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In vitro SAFETY EVALUATION OF PALM TOCOTRIENOL-RICH FRACTION NANOEMULSION FOR TOPICAL APPLICATION

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ABSTRACT

The application of nanotechnologies in cosmetics industry has resulted in the introduction of new nanomaterials for topical applications. Nanomaterial overcomes issues of limited penetration and low bioavailability of non-soluble bioactive agents. While there are many advantages of using nanomaterials, recent development in using submicron particles for enhance skin penetration has raised the concern of safety including the increase potential to induce skin irritation and allergic reactions on the skin. The studies on irritation potential of palm tocotrienol-rich fraction (TRF) nanoemulsion using in vitro *ocular and dermal irritection assays, reconstructed human epidermis and human corneal epithelium tests were investigated. Palm TRF nanoemulsion did not exhibit any potential skin irritation in the* in vitro *ocular and dermal irritation assessment. The studies showed that when reconstructed human corneal epithelium and human epidermis models were treated with the tocotrienol macroemulsion and nanoemulsions, no indication of irritancy to the eyes or dermal tissues were observed giving a mean tissue viability of more than 60% and 50%, respectively. The non-irritant category is classified as Category 1 according to United Nations Globally Harmonised System of Classification and Labelling of Chemicals.* In vitro *studies showed no ocular or dermal irritation potential indicating possible topical application of palm TRF nanoemulsion.*

Keywords: safety evaluation, tocotrienol-rich fraction, nanoemulsion, ocular irritation, dermal irritation, reconstructed human epidermis, reconstructed human corneal epithelium.

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INTRODUCTION

Safety evaluation of an ingredient used in skin care and cosmetic products is important to protect the consumers who will be using the products. When new ingredients become available in the cosmetics and personal care industry, new cosmetic and skin care products are being formulated to capture new and niche market segments. According to Scientific Committee for Cosmetics and Non-Food Product of the European Union Commission, several basic requirements or dossier must be ascertained before an ingredient or product can be commercialised (Anon, 2002). The requirements needed to prepare a dossier are: chemical identification, physical form, molecular weight, purity, characterisation of impurities, solubility, partition coefficient and *in vitro*/*in vivo* irritation/sensitisation studies. All the requirements except the *in vitro*/*in vivo* studies can be made available through chemical elucidation and experimentation.

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The toxicity of a substance is a concern, and may limit its application in cosmetic and skin care and if it is highly detrimental, it may have to be abandoned altogether. In order to assess the safety of a cosmetic ingredient, a tiered-approach has been suggested (Salminen, 2002). Although international cosmetics regulations vary greatly, most countries require that cosmetics manufacturers need to ensure that their products are safe for their intended use. This often requires compilation of toxicity data on the ingredients and/or the cosmetics products. The first level gathers the available toxicity data on compounds with similar chemical structures that are expected to exhibit similar toxicological properties. Ingredients with immediate toxicity levels are eliminated from further development. The second level evaluates the ingredient in toxicity tests (skin irritation or dermal sensitisation) after it has passed the initial performance testing. The final level involves conducting all the necessary toxicity tests for full safety assessment for the ingredient.

Vitamin E, a naturally occurring antioxidant is an essential lipid soluble vitamin which describes bioactivities of both tocopherol and tocotrienol derivatives. While tocopherols are generally present in nuts and common vegetable oils, tocotrienols are mainly concentrated in palm oil (Ahsan *et al*., 2015). There are several isolated reports of skin irritation or allergic reactions from exposure to vitamin E, particularly tocopherols and their derivatives. Tocopherol at 1% concentration was reported as a weak primary skin irritant in rabbits and weak cumulative irritant in guinea pigs (Andersen, 2002). The study also reported that tocopheryl acetate produced weak to moderate conjunctival irritation in rabbits. Sibyl *et al*. (2014) studied acute toxicity of parenterally administered vitamin E on mice and observed that above 200 mg kg^{-1}, a mild to moderately severe dermatitis at the injection site. There are also reports of skin irritation reactions such as erythema, rash, dermatitis and desquamation of skin after application of cosmetic products containing α-tocopherol (Santos *et al*., 2008; Ohko *et al*., 2012), vitamin E acetate (Corazza *et al*., 2012) and tocopheryl nicotinate (Oshima *et al*., 2003). These reports are considered isolated cases and may be due to hypersensitivity of the subjects.

There are no reports on adverse skin reactions on the topical application of tocotrienols. Zafarizal *et al*. (2008) showed that undiluted palm tocotrienol-rich fraction (TRF; composed of 50% tocotrienol/tocopherol complex, with 20% d-γ-tocotrienol, 5% d-δ-tocotrienol, 13% d-α-tocotrienol, and 12% d-α-tocopherol) was practically non-irritating to rabbit skin. At concentration of $\leq$ 5%, TRF was not an irritant in a patch test or a sensitiser in human repeated insult patch test. In 2010, United States Food and Drug Administration has reviewed and concluded that TRF is generally recognised as safe (GRAS) for use as an ingredient in food (USFDA, 2010).

Tocopherols and tocotrienols have been extensively used as an active ingredient in cosmetics due to their potent antioxidant properties. However, lipophilic molecule does not favourably partition out of the stratum corneum into the more aqueous viable epidermis (Potts *et al*., 1992), preventing deeper diffusion and further capillary uptake into circulation. In comparison, hydrophilic substances penetrate across the skin harder than lipophilic penetrants and as low as 10^4-fold differences in flux were noted (Sznitowska *et al*., 1998). In order to overcome the epidermal barrier as to increase transdermal transport, various transdermal carrier systems have been developed including nanoemulsion.

Advanced submicron delivery systems such as nanoemulsion and nanostructured lipid carrier may deliver more active ingredient into skin. However, the same properties that make nanomaterials desirable in these various applications have the potential to alter the biological properties that impact the environment, health, and safety of these materials (Boverhof and David, 2010; Nel *et al*., 2006).

Although nanomaterials are chemicals, their crystalline structure, water insolubility, and slow dissolution mean that molecules do not as easily reach the biological systems as they do for soluble chemicals (Boverhof and David, 2010). The submicron particles which penetrate deeper skin layer may impart higher safety risk. Thus, it warrants further safety assessment for these new delivery systems to ensure safe delivery of active ingredient.

MATERIALS AND METHODS

Materials

Palm TRF was purchased from Sime Darby Research Sdn Bhd, Malaysia. Materials for base emulsion: polyoxyethylene (20) sorbitan monolaurate (Sigma, USA), capric/caprylic triglyceride (KLK Oleo Sdn Bhd, Malaysia) and Phenonip (Clariant, UK). The SkinEthics™ Reconstructed Human Epidermis (RHE) and Human Corneal Epithelium (HCE) models and the culture maintenance medium were purchased from EpiSkin SA, Lyon, France. The 3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide reagent (MTT), Dulbecco's phosphate-buffered saline (PBS) and sodium dodecyl sulphate (99%) were purchased from Sigma Aldrich, Steinheim, Germany. Methyl acetate (MA) was purchased from Acros Organivs,

New Jersey, USA, hexane (HPLC grade, 99%) and isopropanol (HPLC grade, 99%) were purchased from Friedmann Schmidt, Parkwood WA, Australia.

Methods

High performance liquid chromatography (HPLC). The method by Sundram and Nor (2002) was adopted with slight modification to determine the composition of tocopherol/tocotrienol fractions. A 0.5 ml of sample was pipetted into a capped centrifuge tube. The sample was mixed with 4 ml hexane, cap, and vortex the solution in the tube for 2 min. The solution was then subjected to centrifugation at 3000 rpm for 2 min to separate the phases and the upper layer was removed. The removed upper layer was then re-extracted with an additional 4 ml hexane and all the extracts were combined. The extract was evaporated under nitrogen and the solvent was reconstituted in 100 µl hexane and sonicated for 60 s. The sample was transferred to sample vials for HPLC (Agilent 1100 Series, USA) analysis. *Table 1* shows the HPLC method specifications.

Preparation of pre-mix emulsion. The raw materials for the preparation of emulsion were divided into two groups, the water and oil phases based on the nature of the substances. The pre-mix emulsion was prepared using 1% (w/w) palm TRF, 14% (w/w) capric/caprylic triglyceride, 5% (w/w) polyoxyethylene (20) sorbitan monolaurate and deionised water as the remaining diluent. Another formulation with 5% (w/w) palm TRF and 10% (w/w) capric/caprylic triglyceride with similar amount of polyoxyethylene (20) sorbitan monolaurate and deionised water was also prepared. The combined water soluble ingredient was heated to 75°C with stirring until all ingredients have dissolved. The combined oil soluble ingredients were also heated to 75°C with stirring until all ingredients melted as one clear phase. The water phase was then homogenised at 10 000 rpm for 2 min using a rotor stator (Polytron, PT3100) and the oil phase was then added until both phases formed an emulsion. After 2 min of homogenisation, the sample was then subjected to high shear homogenisation for the preparation of nanoemulsion.

TABLE 1. THE CONDITIONS FOR HPLC ANALYSIS FOR DETERMINING PALM TOCOTRIENOL-RICH FRACTION COMPOSITION

Specification	Technical description
Column	Normal phase silica column (Supelco Lichrosorb SI-60-5 µm,
Mobile phase	Dimension: 250 x 4.6 mm)
Flow	Hexane: isopropanol (99:1)
Injection	1 ml min^{-1}
Wave length	20 µl
Detector	295 nm and 330 nm fluorescence

Note: HPLC - high performance liquid chromatography.

Preparation of palm TRF nanoemulsions. For the preparation of nanoemulsion, the freshly prepared pre-mix emulsion was directly poured into a high-pressure homogeniser (Microfluidiser 110P, Microfluidics, USA). The sample was then homogenised at a pre-set pressure of 15 000 psi. For each cycle of homogenisation, the resultant nanoemulsion was then subjected to a pre-set thermostated chamber at 15°C to reduce the nanoemulsion temperature to 15°C. The homogenisation was repeated for a few cycles to ensure a homogenised and narrow distribution of nanoemulsion particle size was achieved. The final products were put into sample bottles and wrapped with aluminum foils.

Determination of encapsulation efficiency. The encapsulation efficiency was determined according to Davidov-Pardo and Mcclements (2015) with slight modification. The TRF nanoemulsion was filtered using polyethersulfone membrane (0.22 micron, Sartorius) and centrifuged at 4500 rpm for 15 min. The sample was then subjected to solvent extraction with hexane. The amount of encapsulated TRF was determined using HPLC. The encapsulation efficiency was calculated as the amount of TRF present in the nanoemulsion compared with the TRF initially used in the preparation.

Particle size measurements. The mean droplet size and zeta potential were determined by dynamic light scattering and laser-doppler anemometry, respectively, using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, United Kingdom). The size analyses were performed at a scattering angle of 173°, after dilution of the samples in ultrapure water (Purelab Flex 3, Elga, Germany). For zeta potential analysis, the samples were diluted in ultrapure water and placed in electrophoretic cells where a potential of ±150 mV was applied. The zeta potential values were calculated as mean electrophoretic mobility values by using Smoluchowski's equation.

In vitro ***ocular and dermal irritation assay.*** The *in vitro* ocular and dermal irritation assays are based on the principle that chemical compounds will promote measurable changes in target biomolecules and macromolecular structures. Miyazawa *et al*. (1984), Hayashi *et al*. (1994) and Vinardell *et al*. (1999) had demonstrated that the induced processes of protein denaturation and disaggregation in *in vitro* techniques are indicators of irritations when surfactants are applied to the ocular or dermal membrane. Consequently, *in vitro* test based on proteins denaturation may be employed to

predict the *in vivo* toxic effect of chemicals and formulations.

The induction of corneal or dermal irritancy by any substance or ingredient is related to its denaturation and disruption of corneal proteins or alteration to the structure of keratin, collagen and other dermal proteins. The ocular and dermal irritection assay (*In Vitro* International, Irvine, CA) that mimics biochemical phenomena is an alternative method to animal irritancy studies (Draize test). The *in vitro* irritation assays have been reported to correlate well with the *in vivo* irritancy tests (Sina *et al*., 1995).

The Irritection Assay (*In Vitro* International, Irvine, CA) for both ocular and dermal test requires two essential components: a membrane disc that permits controlled delivery of the test material to a reagent solution, and a proprietary reagent solution of proteins, glycoproteins, lipids and low molecular weight components that self-associate to form a complex macromolecular matrix. The changes in protein structure induced by the test material were readily quantified by the changes in turbidity at OD405 of 405 nm of the reagent solution for ocular irritation, while in the dermal irritation assay, the extent of dye release and protein denaturation was quantitated by measuring the changes in optical density of the reagent solution at OD450 of 450 nm.

The ocular irritancy potential and the dermal irritancy potential of a test sample is expressed as an Irritation Draize Equivalent (IDE) and Human Irritancy Equivalent (HIE), respectively. The values of IDE and HIE have been reported to correlate well with *in vivo* investigations by the Draize method and human test, respectively. The predicted *in vivo* classifications, based on these scoring systems, are shown in *Tables 2* and *3*.

For ocular or dermal irritation assays, a standard volume dependent dose response was obtained. The following doses of neat sample were applied for the analyses: 50, 75, 100 and 125 mg were placed on the membrane discs. Reagent and blank buffer were added to a 24-well assay plate. The assay plate was incubated at 25°C for 24 hr. The membrane was then removed from the assay plate and 250 µl reagent and buffer were transferred to a 96-well reading plate. The plate was then inserted into a plate reader (MRX Microplate Reader, Dynex Technologies, Inc., Chantilly, VA) which read the optical density of the samples.

TABLE 2. CLASSIFICATION OF IRRITATION DRAIZE EQUIVALENT (IDE) SCORE TO *in vivo* IRRITANCY CLASSIFICATION (Draize test)

IDE score	Predicted ocular irritancy classification
0 – 12.5	No or minimal irritant
12.5 – 30.0	Mild irritant
30.0 – 51.0	Moderate irritant
> 51.0	Severe irritant

TABLE 3. CLASSIFICATION OF HUMAN IRRITANCY EQUIVALAENT (HIE) SCORE TO *in vivo* IRRITANCY CLASSIFICATION

HIE score	Predicted dermal irritancy classification
0 – 0.90	Non-irritant
0.90 – 1.20	Non-irritant/irritant
1.20 – 5.00	Irritant

RHE test. Each test substance (test material, negative and positive controls) was topically applied concurrently on three tissues replicates for 42 min at room temperature (RT, comprised between 18°C to 24°C). The exposed tissues were then rinsed with PBS and air-dried. Epidermis were then transferred to fresh medium and incubated at 37°C for 42 hr. Tissue viability was assessed by incubating the tissues for 3 hr with 0.3 ml MTT solution (1 mg ml^{-1}). The formazan crystals were extracted from the RHE using 1.5 ml isopropanol for 2 hr at RT and quantified by spectrophotometry at 570 nm wavelength. Sodium dodecyl sulphate (SDS 5% solution), and PBS treated epidermis were used as positive and negative controls, respectively. For each treated tissue, the tissue viability is expressed as the percentage of the mean negative control tissues. Treated tissues giving values of tissue viability of below 50% are considered as being treated with 'an irritant' test substance.

HCE test. The SkinEthic™ HCE model comprised of immortalised human corneal epithelial cells cultured in a chemically defined medium and seeded on a synthetic membrane on the air-liquid interface. The tissue structure obtained was a multilayered epithelium resembling the *in vivo* epithelium representing about 5-7 cell layers and a surface area of 0.5 cm^2. The tissue constructs which are made of at least four viable layers comprising of columnar basal cells, transitional wing cells and superficial squamous cells. The structural features of the corneal tissues, such as the presence of mature desmosomes and intermediate filaments, as well as the expression of corneal specific cytokeratin were similar to that of the normal HCE (Nguyen *et al*., 2003).

The tissues were shipped in an agarose semi-solid culture medium. Upon arrival, they were transferred to a new maintenance medium, 1 ml/well in a 6-well plate and incubated at 37°C with 5% CO_2 in a humidified incubator for overnight. After incubation, the medium was renewed and the tissues were topically exposed to 30 µl of 10% (w/v) tocotrienol macroemulsion or nanoemulsion for 30 ± 2 min at 37°C at 5% CO_2 humidified incubator. Three tissues were used for a single test substance.

PBS was used as the negative control while methyl acetate was used as the positive control. After 30 min of treatment, tissues were rinsed at least two times with 10 ml PBS to remove the residual test substance from the tissue surface. The tissues were then immersed into 1.5 ml of fresh maintenance medium for 30 ± 2 min incubation in standard culture conditions. The duplicate tissues were assessed for tissue viability.

Tissue viability assessment. After 30 min of incubation, tissues were carefully rinsed with PBS and were assayed for viability using MTT assay (Mossman, 1983). Each tissue was transferred to a new well containing 300 µl of freshly prepared MTT (1 mg ml^{-1}) solution for 3 hr ± 15 min incubation. The tissue inserts were then rinsed with 300 µl PBS and transferred into new plates containing 1.5 ml isopropanol per well for 4 hr at room temperature to extract the reduced MTT (formazan crystals). Formazan solution aliquots of 200 µl were transferred to a 96-well plate for optical density measurement at 570 nm using a microplate reader (Synergy H1, BioTek, USA). Isopropanol was used as a blank. The percentage viability of each of the treated culture was calculated from the percentage of MTT conversion relative to the corresponding negative controls (100% viability). Results were expressed as mean optical density (OD) and mean % viability and the difference of viability between the two replicate tissues.

Interpretation of Results and Prediction Model

The OD values obtained with the replicate tissue extracts for each test chemical should be used to calculate the mean percent tissue viability (mean between tissue replicates) normalised to the negative control, which is set at 100%. For reconstructed HCE model, the percentage tissue viability cut-off value distinguishing classified from non-classified test chemicals is 60% (OECD TG492, 2015) as shown in *Table 4*. The percentage of tissue viability cut-off value for reconstructed HCE model is 50% according to OECD TG439, 2015 as shown in *Table 5*.

Statistical Analysis

Data are reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) accompanied with Student t-test were conducted to identify the significant differences between test samples ($p < 0.05$).

RESULTS AND DISCUSSION

HPLC Assay of Tocopherol and Tocotrienol Isomers

The HPLC analysis was carried out to determine the composition of tocopherol and tocotrienol isomers. The HPLC analysis of palm TRF sample revealed that the isomers of tocopherols and tocotrienols were well resolved using the method adopted from Sundram and Nor (2002) as shown in *Figure 1*.

Table 6 shows the concentration (mg g^{-1}) of each isomer of the palm TRF obtained through HPLC analysis of tocopherols and tocotrienols. The HPLC analysis revealed that the concentration of α-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol detected in the sample were 169.1 mg g^{-1}, 200 mg g^{-1}, 8.5 mg g^{-1}, 245.5 mg g^{-1} and 28 mg g^{-1}, respectively. The percentage of α-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol and δ-tocotrienol detected in the sample were 26%, 30.7%, 1.3%, 37.7% and 4.3%, respectively. The total tocopherol and tocotrienol content was 651.1 mg g^{-1} sample.

TABLE 4. EYE IRRITATION PREDICTION MODEL ACCORDING TO OECD TG492 (2015)

Mean tissue viability (%)	Interpretation	Classification
> 60%	The test chemical is identified as not requiring classification and labelling according to UN GHS	No category
≤ 60%	The test chemical is identified as potentially requiring classification and labelling according to UN GHS	Category 2/Category 1

Note: UN GHS - United Nations Global Harmonisation System.

TABLE 5. SKIN IRRITATION PREDICTION MODEL ACCORDING TO OECD TG439 (2015)

Mean tissue viability (%)	Interpretation	Classification
> 50%	The test chemical is considered as non-irritant to skin in accordance with UN GHS	No category
≤ 50%	The test chemical is identified as potentially requiring classification and labelling according to UN GHS	Category 2/Category 1

Note: UN GHS - United Nations Global Harmonisation System.

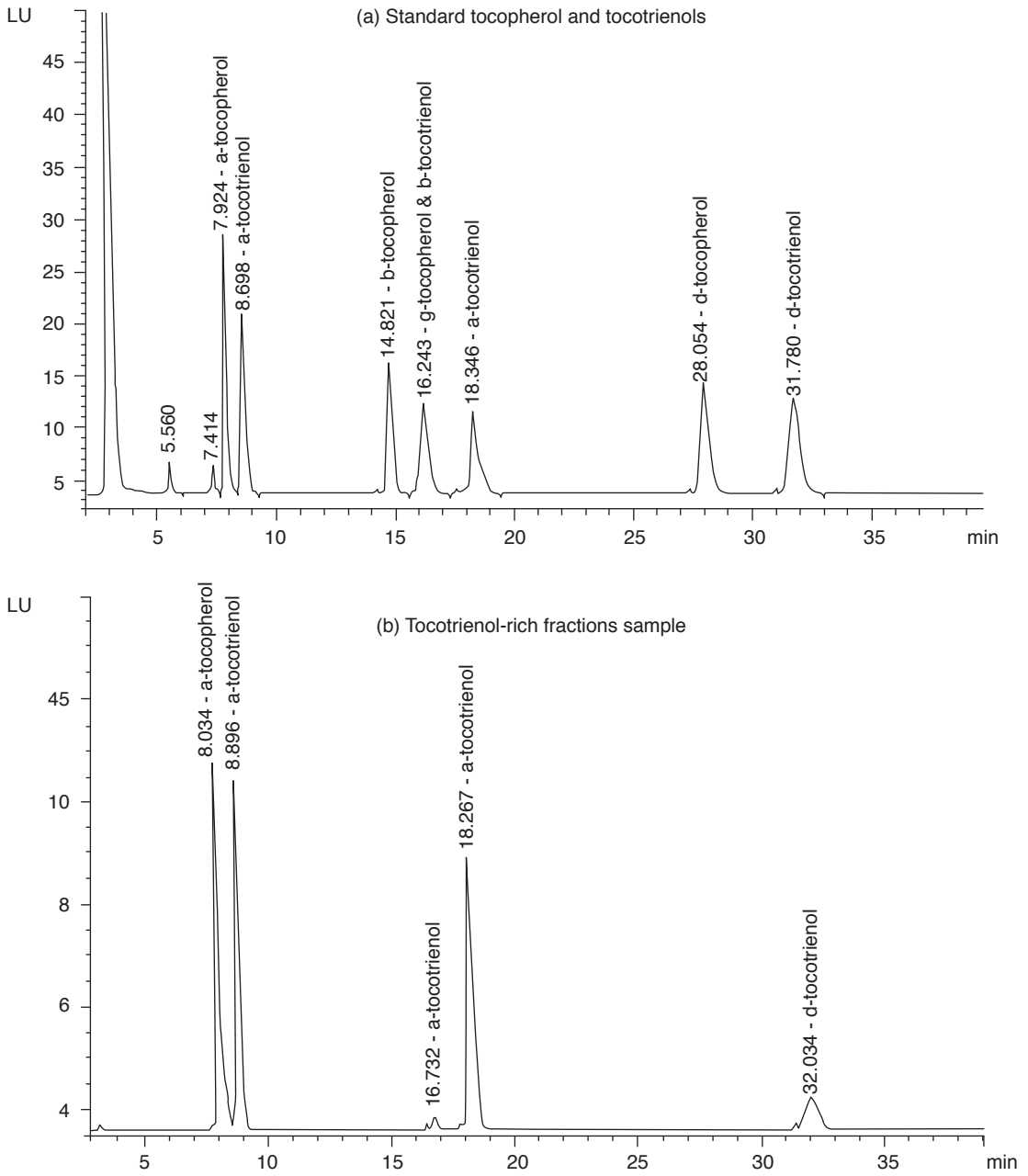
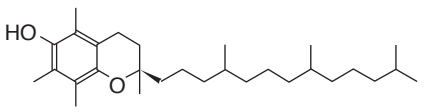
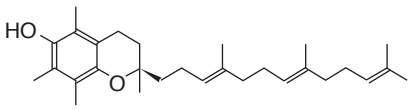
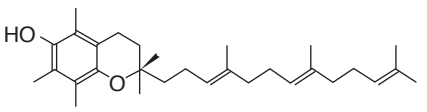
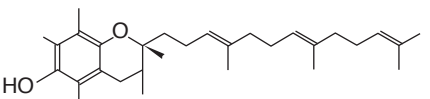
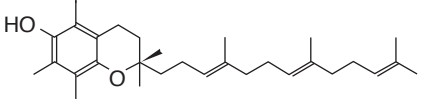


Figure 1. High performance liquid chromatography (HPLC) analysis of tocopherol and tocotrienol isomers. (a) Profiles of standard tocopherol and tocotrienol isomers mixture and (b) profiles of palm tocotrienol-rich fraction (TRF).

Palm TRF macroemulsion and nanoemulsion. *Figure 2* and *Table 7* summarise the physico-chemical characteristics of palm tocotrienol nanoemulsion and macroemulsion. The average size of palm tocotrienol macroemulsion and nanoemulsion were 4335 ± 746 nm and 137 ± 3.8 nm, respectively. The polydispersity index (PDI) of both macroemulsion and nanoemulsion were between 0.15 – 0.22 with mean zeta potential of -20.1 and -24.9, respectively. The low PDI for both emulsions indicated that monodisperse colloidal dispersions were obtained. Zeta potential, which describes the surface charge of the oil droplets, varied from −20.1 to −24.7 mV, giving an indication of the physical stability of the colloidal dispersions (Mishra *et al*., 2009; Mitri *et al*., 2011). The TRF nanoemulsion encapsulation efficiency was at 94.7% and 88.2% for 1% and 5% TRF nanoemulsion, respectively.

The ultimate size of a homogenised emulsion is determined by the balance between two opposing processes; droplet break-up and recoalescence (Meleson *et al*., 2004; Vankova *et al*., 2007). Both of these processes are highly dependent on the intense shear that occurs within a high shear homogeniser such as the Microfluidiser. Henry *et al*. (2009) have studied droplet coalescence in formation of nanoemulsions using a Microfluidiser and was shown to be minimal. Droplet break-up

TABLE 6. CONCENTRATION OF TOCOPHEROL AND TOCOTRIENOL ISOMERS IN PALM TOCOTRIENOL-RICH FRACTION (TRF)

Molecule	Molecular structure	HPLC assay, mg g^{-1} (%)
α-Tocopherol		169.1 (26.0)
α-Tocotrienol		200.0 (30.7)
β-Tocotrienol		8.5 (1.3)
γ-Tocotrienol		245.5 (37.7)
σ-Tocotrienol		28.0 (4.3)
	Total concentration of tocopherol and tocotrienol in palm TRF	**651.1 (100)**

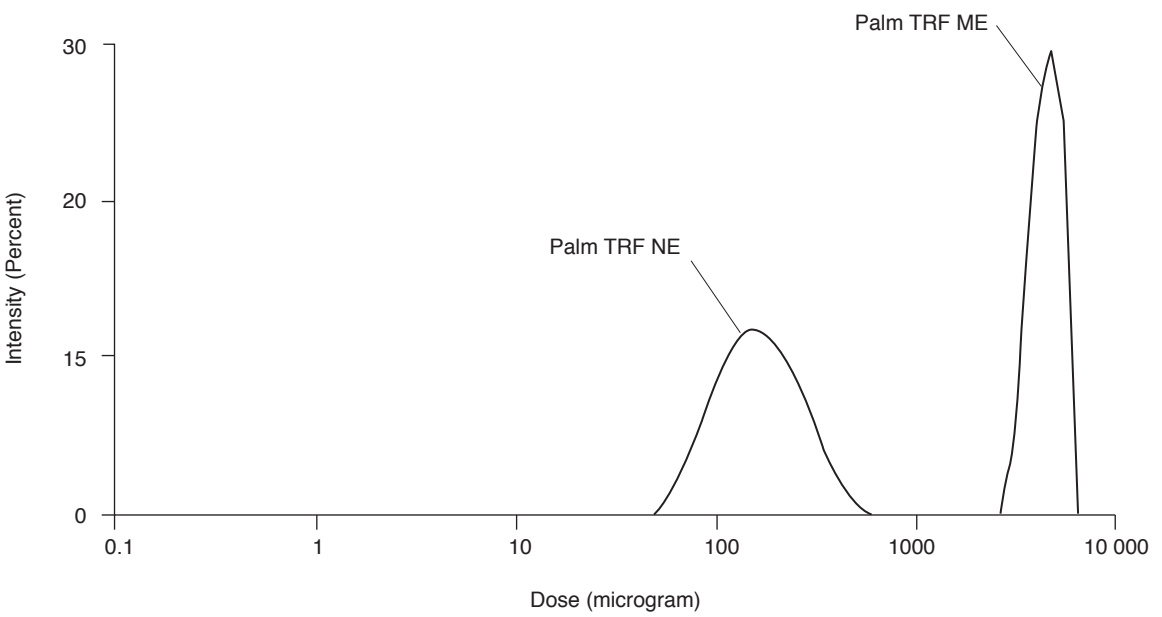


Figure 2. Particle size distribution of palm tocotrienol-rich fraction (TRF) nanoemulsion and macroemulsion.

TABLE 7. PHYSICO-CHEMICAL CHARACTERISATION OF PALM TOCOTRIENOL-RICH FRACTION (TRF) NANOEMULSION AND MACROEMULSION

Samples	Size (nm)	Polydispersity index	Zeta potential (mV)
5% Palm TRF nanoemulsion	137 ± 3.8	0.22	−24.9 ± 2.2
5% Palm TRF macroemulsion	4 335 ± 746	0.15	−20.1 ± 2.6

Note: Results are expressed as mean ± SD of three independent analyses.

occurs when the applied shear is greater than the Laplace pressure of the emulsion. Previous studies have shown that by increasing the homogeniser pressure to 15 000-20 000 psi will result in significant reduction in average droplet size (Jafari *et al*., 2006; Qian and McClements, 2011).

Safety Assessment of Palm TRF Emulsions

In vitro *ocular and dermal irritaction assay.* In this study, control nanoemulsion and tocotrienol nanoemulsion were subjected to both *in vitro* dermal and ocular irritection assays to determine the potential of the nanoemulsion system to induce skin irritation on *in vitro* ocular and dermal membranes. *Figure 3* shows the result of *in vitro* dermal irritation assay of palm TRF emulsions. The mean HIE scores for palm TRF macroemulsions obtained for various dosages of 50 mg, 75 mg, 100 mg and 125 mg were 0.06, 0.07, 0.10 and 0.12, respectively. While the mean HIE scores for palm TRF nanoemulsions were 0.02 for all dosages. The HIE scores for the positive control, sodium lauryl sulphate solution (0.5%, w/v) at 50 mg, 75 mg, 100 mg and 125 mg were 1.34, 2.24, 2.17 and 2.18, respectively. It was observed that both palm TRF macro and nanoemulsions fall under the non-irritant category as the mean HIE values were below 0.90 for all the dosages tested. The HIE score for sodium lauryl sulphates solution falls in the category of irritant as the HIE values were beyond the threshold level of 1.20, which may cause skin irritation on the dermal membrane.

Figure 4 shows that the mean IDE scores of palm TRF macroemulsion at 50, 75, 100 and 125 mg were 2.90, 3.53, 5.83 and 6.37, respectively. Nevertheless, palm TRF nanoemulsion of dosage concentrations of 50, 75, 100 and 125 mg exhibited mean IDE scores of 2.57, 1.33, 1.30 and 2.67, respectively. The mean IDE scores for the positive control, sodium dodecyl sulphate solution (0.5%, w/v) at 50 mg, 75 mg, 100 mg and 125 mg were 24.67, 24.70, 24.30 and 26.77, respectively. Both palm TRF emulsions exhibited an average IDE scores for all dosages studied between 0 and 12.50 which falls under the category of minimal ocular irritation. The positive control, the 0.5% sodium lauryl sulphates solution exhibited IDE scores that fall in the category of minimal irritation indicating that the test material may induce mild ocular irritation.

The *in vitro* dermal and ocular irritation test results provide evidence that both palm TRF emulsions in the range of the tested dosages may not induce any irritation on the ocular and dermal system. Therefore, it is an indication that both the palm TRF emulsions are safe to be used as cosmeceutical bioactive for topical application. The *in vitro* dermal irritation results indicated that palm TRF macroemulsion may not induce any skin irritation.

The results are in agreement with the *in vivo* work by Zafarizal *et al*. in 2008 which showed that

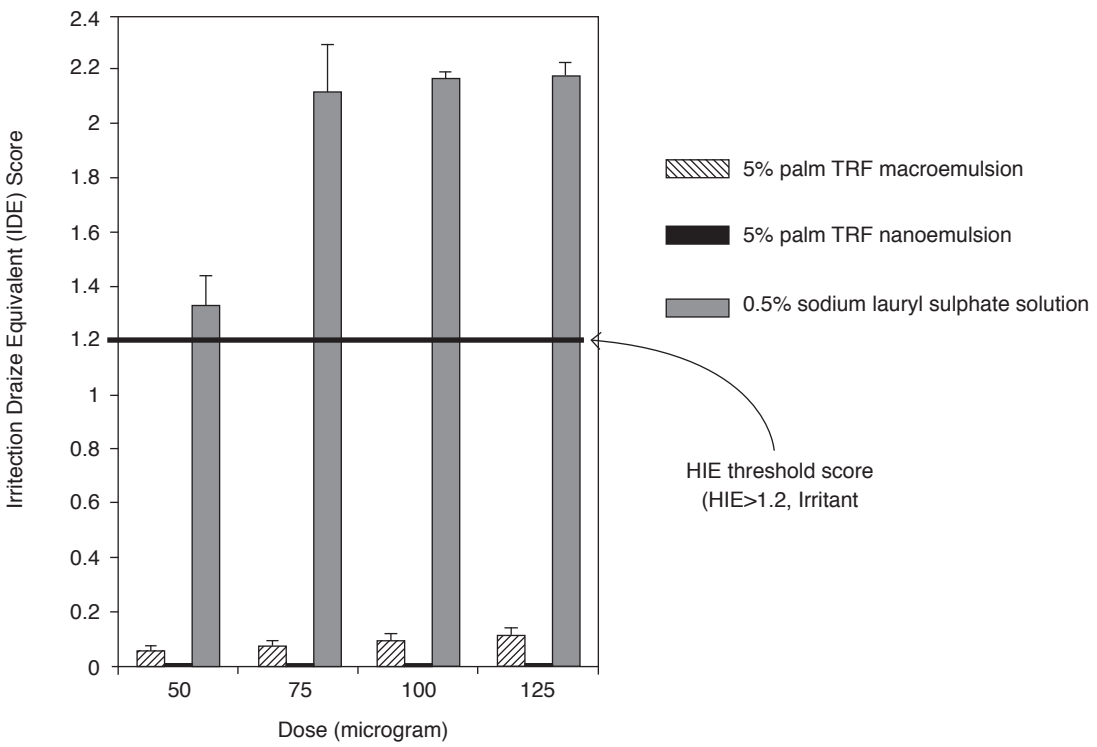


Figure 3. Mean Human Irritancy Equivalent (HIE) scores of palm tocotrienol-rich fraction (TRF) macro and nanoemulsion against sodium lauryl sulphate solution (0.5%, w/v).

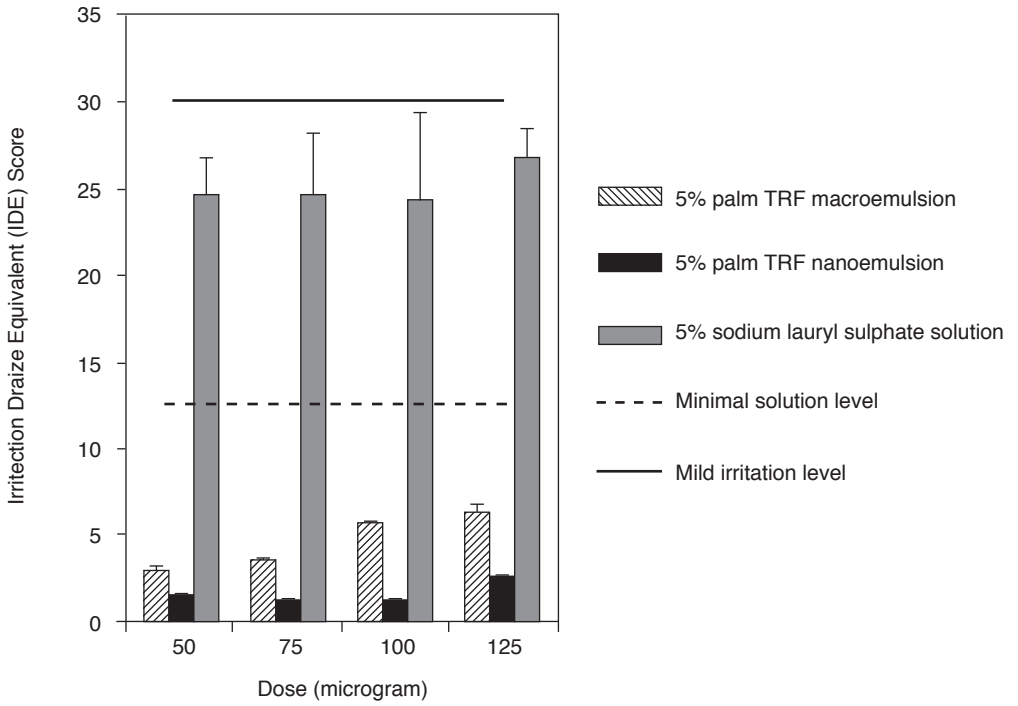


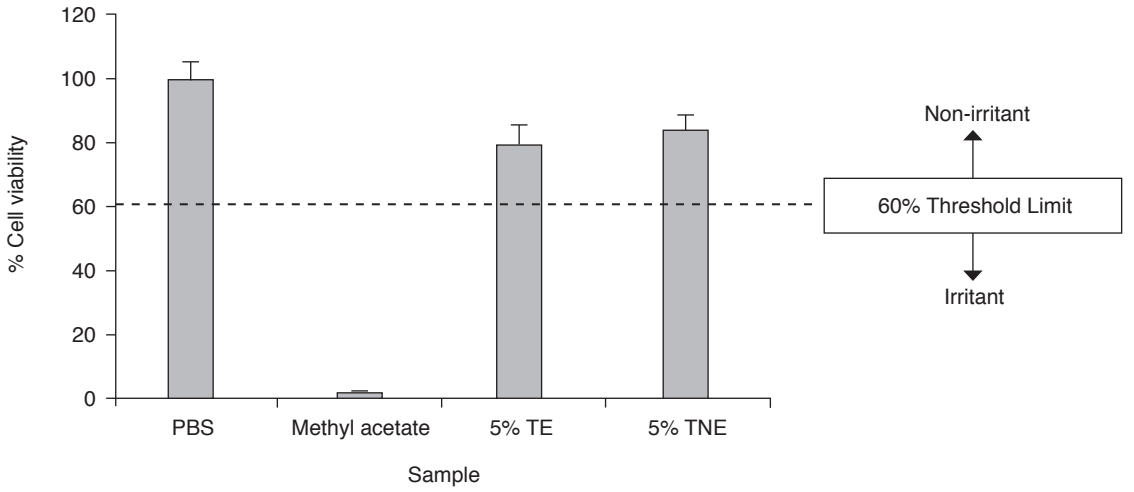
Figure 4. Mean Irritation Draize Equivalent (IDE) scores of palm tocotrienol-rich fraction (TRF) macro and nanoemulsion against sodium lauryl sulphate solution (0.5%, w/v).

palm TRF itself and palm TRF macroemulsion did not induce any skin irritations. These results also showed that reducing the particle size into submicron level may increase the bioavailability of palm TRF. Our study showed that increase bioavailability of palm TRF may not induce adverse dermal irritation.

In vitro *HCE*. The tissue viability assessment of palm TRF emulsions on reconstructed HCE is shown in *Figure 5*. Exposure to both 5% palm TRF macroemulsion and 5% palm TRF nanoemulsion resulted in mean tissue viability of 96.59% and 102.64%, respectively. The negative control, PBS showed mean tissue viability of 97.80% and positive control, methyl acetate resulted in very low tissue viability of 2.02%. The very low tissue viability of methyl acetate was in accordance with other report which recorded a consistent tissue viability of between 1.4%-12.1% (Alépée *et al*., 2015). A chemical can be classified as causing serious eye damage/eye irritancy if the mean tissue viability is less than 60% and *vice versa* for chemicals that do not affect the eye irritation (OECD TG492, 2015). Thus, methyl acetate is considered an irritant to the ocular membrane. Phosphate buffered saline, 5% palm TRF macroemulsion, and 5% palm TRF nanoemulsion are non-irritant to the ocular membrane.

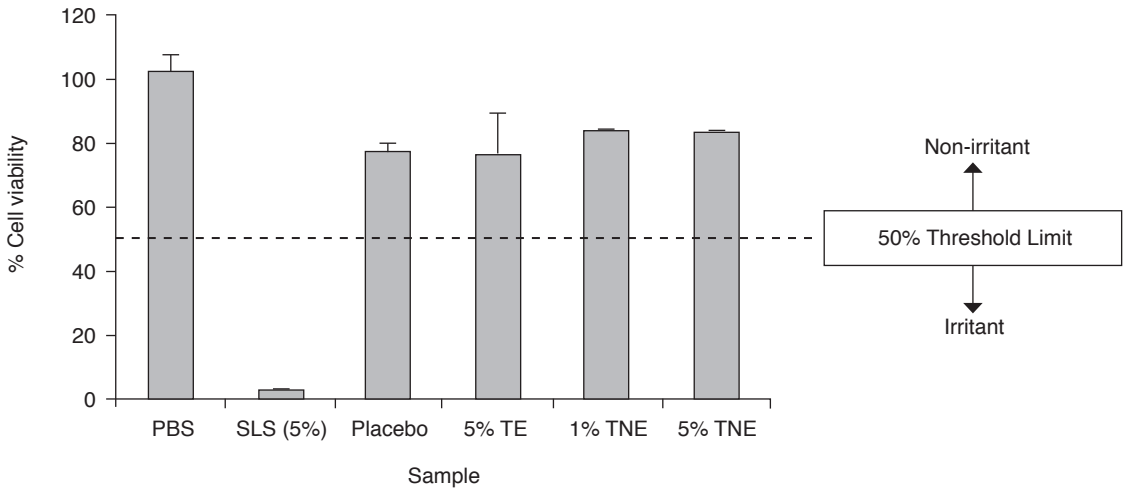
In vitro *RHE*. The tissue viability of palm TRF emulsions on RHE is shown in *Figure 6*. Exposure of the RHE to both placebo and 5% palm TRF macroemulsion resulted in mean tissue viability of 77.62 % and 76.69 %, respectively. On the other hand, exposure of the RHE to 1% palm TRF nanoemulsion and 5% palm TRF nanoemulsion resulted in a better tissue viability giving mean tissue viability of 83.21% and 83.22%, respectively. The *in vitro* treatment of RHE by a negative control, phosphate buffered saline showed mean tissue viability of 102.41% and that of the positive control, a 5% sodium lauryl sulphate solution showed tissue viability of 1.94%. According to *OECD Test Guideline TG439*, a test chemical is considered to be irritant to skin if the tissue viability after exposure and post-treatment incubation is less than or equal to 50%. On the other hand, according to OECD TG439 (2015) and Cotovio *et al*. (2005), a test chemical is considered as non-irritant to skin if the tissue viability after exposure and post-treatment incubation is more than 50%. Phosphate buffered saline, placebo, 5% palm TRF macroemulsion, 1% and 5% palm TRF nanoemulsion, had mean tissue viability of more than 50% indicating that these samples are considered non-irritant to skin. These results further confirm that 5% palm TRF macroemulsion and 5% palm TRF nanoemulsion may not induce adverse skin reactions.

As expected, the positive control of 5% sodium lauryl sulphates which is a known skin irritant (Lee and Maibach, 1995), had mean tissue viability of less than 50% indicating that SLS is considered



Note: PBS - phosphate buffered saline.

Figure 5. Tissue viability (mean ± SD, n = 2) of reconstructed human corneal epithelium (HCE) incubated with 5% palm tocotrienol-rich fraction (TRF) macroemulsion (5% TE) and 5% palm TRF nanoemulsions (5% TNE).



Note: PBS - phosphate buffered saline; SLS - sodium lauryl sulphate.

Figure 6. Tissue viability (mean ± SD, n = 3) of reconstructed human epidermis incubated with 5% palm tocotrienol-rich fraction (TRF) macroemulsion (5% TE), 1% palm TRF nanoemulsion (1% TNE) and 5% palm TRF nanoemulsion (5% TNE).

irritant to skin. The mechanisms behind the surfactant-induced barrier perturbation are not fully understood; however, increased hydration (Wilhelm *et al*., 1993) and disorganisation of the lipid bilayers of the epidermis (Fartasch *et al*., 1998) have been reported. Disruption of the skin barrier could result in the induction of a danger signal. Studies have indicated that, following disruption of the skin barrier, increased levels of immunological active signal molecules, interleukins (IL-1α and IL-1β), tumour necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are present within the skin (Wood *et al*., 1992). Thus, perturbation of the skin barrier itself could actually initiate an immunological stress signal leading to the subsequent development of an inflammatory reaction locally in the skin.

Froebe *et al*. (1990) studied the relationship between stratum corneum lipid removal and *in vivo* irritation. It was found that solubilisation of stratum corneum lipid by anionic surfactant SLS only occurred above the critical micelle concentration of sodium lauryl sulphate which is at the concentration of 0.24%. Cholesterol and free fatty acid were the major components removed from the stratum corneum lipids. In this study, sodium lauryl sulphate at 5% concentration was used as positive control which is above the critical micelle consideration of sodium lauryl sulphate. Thus, the sodium lauryl sulphate solution disrupted the RHE tissue barrier by solubilising the stratum corneum lipids. Perturbation of the RHE tissue may lead to release of cytokines which induce inflammatory reaction (Spielmann *et al*., 2007;

Hoffmann, 2006). The damaged cells may either release inflammatory mediators or induce an inflammatory cascade which in reconstructed human skin models is monitored via tissue viability readout.

CONCLUSION

Based on *in vitro* ocular and dermal irritation assay evaluation, palm tocotrienol nanoemulsion does not induce any irritation to the eye or skin. It is further confirmed by *in vitro* reconstructed HCE and RHE assessment, in which the cell viability are above 50% and 60%, respectively in accordance to the OECD TQ492 and TG493 test methods. Hence, palm tocotrienol nanoemulsion is safe to be applied topically.

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