65.33 to 91.67 dPa.s. The foam stabilities ranged from 65.94 to 69.40%, and the formulas demonstrated effective cleaning ability. The results of the HET-CAM irritation test show that the irritation score of the positive control (SLS 30%) was 12.6 (strong irritation category), that of the negative control (water for injection), and that of all FCEE and FCEA facial cleansing gel formulas, which have an irritation score of 0 (no irritation category). The study presents quantitative and qualitative data in evaluating facial skin cleansers. These results demonstrate safe formulation.

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INTRODUCTION

The facial integument serves as a primary human interface that is frequently compromised by environmental stressors, including pollution-derived free radicals and ultraviolet radiation. Such prolonged exposure disrupts structural integrity and impairs protective functions, leading to chronic cutaneous issues[1]. Furthermore, the proliferation of microorganisms within the sebaceous glands can trigger inflammatory responses, particularly affecting the stratum germinativum and stratum corneum[2]. These concerns are reflected in a demographic survey of 9,010 female respondents in Indonesia, aged 12–66 years, which identified facial dullness as a predominant concern, followed by comedones (57.1%), enlarged pores (51.0%), oily skin (38.9%), rhytides or fine lines (30.3%), and varying degrees of sensitivity or dryness[3]. To address these issues, facial cleansing gels serve as essential cosmetic vehicles designed to purify the skin and neck, effectively removing sebaceous blockages, cellular debris, and cosmetic residue while preserving essential moisture[4]. Formulations containing

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surfactants generally exhibit superior cleansing efficacy compared to water- or alcohol-based foams[5]. These products increasingly incorporate natural bioactives as functional agents to mitigate aging and dullness, leveraging surfactants' ability to reduce staphylococcal colonization and improve overall skin hygiene[6,7].

Phytochemical investigations reveal that Limau citrus (*Citrus amblycarpa* (Hassk.) Ochse) peel is rich in phenolic acids (including caffeic, p-coumaric, ferulic, and sinapic acids) alongside flavanones and flavones that are intrinsically linked to the fruit's cell walls[8,9]. The ethyl acetate fraction is particularly noted for its concentration of flavonoids, polyphenols, and alkaloids[10]. The antioxidant potency of these extracts is quantified by EC_{50} values, where the methanol extract (94.01 µg/mL) and ethyl acetate fraction (134.02 µg/mL) fall into strong and moderate antioxidant categories, respectively, demonstrating inhibition rates up to 89%[11]. When integrated into cosmetic topicals, these phenolic compounds offer a promising alternative for preventing oxidative damage and premature aging[12]. However, there remains a notable research gap regarding whether these specific fractions maintain their biological efficacy and cleanability when incorporated into functional cosmetic bases. While natural in origin, their interaction with synthetic surfactants may still elicit cutaneous irritation, and safety data regarding ocular tolerance remains sparse. This study addresses this gap by utilizing the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) method, prioritizing modern alternative protocols over traditional animal models to provide high-sensitivity compatibility data for conjunctival tissue.

For topical cosmetics, particularly surfactant-based cleansers with specific claims, comprehensive safety evaluations are mandatory for regulatory approval[13,14]. The HET-CAM assay serves as an effective *in vivo* alternative that significantly reduces animal use[15]. This method utilizes the highly vascularized chorioallantoic membrane of chicken embryos, which serves as a reliable model for human conjunctival tissue[16,17]. Given that bioactive compounds in *C. amblycarpa* peel have demonstrated potential to reduce inflammation and promote radiant skin appearance, this study aimed to formulate and evaluate facial cleansing gels using both ethanol (FCEE) and ethyl acetate (FCEA) fractions[18]. This research compares the quality of FCEE and FCEA formulations based on organoleptic properties, pH, homogeneity, viscosity, and cleanability, and rigorously assesses their irritation potential using the HET-CAM to ensure consumer safety and therapeutic efficacy.

MATERIALS AND METHODS

Materials

The primary botanical sample for this study consisted of *C. amblycarpa*, which was formally identified and authenticated by the Indonesian Institute of Sciences, Bogor (Reference No. B-957/V/DI.05.07/12/2021), and the Biology Laboratory at Universitas Tanjungpura, Pontianak. Chemical reagents and excipients utilized in the formulations included sodium lauryl sulfate, sodium carboxymethyl cellulose, sodium chloride, sodium benzoate, and distilled water. Solvents and specialized aqueous components comprised technical-grade *n*-hexane, 96% ethanol, ethyl acetate, and water for injection sourced from Sanbe Farma (Indonesia), alongside oleum lemon acquired from Alkamid Co. (Tehran, Iran). The experimental biological model utilized Leghorn chicken eggs to assess the intended parameters.

Comprehensive laboratory processing and analytical measurements were facilitated by a suite of specialized instrumentation. Precise environmental and thermal control were maintained using a Dragon LAB RE-10 Pro rotary evaporator, a Memmert ICO50 incubator, and a Gemmyco digital oven (#YCO-N01). Analytical assessments were conducted utilizing a Sartorius BL 210S analytical balance, a Smart Sensor AS218 pH meter, a Brookfield viscometer (Ntech®), and a Sigma-Aldrich thermometer (St. Louis, USA). Quantitative and structural evaluations were supported by Vernier calipers, a high-precision stopwatch, and calibrated weights (AT CB: 50 g, 100 g, and 200 g). General laboratory preparation and surgical procedures involved the use of maceration jars, filter paper, mortar and pestle, Pyrex glassware (beakers, measuring flasks, test tubes, and stirring rods), a Miyako® blender, watch glasses, stainless steel spoons, surgical scissors, tweezers, and 1 cc syringes.

Methods

Sample collection and phytochemical processing

The raw materials for this study, specifically *C. amblycarpa* fruits, were sourced from a plantation located in the Sungai Ambawang District of West Kalimantan, Indonesia. During processing, the fruit peels were meticulously separated from

the flesh, yielding 6.7 kg of wet material, which was subsequently chopped and dehydrated in a controlled oven at 40°C to produce 0.489 kg of dry simplicia. This dried material was pulverized in a mechanical blender and macerated in 96% ethanol. The resulting extract was concentrated via a rotary vacuum evaporator to yield 150 g of crude *C. amblycarpa* peel extract[19]. To further isolate active fractions, a liquid-liquid sequential partition was performed using an *n*-hexane/ethyl acetate (1:1) solvent system, yielding distinct ethanol and ethyl acetate fractions. These were further concentrated using a rotary vacuum evaporator to obtain yields of 37.29 g and 17.16 g, respectively[10]. Ethical oversight for the study was provided and approved by the Ethics Committee of the Institut Teknologi dan Kesehatan Muhammadiyah Kalimantan Barat.

Formulation and physicochemical evaluation of FCEE and FCEA

The development of the facial cleansing gel was guided by a pre-formulation study and a foundational base formula, as detailed in **Table I**, conducted in triplicate to ensure reproducibility. The gel base was prepared by dispersing CMC sodium in distilled water at 80–90°C (1:20 ratio) for 30 minutes (Mixture A). Concurrently, SLS, sodium benzoate, and NaCl were dissolved in distilled water, followed by the incorporation of FCEE or FCEA at varying concentrations (Mixture B). Mixture B was integrated into Mixture A under constant stirring until a homogeneous consistency was achieved, after which oleum lemon was added as a fragrance[4].

Table I. Facial cleansing gel formulations involving FCEE and FCEA.

Ingredients	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 (%)	F6 (%)
FCEE	0.20	0.35	0.50	-	-	-
FCEA	-	-	-	0.20	0.35	0.50
SLS	1	1	1	1	1	1
CMC sodium	3	3	3	3	3	3
NaCl	2	2	2	2	2	2
Sodium benzoate	0.5	0.5	0.5	0.5	0.5	0.5
Oleum lemon	0.03	0.03	0.03	0.03	0.03	0.03
Distilled water	ad 100	ad 100	ad 100	ad 100	ad 100	ad 100

The resulting gels underwent comprehensive evaluation, beginning with organoleptic testing for color, odor, and texture performed visually by ten trained panelists using a structured questionnaire[20]. The pH was quantified using a digital pH meter by dissolving 1 g of the sample in 10 mL of distilled water[21]. Physical uniformity was assessed through visual homogeneity tests on glass slides, while rheological properties were determined using a Brookfield viscometer equipped with spindle No. 4 at 10 rpm[19]. Foam stability was evaluated by agitating 1 g of the formula in 10 mL of water within a 25 mL graduated cylinder for 20 s. Stability was calculated by measuring foam height initially and after a 5-minute rest period, with a target stability range of 60%-70%, indicating optimal performance[22,23]. The qualitative cleaning efficiency was tested by applying 0.035 g of liquid foundation and lipstick to glass slides, allowing them to set for 5 minutes. The gel (0.5–1 mL) was applied with gloved hands using a standardized stroke count (10 for lipstick and 25 for foundation), followed by a water rinse, with water alone as a negative control[24].

Data analysis

Biocompatibility was assessed using the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) method. Fertilized white leghorn eggs were obtained from Alfares Farm (Sambas, Indonesia) and incubated at 37.0 ± 0.1°C with 65 ± 2% relative humidity, including thrice-daily manual rotations. On the third day of incubation, eggs were prepared for eight treatment groups (F1–F6, positive control, and negative control) in triplicate. The shell was carefully breached to expose the chorioallantoic membrane (CAM), which was moistened with 0.9% NaCl and cleared of the internal membrane[25,26]. After a 20 minute stabilization period, test substances (0.1 g of FCEE/FCEA) and controls (30% SLS as positive and water for injection as negative) were applied. The membrane was observed for vascular reactions, specifically hyperemia (vasodilation), hemorrhage (bleeding), and coagulation or lysis (vessel disappearance)[14,16]. The irritation index (I) was calculated using the following **Equation 1**. The resulting scores were categorized according to standardized irritation scales: ≤ 0.9 indicating no or mild irritation, 1.0–4.9 for moderate irritation, 5.0–8.9 for significant irritation, and 9.0–21.0 representing strong irritation[15,27].

$$T = \frac{301 - H}{300} \times 5 + \frac{301 - L}{300} \times 7 + \frac{301 - C}{300} \times 9 \quad [1]$$

RESULTS AND DISCUSSION

Taxonomic authentication was performed to verify the precise identity of the plant material, ensuring the scientific validity of the study, and confirming the specimens as *C. amblycarpa* (Hassk.) Ochse. The fruits were separated from their peels, dried in a controlled oven at 40°C to preserve thermolabile constituents, and subsequently pulverized. This process yielded 489.68 g of dry simplicia, representing a 7.31% recovery relative to the starting material. Quantifying this yield is essential for assessing the concentration of biomass remaining after post-harvest processing and determining the overall effectiveness of the drying phase[28]. The material was then macerated in 96% ethanol and concentrated using a rotary vacuum evaporator at 60°C and 100 rpm, yielding a crude extract with a 45.65% yield. To isolate metabolites based on polarity, the extract was partitioned successively with *n*-hexane and ethyl acetate, yielding ethanol and ethyl acetate fractions with yields of 24.86% and 11.44%, respectively. These fractions serve as the primary active ingredients for the development of a specialized facial cleansing gel formulation[10].

The formulation and evaluation of FCEE and FCEA facial cleansing gels use SLS as the primary surfactant, categorizing the preparations as foaming cleansers due to their superior foam generation[5]. Within these cosmetic matrices, NaCl serves as both an isotonic agent and a thickener, while sodium benzoate is incorporated as a preservative to inhibit microbial proliferation in the high-water-content environment of the gel[29]. Organoleptic evaluation of the ethanol and ethyl acetate fractions derived from *C. amblycarpa* peel assessed the physical appearance, odor, and texture of the preparations[30]. The resulting greenish-yellow to brownish-yellow hues were directly influenced by the inherent blackish-green color of the plant fractions used, while the final texture remained soft with a characteristic oleum citrus aroma.

pH assessment is critical to ensure the preparation's acidity is compatible with human skin; overly acidic gels may irritate, while excessive alkalinity can lead to skin dryness[31]. All formulations (**Table II**) remained within the acceptable range for facial cosmetics (pH 3–9) and aligned with skin physiological standards (pH 4.5–6.5), with F2 recording the lowest value (5.66 ± 0.32) and F4 the highest (6.33 ± 0.02). At these physiologically compatible conditions, the preparations are more readily tolerated, minimizing the risk of irritation or tissue injury[32]. Aligning with previous research suggesting healthy skin typically falls between pH 5.5 and 7.5, these SLS-based formulations are optimized to clean effectively without stripping essential lipids and proteins[22,33,34]. By maintaining a pH between 5.5 and 7.0, the products help preserve the stratum corneum and normal skin flora, preventing the infection risks associated with alkaline detergents[31,35].

Table II. pH result test.

Formulas	pH ± SD
F1	5.90 ± 0.55
F2	5.66 ± 0.32
F3	6.16 ± 0.06
F4	6.33 ± 0.02
F5	6.27 ± 0.01
F6	6.20 ± 0.02

Homogeneity testing confirmed that all six formulas were evenly mixed, a factor influenced by the hydration process of the gelling agent and the solubility of the fractions. A consistent gel structure indicates successful incorporation of the surfactant and active plant components, ensuring the absence of lumps or coarse grains. Similarly, viscosity measurements determined the thickness and ease of application; all formulas met the 40–100 dPa.s requirement (**Table III**). F5 recorded the lowest viscosity (65.33 ± 10.12 dPa.s), whereas F6 exhibited the highest (96.00 ± 1.73 dPa.s). The stability of these rheological properties is largely governed by CMC sodium, which maintains a stable viscosity within the pH range of 4–10[36].

Foam stability is a critical attribute for both cleansing efficacy and consumer psychological acceptance[18]. All formulations demonstrated stable foam heights within the 60% to 70% range, satisfying established standards (**Table IV**)[37]. SLS serves as both the primary surfactant and a foam stabilizer; the generated foam sequesters dirt, preventing its redeposition onto the skin surface during rinsing[38].

Table III. Viscosity result test.

Formulas	Viscosity (dPa.s) ± SD
F1	68.00 ± 4.36
F2	80.33 ± 4.93
F3	91.67 ± 2.52
F4	72.67 ± 5.86
F5	65.33 ± 10.12
F6	96.00 ± 1.73

Table IV. Foam stability result test.

Formulas	Foam stability (%) ± SD
F1	65.94 ± 4.24
F2	69.40 ± 0.51
F3	66.69 ± 5.08
F4	67.0 ± 2.9
F5	69.2 ± 0.4
F6	68.6 ± 1.0

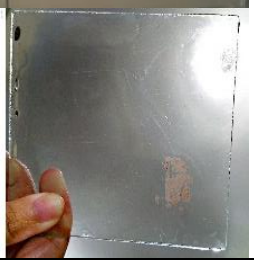
Cleansing efficiency, or detergency power, was evaluated by the removal of foundation and lipstick stains[39]. All formulations successfully cleared these contaminants, as surfactants reduce the interfacial tension between nonpolar soil and rinsing water (**Table V**). The negative charge in the SLS structure facilitates the formation of micelles that trap oil and dirt particles for easy removal[40]. While previous studies have used oil-based cleansers, such as rubber seed or tea seed oil, to emulsify water-resistant makeup[6,18], the FCEE and FCEA gel formulations use polar and semipolar active ingredients to enable the efficient removal of water-based residues.

The irritation potential of the formulated facial cleansing gels was rigorously evaluated using the HET-CAM method, conducted following ethical approval from the Ethics Committee of ITEKES Muhammadiyah West Kalimantan (No. 26/II.I.AU/KET.ETIK/I/2024). This methodology incubates White Leghorn chicken eggs for 10 days at 37°C, providing an alternative to the *in vivo* Draize test by using CAM as a surrogate for conjunctival tissue. The CAM is a highly vascularized membrane containing complex networks of arteries, veins, and capillaries that respond to inflammatory stimuli, as in rabbit eye tissues[15,16]. In this study, a semi-quantitative scoring system was employed to assess vascular responses, specifically hemorrhage, lysis, and coagulation, over a 300-second observation period, with a maximum possible irritation score of 21[25,41]. Positive and negative controls were established using 30% SLS and water for injection, respectively, to ensure assay sensitivity and reliability.

As summarized in **Table VI**, all facial cleansing gel formulas containing FCEE and FCEA of *C. amblycarpa* peel yielded an irritation score of 0, placing them in the "no irritation" category. Conversely, the positive control exhibited a score of 12.6 ± 3.0, categorized as "strong irritation" due to pronounced coagulation and lysis triggered by the dissolution of protective sebum[36]. These findings align with previous cosmetic research on herbal formulations, such as *Syzygium polyanthum* and *Phyllanthus amarus* gels, which reported similar non-irritating profiles[42,43]. The safety of the current formulas is largely due to the use of non-irritant excipients, such as CMC sodium and NaCl. While sodium benzoate and SLS can induce dermatitis or corneal damage at high concentrations, the SLS in the FCEE and FCEA formulas was maintained within a low, safe range of no more than 2.5% as an additive[14,25,43]. Although interactions between surfactants and other ingredients can influence micelle stability and corneal permeability, the consistent zero-score results across all test formulas indicate that the ingredients are well-tolerated. Ultimately, these HET-CAM results confirm that the facial cleansing gel incorporating *C. amblycarpa* peel fractions is safe for facial and ocular contact.

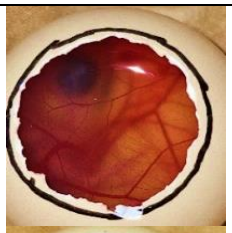
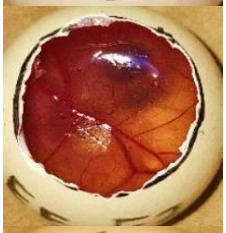
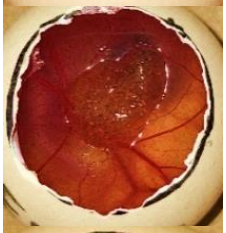
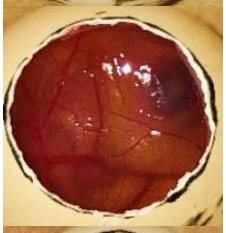
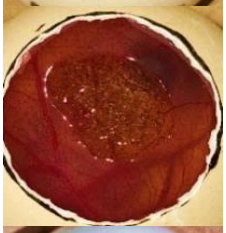
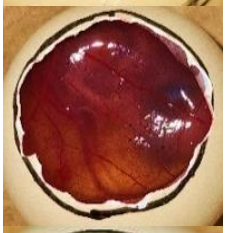
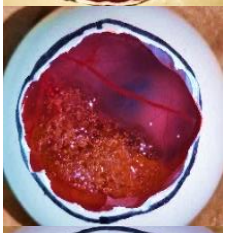
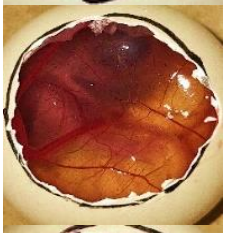
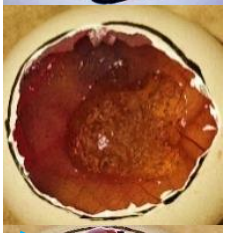
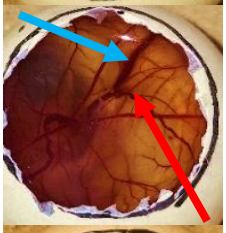
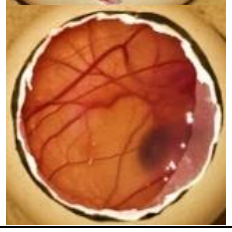
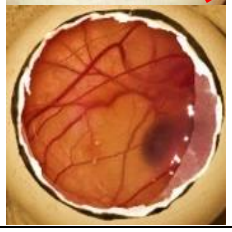
The successful integration of high removal efficiency with an exceptional safety profile underscores the potential of *C. amblycarpa* peel as a high-performance functional cosmetic ingredient. This synergy suggests that the specific bioactive profiles of the FCEE and FCEA fractions effectively complement the surfactant system without increasing the risk of mucosal sensitivity. Such results represent a distinct advantage over conventional synthetic cleansers, which frequently prioritize harsh cleaning power at the expense of dermatological comfort[44]. These findings provide a robust biochemical justification for utilizing natural citrus-derived fractions in the development of sensitive skin care products. Yet this research establishes a solid foundation for creating innovative, plant-derived facial cleansers that meet rigorous standards of efficacy and ocular safety.

Table V. Cleansing result test.

Formulas	Before	After
F1		
F2		
F3		
F4		
F5		
F6		
C-		

Note: C-: water; Yellow: liquid foundation; Orange: lipstick.

Table V. Cleansing result test.

Formulas	Before	After	Irritation score/Category
F1			0/ no irritation
F2			0/ no irritation
F3			0/ no irritation
F4			0/ no irritation
F5			0/ no irritation
F6			0/ no irritation
C+			12.6 ± 3.0/ Strong irritation (blue arrow: coagulation; red arrow: lysis)
C-			0/ no irritation

Note: C+: 30% SLS; C-: water for injection.

CONCLUSION

The comprehensive evaluation of FCEE and FCEA facial cleansing gel formulations indicates that both series exhibit favorable organoleptic properties, including a stable yellowish color, a pleasant citrus aroma, and a consistently soft texture. Each formula maintained a pH profile between 5.66 and 6.33 and demonstrated high homogeneity, confirming their suitability for application on human skin without disrupting its natural acid mantle. Technical performance remained strong across all batches, with viscosities ranging from 65.33 to 96.00 dPa.s and foam stability exceeding 65%, ensuring both effective cleaning ability and a stable user experience. Crucially, toxicological screening through the HET-CAM assay yielded an irritation score of zero for all formulas, significantly outperforming the 30% SLS positive control and matching the safety profile of sterile water. These results collectively signify that the FCEE and FCEA preparations are effective removal agents that are safe and non-irritating for topical use. While these preliminary findings are highly positive, human patch test validation is essential to confirm the clinical safety and dermatological compatibility of these natural cleansing gels.

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AUTHORS' CONTRIBUTION

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Visualization: -
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DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declared no conflict of interest related to this research.

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